Evaluation of Genotoxicity Induced by Low Dose Oral Exposure of Deltamethrin in Mice Model

Anupama Sharma[1*], Jatinder Paul Singh Gill[1], Satyavan Rampal[2] and Jasbir Singh Bedi[1]

[1]*School of Public Health and Zoonoses, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, INDIA*
[2]*Department of Veterinary Pharmacology and Toxicology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, INDIA*

Corresponding author: A Sharma; E-mail: anupamasharma.vph@gmail.com

ABSTRACT

The study was conducted to elucidate the genotoxic effect of deltamethrin in *Swiss Albino* male mice following oral exposure, by comet assay and micronuclei induction test. Deltamethrin was administered at the dose rate of 1.5 mg/kg bwt, 1.0 mg/kg bwt and 0.5 mg/kg body weight. Mean head DNA percentage, mean tail DNA percentage, mean tail length and mean olive moment were the endpoints for the comet assay. Deltamethrin resulted in a significant comet formation at 15, 30 and 60 days of oral exposure in a dose and time dependent manner. Mean tail DNA% at 15, 30 and 60 days of exposure to deltamethrin was 59.28, 57.74 and 63.14, respectively. The results were found to be significantly different in treatment groups as compared to the control groups. Micronucleated normochromatic erythrocytes, micronucleated polychromatic erythrocytes and polychromatic erythrocytes percentage were the endpoints for the micronuclei induction test. Deltamethrin induced significantly higher number of micronuclei in treatment group than the negative control. In conclusion, present study indicates that pesticides are slow poison and even a low dose of pesticide can cause genotoxicity and other biological effects when exposed to for longer duration.

Keywords: Blood, Comet, Mice, Micronuclei, Pesticide

Pyrethroid insecticides are amongst the most widely used class of insecticides worldwide because of having wider margin of safety and broad spectrum of activity against insects. These are the synthetic analogues of pyrethrins and are extracted from flower of the genus *Chrysanthemum* (Kim *et al.*, 2004). Pyrethroids are successively surpassing the organophosphorus and organochlorine pesticides due to their advantage of photostability, high effectiveness even at lower concentrations, easy disintegration and low toxicity in birds and mammals (Bradbury and Coats, 1989). Deltamethrin, a synthetic pyrethroid insecticide, is being used widespread in household as well as in agriculture. It is a contact poison and works by paralyzing the nervous system of insects and thus giving a quick knock down effect (Sharma *et al.*, 2013). Deltamethrin has been reported to exert immmunotoxic and genotoxic effect in mammalian species in a number of studies conducted earlier (Bhunya and Pati, 1990; Husain *et al.*, 1996;

Ismail and Mohamed, 2012). Residues of deltamethrin and other pyrethroids have been found in human breast milk and human blood (Bouwman *et al*., 2006; Sharma *et al*., 2014). Deltamethrin is primarily metabolized in the liver through cytochrome P450's enzymatic hydrolysis and the oxidative pathway (Eraslan *et al.,* 2007). This pesticide is accumulated in larger concentration in the liver, lungs and kidneys due to its lipophilic property it has the potential to induce histological alterations in terms of hepatotoxicity, nephrotoxicity as well as pulmonary toxicity (Manna *et al*., 2005). Toxic effects of deltamethrin depend upon a number of factors such as dose, time and duration of exposure (Sharma *et al*., 2013). As this pesticide gets absorbed readily through the oral route, therefore, contaminated food and water are the main

sources of human exposure (Barlow *et al*., 2001). Taking into consideration the potential toxic effects of pyrethroids including deltamethrin, the present study was undertaken to observe the genotoxic potential of deltamethrin in mice exposed to low dose so as to correlate the possible toxic effects in humans. Ninety adult *Swiss Albino* male mice were used in the study**.** The mice

were provided feed and drinking water *ad libitum*. The pesticide (dissolved in groundnut oil) was administered through oral route.

