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CYTOGENETIC EFFECTS OF FLUCHLORALIN IN MOUSE BONE MARROW AND ROOT MERISTEM CELLS OF *ALLIUM CEPA*

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ABSTRACT

Cytogenetic effects of fluchloralin (Flu) were examined in the mouse bone marrow cells and *Allium cepa* root meristem cells. Male albino mice were orally gavaged to 146, 292 and 438 mg kg^{-1}, corresponding to 20, 40 and 60% of LD_{50} (730 mg kg^{-1}), of Flu for 24 h. A group of mice was identically gavaged to sunflower oil for negative control or injected intraperitoneally to cyclophosphamide (20 mg kg^{-1}) for positive control. For *Allium* test EC_{50} concentration of Flu was determind to be 9 ppm and the roots were exposed to 4.5, 9 and 18 mg L^{-1} of Flu for 24 or 48 h. Roots exposed for 24 h after sampling were left in water for recovery and sampled at 24 h post- exposure. Exposure of higher doses of Flu (> 292 mg kg^{-1}) significantly ($p<0.05$) inhibited the mitotic index (MI) and induced clastogenic effects dose-dependently. Chromatid breaks and fragments observed to be major chromosome aberrations (CA) in all the treatment groups. *Allium* roots also revealed significant ($p<0.001$) percentage of CA at 24 h whereas roots exposed to 18 mg L^{-1} of Flu showed no cell division at 48 h. All the test concentrations found to induce mitotic aberrations (MA) and micronucleus formation significantly at 24 or 48 h exposure which persisted even at 24 h post-exposure. Based on the pres-

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ent findings it can be concluded that higher doses of Flu are capable of causing genotoxic effects in mammals and plants. Significant induction of MA in *Allium* cells also indicate the potential of Flu for mitotic poisoning.

INTRODUCTION

Flu (Basalin 45% EC), a pre and post-emergence herbicide, is a dinitroaniline derivative (N-propyl-N-(2-chloroethyl)-2, 6-dinitro-4-trifluoromethyl aniline; CAS-33245-39-5) commonly used in India on various crops (Worthing, 1987). Acute oral LD_{50} observed in mammals indicate that the compound is less toxic to mammals which possibly led to the indiscriminate use of Flu in agricultural practices.

Flu is readily absorbed by the roots, shoots and affects seed germination and various physiological processes in plants, particularly in radicle (Ashton and Crafts, 1981). Residues of Flu inhibit the root growth and induce swelling of root tips (Panneerselvam *et al*. 1995). In vitro exposure of Flu has been demonstrated to induce significant frequencies of chromatid/isochromatid breaks, micronucleus formation and SCE apart from inhibition of DNA synthesis in CHO cells (Sinha *et al*. 1998). Significant frequencies of chromosome breaks and micronucleated cells have been shown in cultured human lympocytes following exposure of Flu (Panneerselvam *et al*. 1995). A report published on the cytogenetic effects of Flu on *Allium cepa* showed induction of chromatid breaks, anaphase bridges and micronucleus formation (Rao *et al*. 1990). However report contained the results of exposure only whereas recovery of aberrations in subsequent cell cycles was not observed. The literature on genotoxicity of Flu is quite meagre. There are no reports on the in vivo cytogenetic effects of this compound on rodents and microbial test systems. Effects on plant chromosomes have also not examined properly despite that plants are major recipients of this herbicide. Considering the production and indescriminate use of Flu in India the present study was aimed to evaluate the in vivo cytogenetic effects of this compound on mouse bone marrow and root meristem cells of *A. cepa.*

MATERIALS AND METHODS

Chemicals

Commercially formulated Flu (Basalin 45% EC) was procured from BASF India Ltd., India. Other chemicals like fixatives and stains were bought from Glaxo, India and E-Merck, England respectively.

