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Assessment of Clastogenicity of λ-Cyhalothrin, A Synthetic Pyrethroid in Cultured Lymphocytes of Albino Rat

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Abstract: Genotoxic potential of λ-cyhalothrin (LCT) was evaluated *in vivo* in the cultured albino rat lymphocytes on the basis of chromosomal aberration (CA) assay. The LCT was administered to albino rats as repeated oral doses of 18mg/kg bwt. to acute group, 0.6mg/kg bwt. to sub acute group up to 30 days, while recovery group did not receive any dose after 30 days till day 45. The negative control received the vehicle (ground nut oil) only. Mitomycin C (MMC) was used as a positive control (1.5mg/kg bwt.), administered intraperitoneally. Significant clastogenic potential has been observed after 30days sub acute treatment. The types of chromosomal aberrations observed in present study include chromosome gap, chromosome break, chromatid gap, chromatid break and fragments. LCT possesses potential to induce cytogenetic changes in lymphocytes, which are mostly used in defense and cell immunity. These changes inform of chromosomal aberrations are indicative of possibility of the experimental compound to exerts stress at such a higher level of food chain and may be problematic like conventional pesticides of yester years.

Key words: λ-cyhalothrin (LCT) · Chromosomal aberration · Synthetic pyrethroid · Albino rat · MMC

INTRODUCTION

The population of the world is increasing continuously since time immemorial and utilization of natural resources is on increase. The rise of human population has crossed all strides in the beginning of 20th century with the result scientists had to employ all tactics to feed the rising population. During this course, some of the synthesized chemicals not only helped the mankind but also became reasons for his agony. A good number of chemicals in form of pesticides reigned for quite some time, however left many problems that were related to welfare of human beings.

It became essential to look for newer avenues to minimize the misery and plight of human population. In this situation few chemicals were synthesized which were identical to natural origins. The pyrethroids were such in this series. The pyrethroids are grouped into two categories, one, type-I pyrethroid and the other type-II pyrethroid. LCT is a newer third generation type-II synthetic pyrethroid contains α-cyno group and is available in a number of formulations [1,2]. Due to its rapid metabolism and excretion its toxicity for mammal at present is quite low, however it may create problems in non-target species in future if applied indiscriminately.

The genotoxic effects of LCT were investigated in various animal species using chromosomal aberration (CA), Micronucleus formation (MN) and banding pattern analysis [3-8,44]. The bone marrow is most widely used for short-term *in vivo* assay for genotoxic study [7,8], however, in the present study lymphocytes have been used as they are functional in defense mechanism and can easily be obtained from blood of the experimental animal. Although cytotoxic nature of synthetic pyrethroids is a known fact [9, 11, 12, 17, 19, 22-23, 41-44], yet LCT is hereby checked for possessing the cytotoxic potential if any through chromosomal aberrations analysis. Earlier reports emphasize mainly bone marrow [3] for evaluating LCT toxicity however the present studies assess genotoxic potential of LCT in lymphocyte *in vivo*.

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MATERIALS AND METHODS

Test Compound: For the present study λ–cyhalothrin (LCT), a non-systemic pyrethroid insecticide with the trade name 'Karate' (CAS no. 91465-08-06), chemical name (R + S)-F-cyano-3-(phenoxyphenyl)methyl-(1S+1R)-cis-3-(2-2chloro-3,3,3-trifluoroprop-1-enyl)-2-2-dimethyl-cyclopropane-carboxylate of 98% purity, with contact and stomach action and repellent properties was procured from Zeneca-ICI Agro Chemicals, Chennai (India).

Maintenance and Feeding of Experimental Albino Rats: The experimental albino rats (*Rattus norvegicus* [Berkenhout]), procured from inbred colony were acclimated for one month to the laboratory conditions (temperature. 25°±0.5°C, relative humidity 60±5% and photoperiod 12 hr/day) before using them for the experiment. Adult male and female rats of almost equal size and weight were kept in the polypropylene cages and cleaned regularly to avoid any infection or undesirable odour in the laboratory. Each cage was equipped with a metallic food plate and water bottle. The albino rats were offered fresh feed daily throughout the experimentation on Gold Mohar rat and mice feed, manufactured by Hindustan Lever Ltd., India at regular interval and water was provided *ad libitum.*

Random Selection of Individuals: In the present study, for LD_{50} the data were analyzed statistically by log dose/probit regression line method [10]. Oral LD_{50} of male and female rats was found to be 75.85-mg/kg body weight and 56.695 mg/kg body weight respectively [44]. The percent mortality response in the two sexes as per Table 1 did not reveal any significant change ($p>0.05$). Hence, it could be possible to select the individuals randomly (irrespective of sex) for experimentation.