MATERIALS AND METHODS

The genotoxicity testing in the present study was done by two assays. Firstly, *in vivo* alkaline comet assay was conducted to detect direct DNA damage in peripheral blood of mice by the method of Singh *et al.* (1988) with some needful modifications. Secondly, micronuclei assay in mouse bone marrow cells was conducted to detect chromosomal damage associated with the exposure to deltamethrin. Technical grade Deltamethrin with 98.50% purity was obtained from Kilpest India Limited, Bhopal, India. Oral LD50 for deltamethrin in mice is reported to be 30-50 mg/ kg body weight (EMEA, 2001). Ninety *Swiss Albino* male mice were used in the study (Table 1). Animals were kept under controlled conditions of temperature (22 ± 1°C) and humidity (60 ± 5%). They were provided with *ad libitum* pelleted feed (Ashirwad industries, Chandigarh, India) and drinking water. A twelve-hour day and night cycle was maintained. Animal were administered daily dose of deltamethrin (Table 1), dissolved in groundnut oil through oral route. The experimental protocol met the national guidelines on the proper care and use of animals in the laboratory research. The Institutional Animal Ethics Committee (IAEC) approved the experimental protocol. The doses used in the study were in the multiples of maximum residual limit (MRL) values of deltamethrin found in various food products i.e. 0.5 mg/kg for chicken fat, 0.3 mg/kg for chicken muscle (CCRVDF 2019). Thus the three doses used in the study were 0.5, 1.0 and 1.5 mg/ kg bwt/day. The highest dose of deltamethrin was 1/40th of the LD_{50} of the pesticides. Mice were sacrificed after 24 hr of completion of trial period (15/ 30/ 60 days) under ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) anaesthesia, intraperitoneally. Blood samples were collected directly from the heart in heparinised

vials. The bone marrow was collected by gentle flush and aspiration of femurs with fetal calf serum (FCS) and centrifuged at 100 rpm for 5 minutes. The supernatant was removed and sediment was suspended in adequate amount of phosphate buffer saline (PBS, pH 7.4) and stored for further examination. Samples were processed the same day for comet assay as well as for micronuclei induction test. Comet assay/ single cell gel electrophoresis (SCGE) assay was carried out in mice blood for studying the DNA damage. Reagents such as phosphate buffer saline (PBS), lysing solution, electrophoresis buffer, neutralization buffer, staining solution, low melting point agarose (0.5% and 1% in PBS) and normal melting agarose (1% in milli Q water) were required for the process. Conventional one end frosted slides were dipped in methanol and burnt over blue flame to clean. One percent normal melting agarose (NMA) was melted and slides were dipped in hot NMA up to two third the frosted area and removed gently, underside was wiped off with lint free tissue paper to remove agarose. Slides were laid on flat surface to dry and were stored at room temperature in dust free and dry environment. These base slides were prepared one day before use. One percent low melting point agarose (LMPA) was melted in microwave and placed in water bath to maintain a steady temperature of 37°C. Hundred microlitres of 1% LMPA was mixed gently with 10 µl of whole blood and 80 µl of suspension was layered on the base slide in two replicates and cover slips were placed on it. These slides were put on ice pack until agarose gets solidified (5-10 minutes). LMPA (0.5%) was maintained at 37°C. Once agarose got solidified, cover slips were taken off and 80 µl of 0.5% LMPA was added to the slides. Cover slips were replaced and the slides were again put on ice pack for solidification of agarose. Later, cover slips were removed and slides were lowered into an opaque coupling jar containing cool, freshly made lysing solution to avoid exposure to light. Slides were kept in lysing solution for minimum two hours and/or overnight at refrigeration temperature. After refrigeration, slides were gently removed from lysing solution and placed side by side in gel box with agarose ends towards anode and frosted ends towards cathode. Electrophoresis gel apparatus was filled with freshly prepared cold electrophoresis buffer until the liquid level completely covered the slides. Slides were allowed to be in alkaline buffer for 20 minutes to allow unwinding of DNA. After unwinding, electrophoresis was carried out by supplying power of 24 volts and current of 300 milli

amperes, adjusted by raising or lowering the buffer level, for 30 minutes for optimum migration. Slides were gently lifted and placed on dry tray, flooded with neutralization buffer for five minutes. The washing procedure was repeated twice. Slides were stained with 75µl of 1X ethidium bromide and left for 5 minutes. Slides were then dipped in chilled distilled water to remove excess stain. After placing cover slips, slides were scored immediately by fluorescent microscope. Qualitative extent of DNA damage in the cells was evaluated by visual scoring method by observing extent of DNA migration (tail length) and percentage of migrated DNA. Hundred randomly selected cells were analyzed per sample. Quantitative analysis was done by using Open Comet software coupled with J Image interface. Micronuclei assay was carried out in mice bone marrow cells by the method of Chauhan *et al.*, (2000). Reagents required for micronuclei induction test were: Fetal Calf Serum (FCS), Giemsa stain and May-Gruenwald stain. Mice (positive control) were sacrificed 24 hr after administration of cyclophoshphamide @ 40 mg/kg i/p as one time administration.