Test animals and treatment schedule

Male swiss albino mice (7-8 wks, 20±2 g bd. wt.) bred at animal house facility of Industrial Toxicology Research Center, Lucknow, were used in the study. Mice were (5/cage) preconditioned in separate cages, had free access to standard pellet diet (Ashirwad, Chandigarh, India) and fresh water ad libitum. During the experiment mice were maintained at 12 h light/dark period, 22±2^{0}C temperature and 70-80% humidity.

Test compound Flu was initially dissolved in sunflower oil and the test doses were made according to the active ingredient present in the commercial formulation. Mice were orally gavaged to three single doses 146, 292, 438 mg kg^{-1} of Flu which were 20, 40 and 60% of LD_{50} (730 mg kg^{-1}) for 24 h. Each mice was given 0.2 mL fixed volume of test dose or sunflower oil (vehicle control). For positive control mice were intraperitoneally injected to single dose of cyclophosphamide (20 mg kg^{-1}) dissolved in phosphate buffer saline (pH 7.2).

CA assay

Bone marrow aspiration, slide preparations and scoring of the chromosome aberrations were made as described by Adler (1984). In brief, mice were injected to colchicine (4 mg kg^{-1}) 1.15 h before the completion of 24 h exposure and sacrified by cervical dislocation. Both femurs of mice were taken out, cleaned and the bone marrow was flushed out in HBSS (pH 7.2). Cells were centrifuged at 1000 rpm for 5 min and the pellet was dispersed in 0.56% KCl and left in water bath fixed at 37±1^{0}C for 20 min. Cells were again centrifuged and the pellets were dispersed in chilled Carnoy's fixative. The process was repeated 3-4 times, and the cells in Carnoy's were left in refrigerator overnight. Next day cells were dropped over cleaned chilled slides, air dried and stained with 5% Giemsa for microscopic analysis. MI was determined by scoring metaphase cells from at least 3000 nuclei/mouse (1000 nuclei/slide) and expressed in percent. For CA nearly 75 well spread metaphase cells were analysed from each mouse. Chromatid/isochromatid gaps and pulverised cells were also counted but were not included in the total aberrations.

Allium Test
Test organism/growth conditions

Locally obtained common onion bulbs of *Allium cepa* were cleaned under tap water and outer scales were removed. During the treatment bulbs were kept at fixed temperature (20±2^{0}C) in a B.O.D. incubator for proper root growth.

Stock Solution

Stock solution was made in DMSO (dimethyl sulfoxide) and the experimental concentrations of Flu (according to active ingredient) were made by diluting the stock with tap water.

Determination of EC_{50}

EC_{50} concentration of Flu against root growth of *Allium cepa* was determined according to the method described by Fiskesjo (1985). In brief, uninfected clean onion bulbs were exposed to different concentrations of Flu. For each concentration 5 bulbs were exposed and a set of 5 bulbs was run parallel in tap water containing DMSO (0.3%). During the experiment the test concentrations were changed every 24 h after. On the fifth day root lengths from control and experimental groups were measured (10 roots from each bulb). Using control root length as standard average root lengths of test groups were plotted against concentrations and the point showed 50% growth was

marked as EC_{50} concentration.

Exposure schedule and scoring of aberrations

Three concentrations of Flu 4.5, 9 and 18 ppm were selected in which lowest concentration was half and highest one was double of the EC_{50}. Clean bulbs were rooted in tap water and when the roots reached 1-2 cm long they were exposed to aqueous concentrations of Flu for 24 or 48 h. For each concentration 5 bulbs were employed. After completion of exposure 5 root tips from each test group were fixed in Carnoy's. Bulbs exposed for 24 h after fixing few roots were transferred to tap water for recovery and the roots were fixed at 24 h post-exposure. Root tips were fixed in chilled Carnoy's fluid and stained with hematoxylin protocol (Chauhan *et al*. 1986).

