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Lack of genotoxic potential of pesticides, spinosad, imidacloprid and neem oil in mice (*Mus musculus*)

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Abstract

Pesticides, spinosad, imidacloprid and neem oil are widely used both in residential and agricultural environments because of its broad spectrum insecticidal activity and effectiveness. The present study was undertaken to estimate genotoxicity of formulations of some pesticides in mice. Three pesticides of diverse group studied were spinosad (45% w/v), imidacloprid (17.8%, w/v) and neem oil. Animals were exposed 37,4.5 and 50 mg kg^{-1} b.wt. for spinosad, imidacloprid and neem oil, respectively, through oral gavage for 5 consecutive days. A vehicle control group and one positive control (cyclophosphamide; 20 mg kg^{-1}b. wt.) were also selected. The results showed that cyclophosphamide produced 1.12% micronuclei in mice, as against 0.18 in vehicle control, 0.30 in spinosad, 0.28 in imidacloprid and 0.22% in neem oil, respectively. The gross percentage of chromosomal aberration in mice were 28.5% in cyclophosphamide against 6.5% in vehicle control, 8.0% in spinosad, 9.5% in imidacloprid and 7.0% in neem oil, respectively. The overall findings of the present study revealed that all the three pesticide formulations, imidacloprid, spinosad and neem oil at tested dose did not show any genotoxic effect in mice.

Key words

Chromosomal aberration, Genotoxicity, Imidacloprid, Mice, Neemoil, Spinosad

Introduction

Pesticides are widely used to control insects/pests of agriculture to control crop loss and to improve yield and quality (Oerke and Dehne, 2004). Pesticides are biologically active compounds with a component specific to inherent toxicity, and therefore pesticide poses serious concern to humans, animals and environment. Danalas and Eleftherohorions (2011) have reviewed the safety issues and risk assessment indicators of pesticide exposure. Pesticide exposure among human population has revealed varied information on genetic risk (Garaj-Vrhovac and Zeljezic, 2001; Antonucci and De Sylloscolus, 2000).

Imidacloprid, *N-[1-[(6-chloro-3pyridyl)methyl]-4,5-dihydroimidazole-2-yl] nitramide,* a neonicotinoid insecticides works by interfering with transmission of nerve impulses in insects by binding irreversibly to specific insect nicotinic acetylcholine receptor (Tomizawa *et al.*, 2005). Imidacloprid is used in crops, soil and fleas in pets (Tomlin, 2006). Imidacloprid continues to be of great concern to human health due to its continued use worldwide and documented occupational and environmental exposures (Wu *et al.,* 2001). It has been reported that imidacloprid is neither mutagenic nor teratogenic (Tomlin, 2006).

Spinosad a microbial insect control agent derived from *Saccharopolyspora spinosa, a* naturally occurring actinomycete bacteria is composed of spinosad A and D, the two main active components. It is considered as selective insecticide with a novel mode of action involving nicotinic acetylcholine receptor, GABA receptor (Salgado, 1998).

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Azadirachtin is a major biologically active compound of neem, *Azadirachta indica*. Technically, azadirachtin and other related tetranortriterpenoids of neem seed kernel disrupts metamorphosis of insects (Tomlin, 2006). Raizada *et al.,* (2001) and Srivastava and Raizada (2001) reported low acute/subchronic, non-mutagenic/teratogenic effects and lack of postnatal developmental effects of azadirachtin in mammalian species. Neem oil is obtained from pressing of fruits or seeds of neem. Neem oil contain azadirachtin, steroids and many triterpenoids used for skin diseases, inflammation, fever, rheumatic disorders, insect repellent, malaria, tuberculosis and even diabetes (Boeke *et al.,* 2004).

In light of the above, the present study was carried out to investigate *in-vivo* chromosomal aberration and micronuclei in mice exposed to low doses of spinosad, imidacloprid and neem oil.

Materials and Methods

Test organism : Experiments were performed on healthy and mature (six to eight week old) Swiss female albino mice (*Mus muscullus*) weighing 30± 2 g, obtained from Life Science Intelligentsia, Animal house, Patna, India. Ethical approval for the experiment was obtained from Institutional Animal Ethical Committee. Animals were randomly selected and housed in polycarbonate boxes. Animals were segregated into various experimental groups with six animals and maintained in controlled atmosphere for 12 hrs dark/lightcycle, 23±3°C and 40-70% humidity. Animals were given commercial pellet feed (M/s. Ashirwad, Chandigarh, India) and water *ad libitum*.

Test substance and exposure level : Commercial formulation of spinosad (45%SC), imidacloprid (17.8%SL) and neem oil were purchased from local market of Patna, India. These compounds are soluble in water, hence diluted with distilled water to obtain desirable concentration of pesticides. One group of animal was maintained as vehicle control group and 2nd group as positive control (cyclophosphamide; 20mg kg^{-1} b.wt.) without any exposure to these pesticides. Animals of group 3, 4 and 5 were orally administered spinosad (37mg kg^{-1} b.wt.),imidacloprid (4.5 mg kg^{-1} b.wt.) and neem oil (50 mg kg^{-1} b.wt.), respectively.