Table 1: Experimental Design

Pesticides	Groups	Doses	Number of mice		
			15 days	30 days	60 days
Negative Control	I	Groundnut oil	6	6	6
Positive Control	II	Cyclophosphamide	6	6	6
Deltamethrin	III	(1.5 mg/kg)	6	6	6
	IV	(1.0 mg/kg)	6	6	6
	V	(0.5 mg/kg)	6	6	6
		Total	**30**	**30**	**30**

The air dried bone marrow smears were first stained with undiluted May-Grunwald 1:1 (v/v in distilled water) for 2 minutes. Slides were rinsed in distilled water followed by staining with working Giemsa solution for 10 minutes. Slides were rinsed in distilled water, blot dried and cleared in xylene for 5 minutes and then embedded in DPX for scoring. Scoring of slides was accomplished using compound microscope at 1000X magnification and presence of micronuclei was determined by scoring 2000 number each of polychromatic erythrocytes (PCEs) and normochromatic erythrocytes per animal.

RESULTS AND DISCUSSION

Comet assay

In order to assess the DNA damage qualitatively, the visualization of agarose embedded blood cells was done following staining with the ethidium bromide using fluorescent microscope at 200X magnification. The pesticide exposure to mice for different duration affects the appearance and/or shape of comet, which is indicative of DNA damage (Fig. 1, A-E). In this study, head DNA percentage, tail DNA percentage, mean tail length and mean olive moment were the four parameters considered to assess the quantitative DNA damage. There is a positive correlation between the percentage of DNA damage to the percentage of tail DNA and therefore negatively correlated to the percentage of head DNA. The comet tail is made up of relaxed DNA loops and the number of loops in the tail indicates the number of DNA breaks. In this study, it was observed that, with increasing amount of damage, the tail length increased and is determined primarily by the length of the loops. Table 2 shows quantitative analysis of DNA damage in mice on exposure to deltamethrin through oral route.
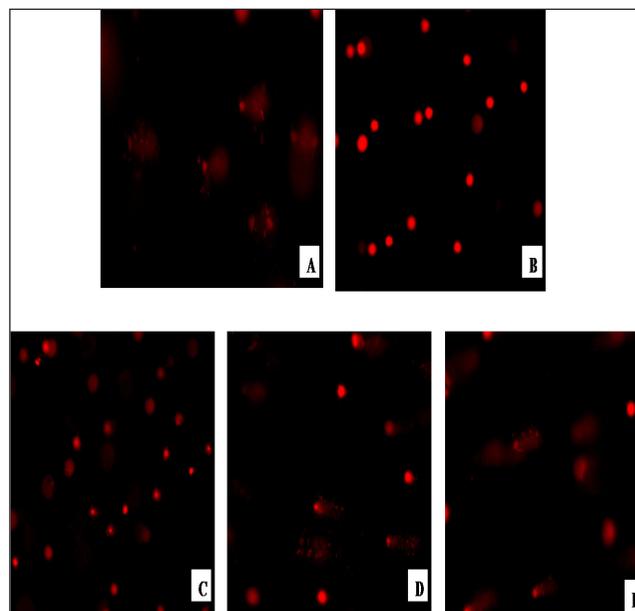
Fig. 1: Qualitative analysis of DNA damage through fluorescent microscopy. Control negative (A), control positive (B), 15 days exposure to deltamethrin (C), 30 days exposure to deltamethrin (D) and 60 days exposure to deltamethrin (E)

Table 2: Quantitative analysis of DNA damage in mice on oral exposure to deltamethrin

Days	Parameters	I	II	III	IV	V
				Groups		
15	Head DNA %	72.61±5.44	37.07±9.01	*40.72±6.48	*43.11±4.04	*50.21±5.39
	Tail DNA %	27.38±5.44	62.93±9.01	*59.28±6.48	*56.89±4.04	*49.78 ±5.39
	Tail length	18.38±3.94	65.2±15.17	17.65±2.30	*,a20.64±2.43	a17.82±2.25
	Olive moment	7.71±1.75	31.34±7.18	8.79±1.23	10.07±1.15	7.58±1.06
30	Head DNA %	82.48±3.34	37.07±9.01	*42.26±7.67	*49.99±5.79	*52.06±5.46
	Tail DNA %	17.52±3.34	62.93±9.01	*57.74±7.67	*50.01±5.79	*47.94±5.46
	Tail length	19.72±2.88	65.2±15.17	*,b82.77±14.48	b,c12.95±3.98	*,c77.66±13.53
	Olive moment	5.32±1.38	31.34±7.18	*,d37.44±7.60	d14.07±1.97	*28.19±5.03
60	Head DNA %	73.33±5.89	37.07±9.01	*,e36.86±3.21	*41.33±2.43	*,e49.09±2.63
	Tail DNA %	26.66±5.89	62.93±9.01	*63.14±3.21	*58.66±2.43	*50.91±2.63
	Tail length	22.38±5.02	65.2±15.17	21.20±2.34	16.70±1.12	15.29±0.86
	Olive moment	9.72±2.98	31.34±7.18	11.01±1.29	8.50±0.57	7.49±0.53

I= Negative/vehicle control, II= Positive control, III= Dose 1, IV= Dose 2, V= Dose 3

Results are expressed in mean ± standard error; Superscript* indicates significant differences with control; Results with common superscripts [a,b,c,d,e] indicate that groups are significantly different from each other.