Statistical Analysis

To determine the significant depression of MI one way analysis of variance (ANOVA) was carried out. Individual group means of test groups were compared with the controls. The significant levels of CA and MA were determined by Chi-square test. In all the cases p value less than 0.5 was considered significant.

RESULTS

Single oral exposure of three doses (160-438 mg kg^{-1}) of Flu inhibited the MI dose-dependently. However, significant ($p<0.01$) inhibition was observed at >292 mg kg^{-1} (Table 1). In vehicle control MI was found to be 3.56±0.76 which in the test group exposed to highest dose (438 mg kg^{-1}) of Flu decreased to 1.84±0.24. All the mice showed CA, though in comparison of vehicle control, significant ($p<0.001$) percentage of aberrations was observed in the mice exposed to 292 mg kg^{-1} or above. Chromatid breaks and fragments were found to be major CA, though ring chromosomes were also noted in mice exposed to higher doses (>292 mg kg^{-1}). A number of cells, with chromatid gaps and pulverized chromosomes, though not included in percent aberrations or aberrant cells, were also noted in all the treatment groups. Further the overall effect of Flu on CA was observed to be dose-dependent.

Allium roots exposed to different concentrations (4.9 to 18 mg L^{-1}) of Flu for 24 or 48 h showed significant and dose-dependent effect on MI (Fig. 1). However root meristem cells exposed to 18 ppm of Flu induced toxic effects at 48 hence no cell division observed. Toxicity of Flu at this dose was manifested by club shaped and stiff root tips.

Treatment of Flu also induced significant and dose-dependent percentage of CA except at 18 mg L^{-1} (Table 2). Chromatid breaks and fragment were found to be major CA (Table 2). Root tips examined at 24 h post-exposure also showed significant frequency of CA with 9 mg L^{-1} despite 47.08% decline in the percent aberrations. Similar dose-dependent effect was observed on mitotic aberrations (Table 3). Except 4.5 mg L^{-1} both the higher concentrations 9 and 18 mg L^{-1} induced significant ($p<0.01$ or 0.001) percentage of mitotic aberrations. Stickiness, chromosome bridges and laggarding chromosomes at

anaphase were major MA in all the treatment groups. Root tip cells exposed for 24 h and left in water for 24 h showed concentration related decline in the frequencies of aberrations (25.54 to 38.72%) however significant frequencies of MA were still found in the cells exposed to 9 mg L^{-1} (Table 3). Exposure of Flu also induced micronucleus formation in concentration-related manner which persisted at 24 h post-exposure (Fig. 2). As the exposure of 18 mg L^{-1} appeared to be toxic no cell division and micronucleus induction was observed at 48 h (Table 3).

DISCUSSION

Significant inhibition of MI in bone marrow and *Allium* root meristem cells indicate the cytotoxic potential of fluchloralin. Flu like oryzalin and trifluralin is a dinitroaniline herbicide that are known to inhibit polymerization of tu-

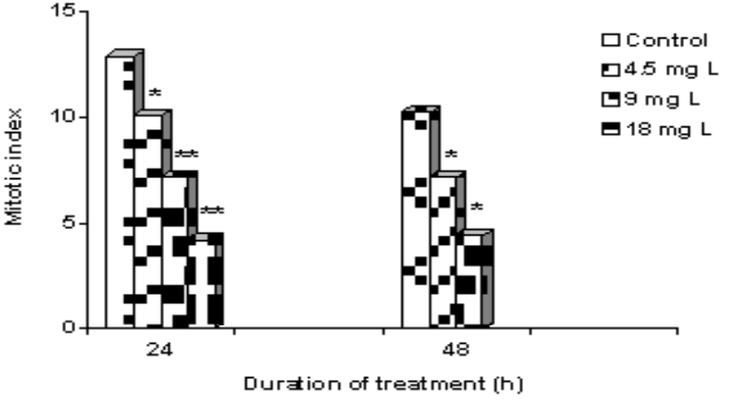

Fig. 1 Inhibition of mitotic index in the root meristem cells of *Allium cepa* exposed to fluchloralin, Data obtained from 5000-6000 cells, *p<0.05, **p<0.001