Screening of chromosomal abnormalities : Animals were sacrificed 24 hr after last treatment (*i.e.*, on 6th day) and slides were prepared for detection of any abnormalities in chromosomes of their bone marrow cells by standard colchicines – acetoalchohol – flame drying – Gimsa staining technique (Preston *et al.,* 1987) with few modifications (Khan and Sinha, 1993). Mice from each group were given colchicine treatment at a dose of 4 mg kg^{-1} b.wt. 1.5 hrs prior sacrificing animals to arrest cells at metaphase stage. Briefly, bone marrow was flushed out from both femurs using Hanks buffered salt solution (pH 7.2). Cells were centrifuged at 1000 rpm for 5 min and pellets were re-dispersed in hypotonic solution of 0.56% (w/v) KCl for 30 min at 37º C to permit osmotic swelling of cells. Swollen cells were fixed in ice-cold Carnoy's fluid, dropped onto slides, and stained with phosphate buffer 5% Giemsa solution. A total of 75 well spread metaphase plates per animal in each group were analyzed for chromosomal aberrations at 100X magnification. Abnormalities in chromosomes were classified into structural and numerical types. Structural aberrations were classified as gap and breaks in chromatid(s), acentric fragments, rings, terminal association, centric fusion, deletion and multiple breaks while numerical aberrations included of polyploidy, hypoploidy, c-metaphase and precocious separations. The incidence of aberrant cells was expressed as percentage of damaged cells (aberrant metaphases).

Micronuclei induction assay : Suspension of bone marrow cells of mice were directly smeared on slide (Schmid, 1975; Das and Kar, 1980). Animals were sacrificed 24 hrs. after the last treatment, and bone marrow cells were collected from their femur in chilled hypotonic KCl solution using Hanks' buffered salt solution, 1% (w/v) bovine serum albumin and 0.15% (w/v) EDTA (pH 7.2). Cell suspension was centrifuged immediately and resuspended in the remaining fluid. Smear of bone marrow cells were made by slide-drawn method, air dried and then fixed in methanol. Slides were first stained in May-Grunewald's solution followed by Giemsa staining rinsed in running tap water and then air-dried again.

A minimum of 2000 (about 1000 PCEs and corresponding number of NCEs encountered in same optical field) erythrocytes were scored for each experimental group. Stained slides containing mature enucleated erythrocytes were screened for the presence of micronuclei (MN). Only non-retractile structures that resembled nuclei in colour and staining intensity and had diameter less than one third of the main nucleus were scored as micronuclei PCEs/NCEs ratio was also taken into consideration for each group to register any cytotoxic effect on the turnover of PCEs

Statistical analysis : Data of all the groups were expressed as mean frequency per 100 cells along with the standard error of percent mean (%± SE).Statistical comparisons were made using Students *t*-test, and $P < 0.05$ was considered significant.

Results and Discussion

Indiscriminate and reckless use of pesticides and presence of their residues in food, water and air beyond the acceptable limit has resulted in large scale exposure of human population to harmful concentrations. Among the various

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toxic effects of these agrochemicals, large scale abuse of genetic material is of particular concern, and hence, the potential genetic hazard of pesticides on man cannot be ignored. The analysis of the observations in the present study revealed that the administration of spinosad, imidacloprid and neem oil at low level did not produce any significant increase in frequency of chromosomal anomalies and micronuclei in mice. Frequencies of total anomalies in chromosomes were 8.0, 9.5, and 7.0% in spinosad, imidacloprid and neem oil treatment, respectively. Statistically these values when compared with control (6.5%) did not show any significant difference. However, if compared to the percent value of total anomalies (28%) in mice with cyclophosphamide (positive control), a significant ($p<0.001$) difference was noticed (Table 1). Absence of a statistical difference was also noted in PCE and NCE micronucleus in bone marrow of mice exposed to spinosad (0.30±0.13), imidacloprid (0.28±0.12) and neem oil (0.22±0.11) in comparison to control (0.18±0.10). However on comparison to cyclophosphamide (positive control) a significant ($p< 0.05$) difference was observed (Table 2).

Chromosomal aberration test of bone marrow cells *in vivo has* become a standard method for testing for the potential mutagenic effect of virus, drugs and chemical pollutants, whereas micronucleus assay is considered as surrogate for corresponding metaphase chromosome analysis (Fenech, 2002a,b). The results obtained through metaphase chromosome analysis can therefore be confirmed by means of micronucleus assay (Khan and Sinha, 1994).The result of the present study, based on chromosomal aberration test in bone marrow cells and micronucleus induction in erythrocytes in mice, exhibited non-significant genotoxic potential at low level of imidacloprid, spinosad and neemoil.