On 15 days exposure to deltamethrin, significant DNA damage was observed in treatment group as compared to the control group in terms of olive moment and tail length. The olive moment is the distance between centroids of the head and the tail of the comet and with increasing damage, mean olive length also keeps on increasing. Significant difference was observed in mean head DNA percentage between control and treatment groups (P<0.05).

Mean tail DNA percentage was also significantly different in control group (Group I and II) and treatment groups at all the three selected doses (Group III, IV and V). The mean tail DNA percentage was significantly higher in the treatment group than the control (Fig. 2A). Differences in mean tail length were also found significant between control and treatment group IV. Group IV and group V also differ from each other significantly with respect to mean tail length. However, in case of mean olive moment, no significant differences were found on comparison amongst control and treatment group. On exposure to deltamethrin for 30 days, mean head DNA percentage as well as mean tail DNA percentage were found to differ significantly between control and treatment groups at all the three doses (Fig. 2B). Mean tail length in group III and V was found significantly higher than the control group. Group IV was also found to be significantly different from group III and

V in a dose dependent manner. Differences were also found significant for mean olive moment in case of group III and V in comparison to the control group (I). Similarly, on 60 days exposure to deltamethrin, all the treatment groups were found to be significantly different from control groups in terms of mean head DNA percentage as well as tail DNA percentage (Fig. 2C). Group III and V were also differing significantly from each other in terms of mean head DNA percentage. Overall, DNA damage was found to be directly proportional to the dose of deltamethrin exposure and the duration of exposure as well.

Micronuclei induction test

A micronucleus is a small structure (1/5 to 1/20 the size of the nucleus) containing nuclear DNA that has arisen from chromosome fragments or whole chromosomes that were not incorporated into daughter nuclei at anaphase of mitosis. It can be found in any tissue but its formation occurs only in the dividing cells. Therefore, in the present study, the mouse bone marrow cells were analyzed for the presence of micronuclei. In the present study, the frequency of micronucleated normochromatic erythrocytes (MN-NCE), micronucleated polychromatic erythrocytes (MN-PCE) and percentage of PCE were the endpoints used for statistical evaluation of genotoxicity

(A)

(B)