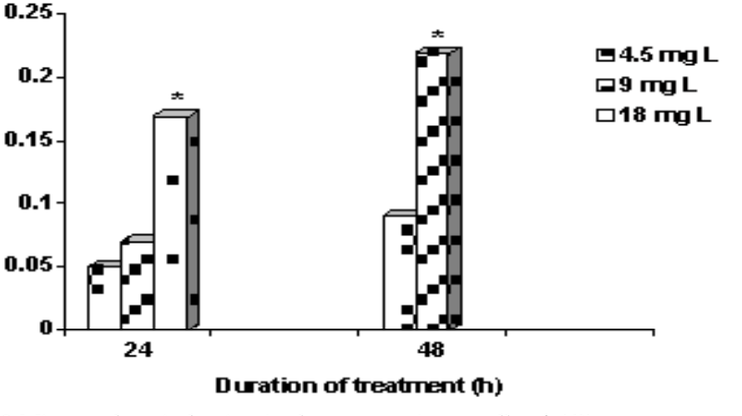

Fig. 2 Micronucleus induction in the root meristem cells of *Allium cepa* exposed to fluchloralin, Data obtained from 5000-6000 interphase cells,
*p<0.01(χ^2 test), **p<0.001 (χ^2 test)

Table 1
Chromosome aberrations in mice following 24 h oral exposure of fluchlorali

Treatment group/ Doses (mg kg^{-1}) Cells/mice	Mitotic[a] Index	No.of Metaphase	Aberrations				Chromatid gaps	Pulverized	Total[b] aberrations (aberrant cells)	[b]aberrations (aberrant cells)
			Chromatid breaks	Fragments	Rings	Exchanges				
Sunflower Oil (Vehicle Control)	3.56 ± 0.76	415/5	-	4 (4)		-	-	4	4 (4)	0.96 (0.96)
Fluchloralin 146	2.41 ± 0.55	408/5	2 (1)	5 (4)	-	-	6 (5)	4	7 (5)	1.71 (1.22)
292	1.99 ± 0.49*	374/5	3 (2)	6 (4)	2 (2)	2 (2)	12 (7)	6	13 (10)	3.47 (2.67)*
438	1.84 ± 0.24*	360/5	3 (2)	5 (5)	3 (3)	3 (3)	8 (6)	8	14 (13)	3.88* (3.61)
Cyclophos phamide 20	2.05 ± 0.94*	378/5	9 (6)	14 (9)	-	3 (3)	16 (10)	9	26 (18)	6.89** (4.76)*

[a]Expressed as group mean ± standard error, [b]Chromatid gaps and pulverized chromosomes excluded, * $p < 0.01$, ** $p < 0.001$

Table 2
Induction of chromosome aberrations in the root meristem cells of *A. cepa* exposed to fluchloralin

Concentrations (mg L^{-1})	Exposure time (h)	Chromatid	Fragments	Total breaks	% Aberrations	% Aberrations aberrations at 24 h	% Recovery of aberrations post exposure
Control	24	1	1	2	1.00	0.66	34
	48	1	4	5	1.60	-	-
4.5	24	5	7	12	4.00*	1.66	58.5
	48	8	10	18	6.00*	-	-
9.0	24	9	8	17	5.67**	3.00*	47.08
	48	9	15	24	8.00**	-	-
18	24	1	3	4	1.34	0.33	75.18
	48	ND	ND	ND	ND	-	-

ND - NOT DETECTED; Mitotic aberrations scored from 500-600 dividing cells., * $p < 0.01$ (x^2 test), ** $p < 0.001$ (x^2 test)

bulin by binding with the alpha-tubulin proteins which arrest the cell division in plant cells (Bartels and Hilton, 1973, Morejohn *et al.* 1987 and Morisette et al., 2004). However, its action on the tubulin proteins of mammaliam cells is not still clear. Flu has also been demonstrated to inhibit DNA synthesis in cultured CHO cells (Sinha *et al.* 1998).