Five healthy adult albino rats (7–8 weeks of age, with average body weight of 150–200 g) were selected randomly for test, control and recovery studies sacrificed after 1, 2, 15, 30 and 45 (recovery) days for the collection of blood in the present investigation. Each rat was assigned a number for convenience prior to experimentation. All the rats of the experimental sets were given doses of LCT orally with the help of gavage tube and those of control sets equal amount of vehicle i.e. ground nut oil.

Selection of Dose: Test agent-An oral dose of 18 mg/kg body weight for acute treatment, while for sub acute treatment 1/30 of acute dose was given for 30 days i.e. 0.6 mg/kg body weight/day by gavage tube. The recovery group did not receive any dose after 30 days of sub acute treatment till day 45.

Positive Control- Mitomycin C (MMC), CAS no. 50-07-7 was used as a positive control. MMC was given as a single dose of 1.5mg/kg bwt via intraperitoneal injection. It is acceptable that a positive control is administered by a route different from or the same as the test agent and that it is given only a single time [11].

Negative Control- Negative controls consisting of vehicle (groundnut oil) were treated per Os with all treatment groups.

Collection of Blood Samples: In the early morning hours (7-8 AM) on the due date of autopsy the rats were warmed under the desk lamp to facilitate bleeding. Enough care was taken to avoid them going into heat shock. Over heated condition was depicted by beads of perspiration on the nose or excessive activity. The rats were placed in a restraining block and swabbed the tail first with 70% ethanol, then with 90% ethanol. After the alcohol was evaporated a small slit was made in the underside of the tail about 1 inch from the base of the tail by a flamed razor blade. First two drops of blood were discarded to avoid contamination; Blood collecting tubes were gently flamed just before and just after the blood was collected. After collection 8-10 drops of blood, the tube was agitated gently to mix the blood and heparin solution. The blood collecting tubes were tightly capped and stored at 37°C.

Table 1: Comparison of percent mortality of LCT in male and female albino rats

S. No.	Group	Dose (mg/kg b.w.)	No. of animals treated	Percent Mortality in Male	Percent Mortality in Female	Significance level
1.	A	20	7	14.28	14.28	P >0.05
2.	B	40	7	28.57	42.85	Non significant
3.	C	80	7	42.85	71.42	
4.	D	160	7	71.42	71.42	
5.	E	320	7	100	100	

Chromosomal Aberration Assay: Chromosomal aberration was performed in cultured lymphocyte according to the methodology proposed by Triman [12] with slight modifications.

Inoculation of Culture: For culture 0.1 ml of the blood-heparin mixture was added to each prepared culture vials containing 0.1 ml phytohemagglutinin (PHA) and 0.8 ml supplemented medium (containing RPMI 1640, 25mM Hepes buffer, fetal calf serum and penicillin-streptomycin solution), to bring the final culture volume to 1.0 ml. The cultures were mixed well and capped tightly. The cultures were incubated at an angle of approximately 5° from horizontal at 37° C for 24 hours.

Culture Growth in Media: The cultures were centrifuged after 24 hours at 200 X g for 8 min. The supernatant was removed and discarded aseptically with a sterile Pasteur pipette. The medium was replaced with 0.8 ml of supplemented medium and 0.1 ml of PHA. The culture was mixed gently; bubbling was avoided, with a sterile Pasteur pipette and returned to the 37° C incubator for another 24 hours. 5-µg of colchicine was added to each culture at 48 hours and mixed gently and thoroughly with a Pasteur pipette. It was incubated at 37° C for an additional 4 hours.

Harvesting and Hypotonic Treatment of Cells: Each culture was transferred to a 15 ml conical centrifuge tube and centrifuged at 200 X g for 8 min. at 52 hours. The supernatant was removed carefully with a Pasteur pipette. 1.0 ml of 0.075m KCl was added slowly to the cells without disturbing the pellet. The culture was mixed gently with a Pasteur pipette and bubbling was avoided. The cultures were returned to the 37°C incubator for 10 min., Centrifuged them at 1200 rpm for 10 min.

Fixation: The supernatant was carefully removed with a Pasteur pipette leaving a volume approximately 0.25 ml including the cell pellet. Chilled fixative (glacial acetic acid:methonal, 1:3) was added very slowly down the inside of tube to avoid clumping of the lysed cells. When the total volume of the culture and fixative become 1.0 ml, mixed it gently by rapid pipetting. 1.0 ml of fixative was then added to bring the volume to 2.0 ml and mixed the culture again. At this point the culture was tightly capped and refrigerated overnight.