(C)
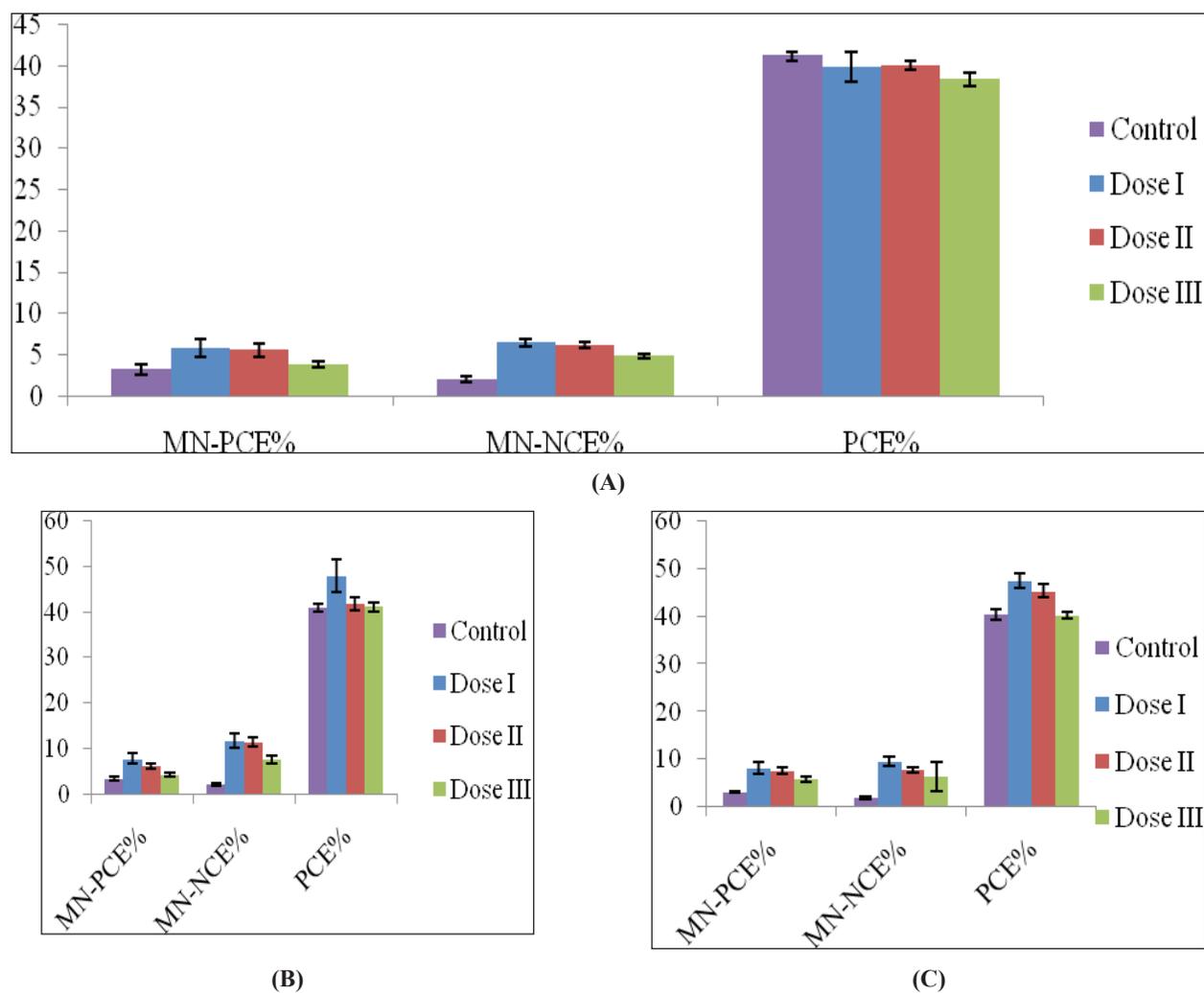
Fig. 2: Comparison of micronuclei induction (MI) test parameters on exposure to deltamethrin for 15 days (A), 30 days (B) and 60 days (C)

by micronuclei induction (MI) test. It was observed that oral exposure of mice to deltamethrin slightly induced micronuclei in bone marrow cells at 15 days of exposure in both PCE as well as NCE in all treatment groups (Table 3, Fig. 3A). Difference, however, was significant only for

MN-NCE, which were dose dependent in all the treatment groups. Deltamethrin did not alter PCE % in any of the treatment group, significantly. At 30 days of exposure to deltamethrin, group III showed significant induction of MN-PCE when compared to group I and IV. Induction of MN-NCE was also found to be significantly high in all the treatment groups in a dose dependent manner (Fig. 3B).

At 30 days of exposure also, deltamethrin did not induce any significant alteration in PCE% in any of the treatment group. At 60 days of exposure, a significant increase was observed in all the treatment groups with respect to mean MN-NCE, MN-PCE and PCE% as compared to control group and this difference was dose dependent (Fig. 3C).