Inhibition of MI as observed in the present study may be due to the effect of Flu on the microtubular proteins and/or DNA synthesis of root meristem cells and mouse bone marrow. Similar inhibition of DNA synthesis associated with the inhibition of cell division has been reported with other herbicides also and attributed to be a characteristic effect of herbicidal chemicals (Seiler, 1987, Chauhan *et al.* 1998 and Badr and Elkington, 1982). Significant percentage of mitotic aberrations and its persistence upto 24 h post-exposure also indicate the effect of Flu on the mitotic apparatus of root meristem cells which is known as primary mode of action of dinitroaniline herbicides (Ashton and Crafts, 1981 & Morejohn *et al.* 1987).

Significant increase in the frequencies of CA in mouse bone marrow and *Allium* root meristem cells indicate the clastogenic potential of Flu. To the best of our knowledge, hitherto *in vivo* cytogenetic effects of Flu have not been reported in rodents. Whereas *in vitro* exposure of Flu has been reported to induce CA, SCE, micronucleus formation in CHO and peripheral lymphocyte cells concomitant with the 50% loss of cell viability (Panneerselvan et al., 1995 and Sinha *et al.* 1998). The interaction of dinitroanilines with DNA is not known but induction of

Table 3
Induction of mitotic aberrations in the root meristem cells of *A. cepa* exposed to fluchloralin

Concentrations (mg L^{-1})	Exposure Time(h)	Stickiness	C-metaphase	Multipolar	Bridges	Laggards	Unequal separation	Polyploid	Disoriented	Cells with Mitotic aberration	%Aberrant cells	%Aberration at 24 h post expos
Control	24	3	-	-	-	-	-	-	-	3	0.83	.80
	48	6	-	-	-	-	-	-	-	6	1.69	
4.5	24	5	3	2	3	1	1	-	1	16	4.15**	3.09*
	48	16	6	1	5	-	2	3	6	39	11.46***	
9.0	24	11	6	2	5	-	9	6	3	42	11.29***	4.66**
	48	18	12	2	2	1	13	2	8	58	16.03***	
18	24	19	10	3	2	-	2	-	16	52	13.75**	6.92**
	48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-

ND – Not detected, * Significant from the control $p < 0.05$ (x^2 test), ** Significant from the control $p < 0.01$ (x^2 test)
*** Significant from the , control $p < 0.001$ (x^2 test)

apoptosis in CHO cells indicates its action on DNA (Sinha *et al*. 1998).

Trifluralin another dinitroaniline herbicide, similar to Flu in chemical structure, has also been shown to induce significant frequencies of SCE (with or without metabolic activation), CA, micronucleus formation along with the DNA damage in lymphocyte cells measured through single cell gel electrophoresis (Gebel *et al*. 1997 and Ribas *et al*. 1995). Further, trifluralin treatment also causes micronucleus induction significantly in mice (Ribas *et al*. 1996). Similar genotoxic effects of Flu are possible as both the compound have similar chemical structure. Result of our in vivo cytogenetic study in mice and root meristem cells of *A. cepa* are in agreement with in vitro effects observed in CHO, human peripheral lymphocyte and *Allium* root meristem cells reported earlier (Rao *et al*. 1990).

CONCLUSION

The observations of present study indicate that the in vivo exposure of Flu can cause genotoxic effects in mammals and non-target plants. Induction of MA in *Allium* root cells also indicate that Flu like other dinitroaniline herbicides affects the spindle apparatus that results in the production of various mitotic aberrations. Further studies in mammalian and microbial test systems are very essential to elucidate the genotoxic potential of Flu and consequent risk to human beings.

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