Slide Preparation and Staining: Before slide preparation culture was centrifuged at 1200 rpm for 5 minutes, removed the supernatants fixative (leaving the volume of 0.5 ml) and added 1.5 ml of fresh fixative. This washing procedure was repeated two more times before using this for slide preparation. Two drops of above suspension were dropped on to a clean, chilled, wet slide. The sides and back of slide were quickly blotted and simultaneously blew once across slide and placed onto slide warmer to dry. Slides were immediately coded with a random number, which had been correlated with the animal number. The dried slides were stained in 4% Giemsa (in phosphate buffer pH–6.8). Slides were dried thoroughly and cover slips were applied.

Metaphase Scoring: Hundred well spread intact metaphases were scored through blind scoring from each animal number under 100 x oil immersion. The abnormalities suggested by the "Ad Hoc Committee of Environmental Mutagen Society and The Institute for Medical Research" [13] were considered which included chromatid and chromosome breaks, fragments of untraceable origin. Chromatid and chromosome gaps were recorded but were not included as aberrant features in the final evaluation [14]. The following values were calculated [46].

$$\text{Frequency of aberrant cells} = \frac{\text{Total aberrant cells}}{\text{Total No. of cells studied}}$$

$$\text{Percentage of aberrations} = \frac{\text{Total aberration (excluding gaps)}}{\text{Total No. of cells}}$$

Statistical Analysis: The data are compared by 't' test and expressed as mean±SE.

RESULTS

The metaphase analysis of the lymphocytes revealed various types of chromosomal aberrations, which consisted of chromatid, chromosome gaps and breaks and fragments. Relatively higher frequencies of gaps and fragments were observed for all the doses tested (Table 2). A quantitative assessment of the distribution of breaks and gaps revealed that the distal regions of the long chromosomes were more vulnerable to the effects of LCT. The frequency of CA is increased with increasing concentrations of LCT (Figure 2) and statistically

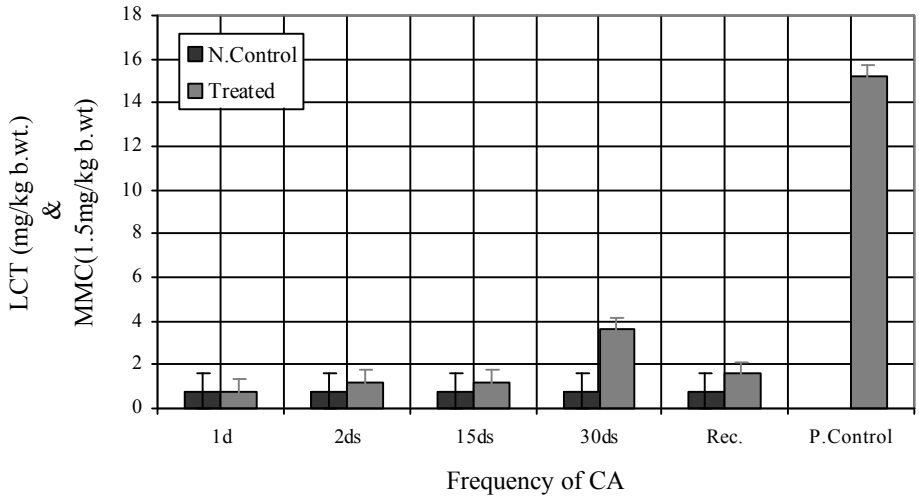

Fig. 1: Frequency of chromosomal aberration (%) in relation to LCT intoxication in albino rats using cultured lymphocyte

Table 2: Chromosomal aberration analysis in peripheral blood lymphocytes of albino rats

S. No.	Treatment	Dose (mg/kg b. w.)	No. of individuals treated	Treatment time (In days)	No. of Cells / animal	Chromosomal aberration: Gap: Chrom-atid	Gap: Chrom-osome	Break: Chrom-atid	Break: Chrom-osome	Fragments	Total: Without Gap	Total: With Gap	% of aberration without gap: Mean± S.E.	% of aberration without gap: Significance	Frequency of aberrant cell: Mean± S.E.	Frequency of aberrant cell: Significance
1	Negative Control		5	01	250/5	1	-	1	-	1	2	3	0.8±0.55		0.004±0.004	
2	Acute	18	5	1	250/5	2	-	1	-	1	2	4	0.8±0.55	p>0.05	0.016±0.008	p>0.05
3	Negative Control		5	2	250/5	1	-	1	-	1	2	3	0.8±0.55		0.004±0.004	
4	Acute	18	5	2	250/5	1	-	1	-	2	3	4	1.2±0.55	p>0.05	0.004±0.004	p>0.05
5	Negative Control		5	15	250/5	1	1	-	1	1	2	4	0.8±0.55		0.008±0.005	
6	Sub-acute	0.6	5	15	250/5	2	1	1	1	1	3	6	1.2±0.55	p>0.05	0.012±0.009	p>0.05
7	Negative Control		5	30	250/5	1	-	1	-	1	2	3	0.8±0.55		0.008±0.005	
8	Sub-acute	0.6	5	30	250/5	1	1	2	3	4	9	11	3.5±0.55	p<0.05	0.036±0.008	p<0.02
9	Negative Control		5	45	250/5	2	-	-	1	1	2	4	0.8±0.55		0.008±0.005	
10	Recovery	0.0	5	45	250/5	1	1	1	1	2	4	5	1.6±0.84	p>0.05	0.008±0.005	p>0.05
11	Positive Control	1.5	5	2	250/5	13	11	15	12	11	38	62	15.2±1.2	p<0.001	0.120±0.050	p<0.01