Thus, in this study, on analysis of DNA damage through comet assay, it was observed that the parameters of DNA damage *viz*; mean tail DNA percentage, mean head DNA percentage, mean tail length and mean olive moment were found to be higher in the treatment groups as compared to the control groups at all the dose levels and at all the
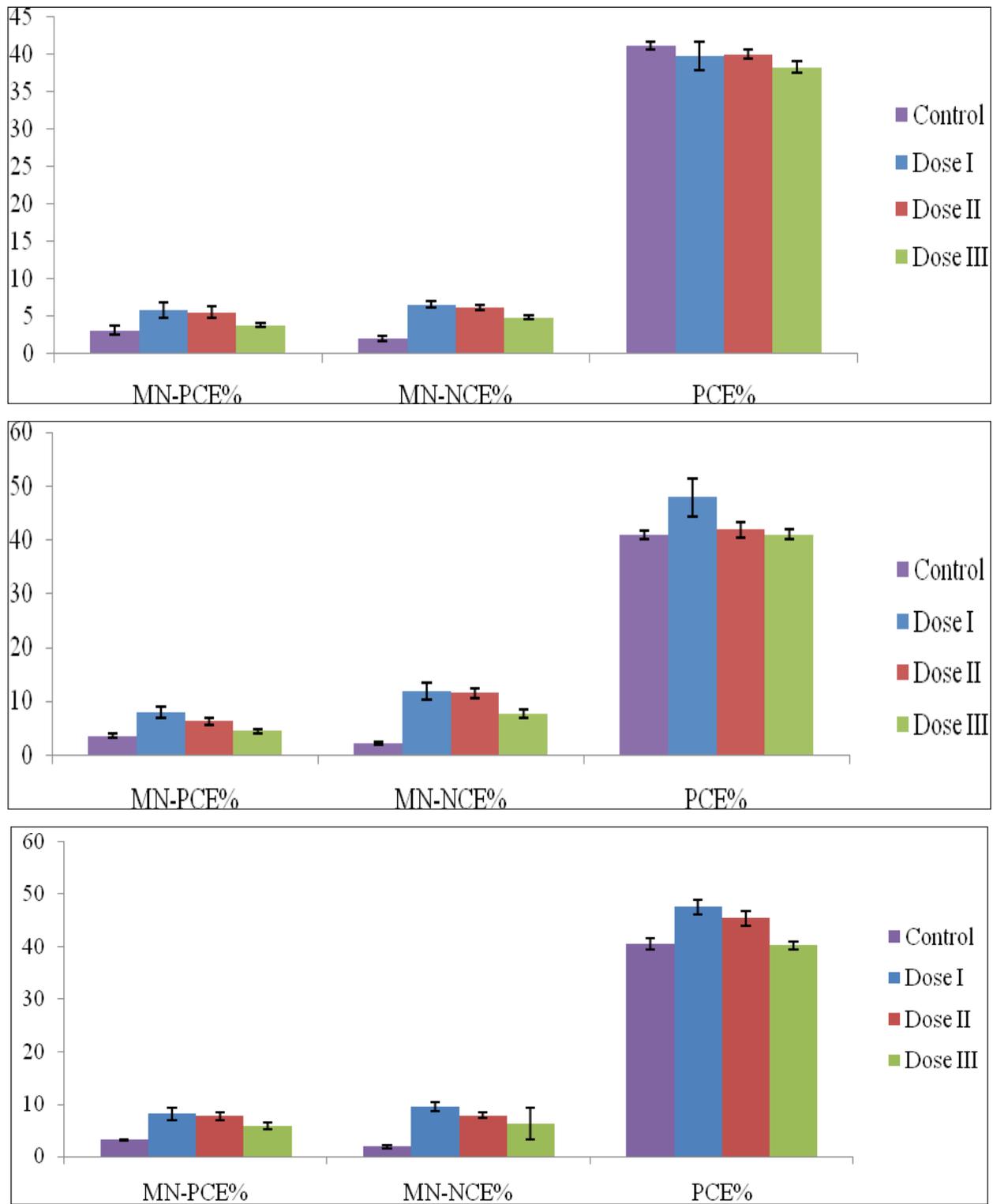
Fig. 3: Comparison of micronuclei induction (MI) test parameters on exposure to deltamethrin for 15 days (A), 30 days (B) and 60 days (C)

duration of exposure to deltamethrin suggesting the ability of this pesticide to cause genotoxicity (Fig. 4, A-C).

A dose dependent effect of deltamethrin exposure has been observed in mice blood cells in terms of DNA damage at all the durations of exposure. DNA damage at various treatment doses (1.5 mg/kg b.wt., 1 mg/kg b.wt. and 0.5 mg/kg b.wt) and duration (15, 30 and 60 days) is further supported by micronuclei induction at the respective doses and durations and doses of exposure. Genotoxicity of deltamethrin in the present study is supported by a study conducted earlier by Issam *et al*. (2012) which also