It is interesting to note that fansidar, an antifolic agent, produces moderate clastogenic effects (Praveen *et al.,* 2011) in human lymphocyte chromosomes *in vitro*, whereas fludrocorticosone does not have any toxic effect on human lymphocyte chrosomes at wide range of concentration (Shadab *et al.,* 2006). Similarly, induction of chromosomal aberration and micronucleated erythrocytes due to high exposure level of imidacloprid (50 and 100mg kg^{-1} b.wt. to

Table 1 : Frequencies of structural and numerical aberrations (numbers and %) in bone marrow of mice exposed to spinosad, imidacloprid and neem oil

Experimental group	Structural aberration		Numerical aberration		Gross aberration	
	No.	% ± SE	No.	% ± SE	No.	% ± SE
Vehicle control	09	4.5±1.46	04	2.0±0.98	13	6.5±1.74
Positive control cyclophosphamide (20 mg kg^{-1} b.wt., ip)	37	18.5*±2.74	20	10.0*±2.12	57	28.5*±3.19
Spinosad (37 mg kg^{-1} b.wt.)	11	5.5±1.61	05	2.5±1.10	16	8.0±1.91
Imidachoprid (4.5 mg kg^{-1} b.wt.)	13	6.5±1.74	06	3.0±1.20	19	9.5±2.07
Neem oil (50 mg kg^{-1} b.wt.)	10	5.0±1.54	04	2.0±0.98	14	7.0±1.80

Values are mean ± SE; * significance at $p<0.001$ when compared to control and treatment groups

Table 2 : Frequency of micronucleus (MN) induction in polychromatic (PCEs) and normochromatic erythrocytes (NCEs) of mice upon spinosad, imidacloprid and neem oil treatment

Experimental group	Total no. of PCE scored	PCEs with MN		Total no. of NCEs scored	NCEs with MN		PCEs + NCEs scored	PCE+NCE with MN		PCE : NCE ratio
		No.	%±SE		No.	%±SE		No.	%± SE	
Vehicle control	908	02	0.22 ±0.15	792	01	0.10 ±0.11	1700	03	0.18 ±0.10	1.20
Positive control cyclophosphamide (20mg kg^{-1} b.wt., ip)	836	11	1.31* ±0.39	868	08	0.92* ±0.32	1704	19	1.12* ±0.25	0.96
Spinosad (37 mg kg^{-1} b.wt.)	896	03	0.33 ±0.19	765	02	0.26 ± 0.18	1661	05	0.30 ± 0.13	1.17
Imidachloprid(4.5 mg kg^{-1} b.wt.)	903	03	0.32 ±0.18	726	02	0.24 ± 0.17	1739	05	0.28 ± 0.12	1.10
Neem oil (50 mg kg^{-1} b.wt.)	939	02	0.21 ±0.15	863	02	0.23 ± 0.16	1802	04	0.22 ± 0.11	1.08

Values are mean ±SE; * significance at $p<0.05$ when compared to control and treatment groups

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rats for 90 days) were noted in Wistar albino rats indicating its potential for clastogenicity (Karabay and Oguz 2005).The non-significant effect of low exposure of imidacloprid in the present study on mice could be due to low toxicity to vertebrates and low binding affinity to nAch receptors (Huang *et al.,* 1999).

Spinosad has shown no increase in chromosomal aberration in Chinese hamster ovary cell, no increase in micronuclei frequency in bone marrow cells in mouse and negative evidence for mouse forward mutation with or without metabolic activation (USEPA, 1998). It was further suggested that cytogenetic activity of spinosad may refer to its chemical structure and/or certain impurities in the commercial formulation; a matter, which needs further elucidation. Mutagenic chemicals have potential to damage germ line leading to fertility problems and establishing defects due to mutation in future generation (Kumar *et al.,* 2013). Present findings are in agreement with the results reported by Akmoutsou *et al.* (2011) and Gowri *et al.* (2013) who found that spinosad showed either no genotoxic activity or mild genotoxicity at high dose level.

It is well established that neem (*Azadirachta indica*) exhibit antibacterial, antifungal, anti-inflammatory and pesticidal toxicity due to presence of triterpenoids, saponin, glycosides flavonoids and tannin alkaloids. The ethanol extract of neem leaves has been reported to cause chromosomal abnormalities for e.g., strand breakage and spindle disturbances, thereby inducing genotoxic effects in mice (Awasthi *et al.,* 1999). However, there are reports that to suggest that neem products have significant modulating (Subapriya *et al.,* 2005), anti-mutagenic (Vinod *et al.,* 2011) and anti-genotoxic effect (Alabi *et al.,* 2011). The protective effect of neem extract can be ascribed to the antioxidant property (Schaaf *et al.,* 2000; Farah *et al.,* 2006) that might be one of the cause of non-genotoxicity of neem oil in the present study also. As compared to vehicle control animals, number of chromosomal aberrations and micronucleated erythrocytes in neem oil treated animals showed similar results, indicating non-genotoxic activity of neem oil in mice.

Administration of low dose formulations of imidacloprid, spinosad and neem oil over limited period could be one of the reasons for the absence of chromosomal aberration and presence of micronuclei in the present study. The present study further suggests necessity of evaluating combnination of pesticides.

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