non-significant (P>0.05). Differences from the negative control were observed, except at the 30 days sub acute treatment (P<0.05). The mean of the induced CA range from 0.8±0.55 to 3.6±0.55, in different treatment groups, while in recovery groups it is 1.6±0.84 on comparison to controls. Such values are much lower than those induced by the positive control Mitomycin C (1.5 mg/kg bwt) (15.2 ± 1.2).

DISCUSSION

In the present investigation non-significant induction of clastogenic activity of λ-cyhalothrin has been observed in acute, sub acute (15ds) treatment and recovery groups, while in 30ds sub acute treatment clastogenic potential has been observed. Similarly workers of USEPA (15) and WHO (16) working group failed to observe clastogenic potential under LCT stress, while Campana *et al.* [6] Fahmy and Abdalla [4] and Celik *et al.* [3] demonstrated clastogenic potential of λ-cyhalothrin in fish, mouse and wistar rat in red blood cells and bone marrow respectively.

The findings in the present studies are in accordance to previous reports on the clastogenic potential of synthetic pyrethroids as manifested in rodent bone marrow [3, 5, 17-24, 46], in human peripheral lymphocyte cultures [22, 25-31], in CHO cells [30, 32] and in aquatic organisms [6, 33].

The effect of LCT seems to be time of exposure and concentration dependent. In 30ds sub acute treatment a significant difference in aberration has been observed, might be due to the longer duration of treatment. Hence, LCT has greater potential for inducing chromosomal aberration in long duration treatments, while its effect has been found to be non-significant in short term treatment [3, 17, 19, 30, 34 and 35].

Chemicals that cause damage to lysosomes and membranes of cellular system, induce the release of lysosomal or other DNAase into the cytoplasm of damaged cell and induce DNA double strand break and in those cells that survive sub lethal damage, such double strand breaks could have a variety of genotoxic effects such as mutation, chromosome aberration [36,44].

The clastogenic property of LCT may be due to its ability to cause degenerative and necrotic damage to mammalian tissue like other pesticides [37, 38, 46], which may probably induce lysosomal damage and release of hydrolytic enzymes. Release of hydrolytic enzymes as a result of lysosomal damage following cybil intoxication has been observed [39, 46]. Further, cypermethrin has been seen causing major degenerative changes in rat bone marrow cells [40-41].

The frequency of chromosomal aberrations was directly related to the concentration used and duration after exposure to fenvalerate in Swiss albino mice [34], while cytolethality was time; concentration and cell number dependent in rats [35] is already a known fact. Further, Chauhan *et al.* [42] observed greater potential of cypermethrin and deltamethrin as genotoxic agent in mice. It is thus evident that different kinds of mechanism are responsible for toxicity and clastogenicity on one side and DNA breakage and gene mutation on the other side [44].

The non-significant increase in recovery group is also supported by Amer and Aboul-Ela [18] who found that frequency of chromosomal aberration returning to normal levels of control after 14ds recovery following cypermethrin toxicity in mice, while Amer *et al.* [20] revealed percentage of chromosomal aberration to be decreased as time lapses after treatment. Similarly Sharma *et al.* [43] observed that elevation in the frequency was steep up to 10_{th} day, but it was slowed down with an increase in dose and time after 2, 4-DB acid toxicity in mice. Similar mechanism may also be responsible for non-significant increase of chromosomal aberration in recovery group.

The selected cyno group derivative (λ-cyhalothrin, type-II pyrethroid) has a potential to induce genotoxic alterations in peripheral blood leucocytes particularly lymphocytes which forces its regulated application in the ecosystem else the chemical may prove its worth as a future mutagen like the conventional II^{nd} and III^{rd} generation pesticides of yester years.

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