indicated similar findings at 30, 45 and 60 days of exposure of rats to deltamethrin at the dose rate of 0.3mg/kg bwt. Similar results were observed in a study conducted by Ismail and Mohamed, (2012) where deltamethrin (at dose rate of 5mg/kg bwt) was reported to induce significant genotoxicity and testicular injury in rats on oral exposure for 30 days as assessed by comet assay and chromosomal aberrations along with increase in testicular oxygen free radicals, enzymatic activity of poly (ADP-ribose) polymerase (PARP) and lactate dehydrogenase (LDH). Thus the findings in the present study are in agreement
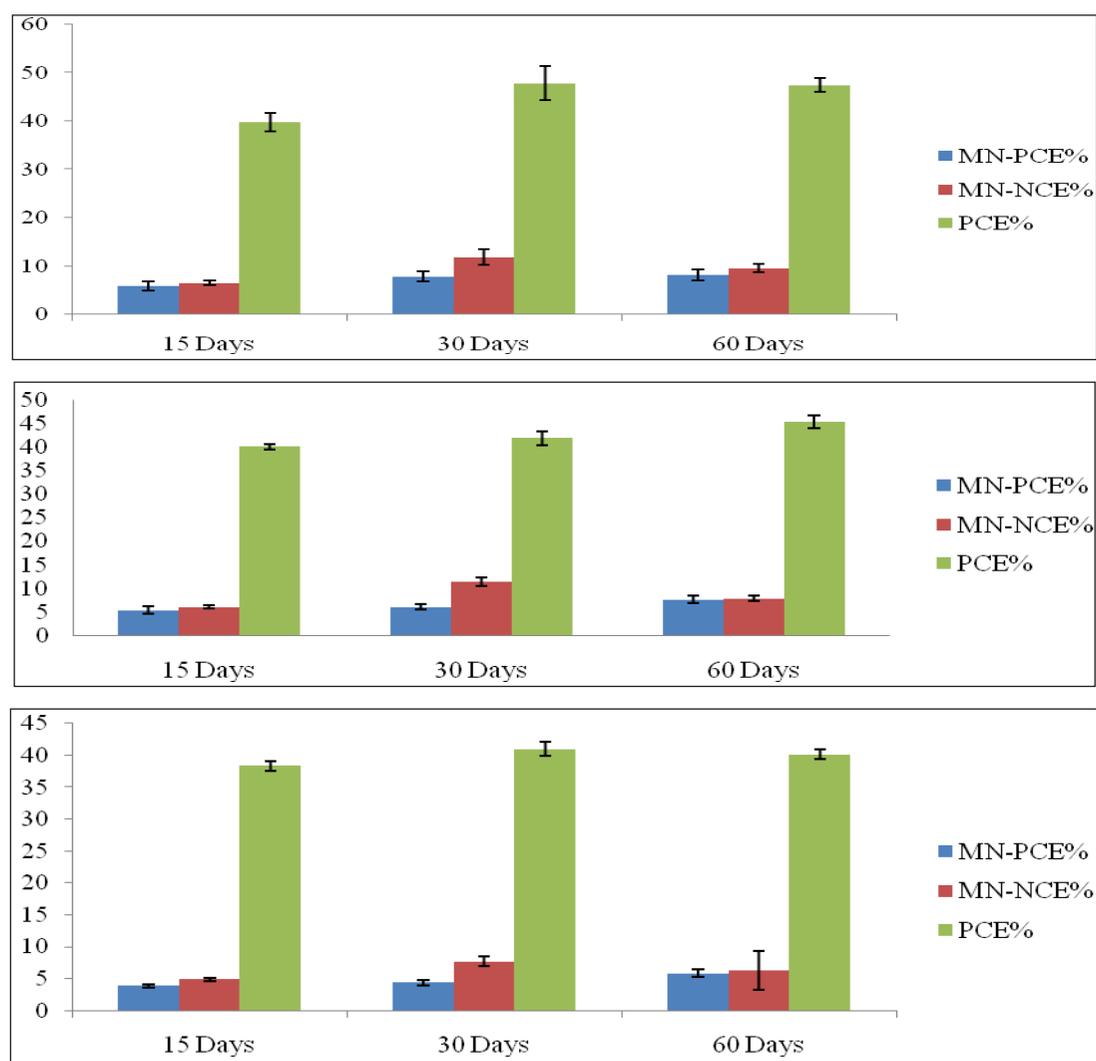
Fig. 4: Comparison of various micronuclei induction (MI) test parameters on exposure to 1.5 mg/kg bwt (A), 1 mg/kg bwt (B) and 0.5 mg/kg bwt (C) of deltamethrin for different periods.

Table 3: Induction of micronuclei in mouse bone marrow cells following deltamethrin exposure

Days	Parameters	Groups				
		I	II	III	IV	V
15	MN-PCE%	3.17 ± 0.60	4.83 ± 0.48	5.83 ± 1.01	5.50 ± 0.81	3.83 ± 0.31
	MN-NCE%	2.00 ± 0.36	2.50± 0.22	*,a6.50 ± 0.43	*6.17 ± 0.31	*,a4.83 ± 0.31
	PCE%	41.17 ± 0.54	48.17 ± 0.79	39.83 ± 1.89	40.00 ± 0.58	38.33 ± 0.80
30	MN-PCE%	3.50 ± 0.34	4.83 ± 0.48	*b7.83 ± 1.11	6.17 ± 0.54	b4.33 ± 0.42
	MN-NCE%	2.16 ± 0.31	2.50 ± 0.22	*,c11.83 ± 1.66	*11.50 ± 0.92	*,c7.67 ± 0.76
	PCE%	40.83 ± 0.83	48.17 ± 0.79	47.83 ± 3.55	41.83 ± 1.40	41.00 ± 1.03
60	MN-PCE%	3.17 ± 0.17	4.83 ± 0.48	*8.17 ± 1.19	*7.67 ± 0.71	5.83 ± 0.60
	MN-NCE%	1.83 ± 0.31	2.50 ± 0.22	9.50 ± 0.88	*7.83 ± 0.54	6.33 ± 3.04
	PCE%	40.50 ± 1.15	48.17 ± 0.79	*,d47.50 ± 1.43	*,e45.33 ± 1.38	d,e40.17 ± 0.70

I= Negative/vehicle control, II= Positive control, III= Dose 1, IV= Dose 2, V= Dose 3

Results are expressed in mean ± standard error; PCE = polychromatic erythrocytes, NCE = normochromatic erythrocytes, MN= micronuclei; Superscript* indicates significant differences with control; Results with common superscripts viz.; a,b,c,d,e indicate that groups are significantly different from each other.

with the findings of the earlier studies with the difference that the dose in present study was 1/10 of the dose used in the earlier study and also even at 15 days of exposure, the lowest dose of deltamethrin has resulted into significant genotoxicity. Extent of DNA damage in an *in-vivo* or *in-vitro* study depends on test methods, live material, route of administration, dose of compound and duration of exposure and many studies have been conducted in the past to find out genotoxicity of deltamethrin and it is claimed that it may cause DNA damage depending on test methods, live material, compound delivery method and dose of the compound (Katsuda, 1999). Deltamethrin, like many other chemicals, execute cellular damage primarily by damaging the cellular membrane as well as lysosomes thereby leading to release of lysosomal and other DNAases into the cytoplasm. These DNAses cause double strand breaks in DNA, ultimately leading to mutation and chromosomal aberrations (Bradley, 1985).

In another study conducted by Bhunya and Pati, (1990) exposure of deltamethrin in mice at dose rate of 10, 15 and 20 mg/kg bwt for 24 hr, 30 hr and 35 days demonstrated a significant increase in chromosomal aberration and micronuclei induction in bone marrow in addition to sperm abnormalities. The lowest dose of deltamethrin used in present study was 1/20 of the lowest dose used in the earlier reported studies and it was observed that even the lowest dose of deltamethrin was able to induce genotoxicity in the present study. Thus the present work expresses the

genotoxic impacts of deltamethrin and commemorate earlier findings. Micronuclei in polychromatic erythrocytes arise due to damage that occurred recently (within past 48 hours) and the presence of micronuclei in the normochromatic erythrocytes exhibits that the damage accumulated over the past months of exposure among the NCE population being in steady state equilibrium in the peripheral blood. Thus for long-term studies, MN-NCE is a good indicator of the damage over MN-PCE. In this study, the comparatively higher number of micronuclei has been found in NCE than PCE indicating that the damage occurred due to historical exposure and thus suggesting the possible role of pesticides in causing genotoxicity following long term exposure. Some studies conducted in the past have shown low dose and long duration clastogenic potential of pyrethroids other than deltamethrin. In a study conducted earlier by Sharma *et al*. (2010), it was observed that oral exposure of λ-cyhalothrin to rats for 30 days had lead to chromosomal damage in terms of chromosomal gaps, chromosomal breaks, chromatid gaps and chromatid fragments, suggesting clastogenic potential of the pyrethroids. Likewise, Ayse and Mehmet, (2009) studied genotoxic potential of commercial formulation of alpha-cypermethrin on human peripheral lymphocytes in terms of sister chromatid exchange (SCE), chromosomal aberrations (CA) and micronuclei induction (MI). It was observed that commercial formulation of alpha-cypermethrin significantly induced SCEs, CAs and MNs

and decreased the proliferation index and reduced both the mitotic index and nuclear division index. Similarly, DNA strand break and increase in tail length was assessed by comet assay after low dose exposure of alpha-cypermethrin in a study by Suman *et al*. (2006). Therefore, results of the present study in terms of micronuclei induction on low dose exposure to deltamethrin for longer duration are in agreement with the studies conducted in the past on exposure to other pyrethroids. Thus, summarily, low dose oral exposure to deltamethrin induced genotoxicity in mice in terms of direct DNA damage and chromosomal abnormalities is evidenced by the comet assay and micronuclei induction tests. The effects were dependent on the dose and duration of exposure of each pesticide.

CONCLUSION

From the finding of the present study, it can be concluded that deltamethrin has toxicity potential to cause alteration in the DNA and can induce micronuclei, even at lower doses. The exposure of these pyrethroids in the body throughout the lifetime can cause permanent alterations in the vital organs of the body. Therefore it is imperative to study the toxic potential of deltamethrin at the levels to which human beings are usually exposed.

DECLARATION OF INTEREST

Authors declare that they have no competing interest associated with this study.

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