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In vitro Cytotoxicity and Genotoxicity Assessments of Carbofuran and Malathion Pesticides on Cat (*Felis catus*) Fibroblast Cells

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Pesticides constitute a different class of chemicals, basically designed for the protection of agricultural crops by controlling a variety of insects, pests, weeds harmful for the agricultural plants. Pesticides are considered as potential chemical mutagens. Experimental data have shown that various agrochemicals have lethal effects, including mutational properties such as chromosomal changes, DNA damage, micronuclei formation or cytotoxicity in the cellular level. This study was designed to examine the cytotoxic and genotoxic effects of carbofuran and malathion pesticides on cat (*Felis catus*) fibroblast cells. The deterrent effects were assessed based on cell viability, chromosomal changes and DNA damage on fibroblast cells in approx. 1×10^5 cells. The cells were exposed to 0.045 mM - 1.08 mM of carbofuran for 24 h, and 5 mM – 45 mM of malathion for 48 h. The effects were evaluated in terms of DNA damage as changes in comet tail length, comet scores, chromosomal aberrations and micronucleus formation. Lethal doses were determined by using MTT assay whereas crystal violet staining was carried out to assess cytotoxic effects. The LC_{50} concentrations of carbofuran and malathion were estimated to be 0.42 mM and 20 mM, respectively. The maximum DNA damage was attained at 1.08 mM of carbofuran and 45 mM of malathion. The genotoxic and cytotoxic effects of the two chemically different pesticides in *F. catus* fibroblast cells was evident. While carbofuran was more toxic in terms of lethal doses and exposure time, malathion seemingly promoted genomic instability, albeit at unusually high concentrations.

Keywords: Carbofuran; Malathion; Genotoxicity; Cytotoxicity; *Felis catus* fibroblast cell line.

Pesticides are the chemical compounds that are used to kill pests, including insects, rodents, fungi and weeds[1]. The active ingredients of these compounds are mainly organophosphorus (OP), carbamates, chlorinated hydrocarbons, and carbamide derivatives[2]. Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) and malathion(diethyl, 2-[(dimethoxyphosphinothioyl] butanedioate) have short half-life in the environment[3,4] and these anthropogenic compounds bring about substantial hazard to the public, environment and animals[5-7]. Carbofuran and malathion poisoning shows various symptoms such as, dizziness, blurred vision, excessive perspiration, salivation, vomiting, diarrhea, aching muscles and a general feeling of severe malaise[8,9].

Organophosphorus and carbamate toxicants share a common mode of action, i.e., both inhibit acetylcholinesterase and butyrylcholinesterase *in vitro* and *in vivo*, and consequently prevent acetylcholine hydrolysis[10,11]. Accumulation of acetylcholine impairs the nervous system and respiratory muscles, and increase the nerve impulse transmission to cause respiratory paralysis[12]. Carbamate is one of the most toxic pesticides and known to exert high toxicity to mammalian systems, and also produce an adverse health problem for humans, animals and wildlife[3]. Malathion, on the other hand, is a commonly applied agrochemical formulation which produces low to moderate toxicity[13]. The epidemiological studies provide evidence that long-term exposure leads to neurological and cardiac dysfunction, retinal degeneration and gastrointestinal problems[5,14].

Chronic and acute exposure of OP compounds can be differentiated by determining their concentrations in blood and urine. Concentrations in a range 10-100 µg/ml in human tissue reflect their acute, accidental or intentional exposure, whereas lower concentrations (0.01-1 µg/ml) represent the chronic environmental exposure[15]. There are also evidences suggesting that upon exposure to profennofos, endosulfan and chlorpyrifos pesticides, the isolated human lymphocytes display extensive chromosomal aberration and DNA damage[16]. Moreover, another study using carbofuran revealed that in human blood cells, the extent of DNA damage reflected in comet assay was directly dependent on the concentration of the test OP pesticide[17]. Carbofuran also induced micronuclei formation in Chinese hamster ovary (CHO_{K1}) cells, an attribute demonstrating genotoxicity[18]. Overall, the effects of pesticides have beenmostly examined on human, and rat lymphocytes and human liver carcinoma (HepG2) cell lines[15-19].

Reactive seizures have been detected as a common neurological disorder in cats intoxicated with OP insecticides[20]. These insecticides are used in pets for treating tick- and lice-infestation of skin. For chronic level intoxication and early diagnosis, genotoxic assays are better option compared to acertylcholiesterase-inhibition assays, which is used in acute cases. To best of our understanding, the genotoxic and cytotoxic assessment of insecticides has not been carried out in a feline model cell lines. Moreover, skin fibroblast cells, the primary target of pesticide exposure, is rarely tested for toxicological analyses.

The present study reports on the deterrent effect of carbofuran and malathion pesticides onviability of cat (*F. catus*) fibroblast cells. Further, the effects were also examined on chromosomal aberrations and DNA damage using comet assays.

MATERIAL AND METHODS

Chemicals

The following chemicals were purchased at their purest grades: carbofuran (Sigma 426008), malathion (Sigma 36143), fetal bovine serum (Hyclonesh30070.70), Dulbecco's Modified Eagle Medium (DMEM; Sigma D 6170), Dulbecco's Phosphate Buffer Saline (DPBS; Sigma D 4031), Typsin-EDTA (Sigma T 4049), L-glutamine (Sigma 8540), gentamicin sulphate (Dsuchefa Biochemical k 0124),giemsa stain solution (Himedia TCL 083), colchicine (Sigma C3915), dimethylsulfoxide (DMSO; Merck C6164), ethidium bromide (EtBr) solution (Promega), cyclophosphamide (Merck BP094), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma 88417), Tris base, Triton-X 100, normal melting agarose (Lonza 50004) and low melting agarose (Himedia RM861).

Establishment of a fibroblast cell line from cat tissue and stock preparation of pesticides

F.catus ear tissue samples were collected from injured 10-month old healthy female cat brought to the Department of Surgery, Nanaji Deshmukh Veterinary Science University, Jabalpur, India, for the treatment. Tissue samples were collected by the veterinary surgeon with the owner's permission at the size of 0.5 × 0.5 cm from the wounded area of the skin. A tissue sample was processed as per the standard protocol of animal cell culture technique[21]. Tissue chopped in approx. 0.1 cm small pieces under biosafety cabinet (Esco standard II)was transferred into T-25cm^2 cell culture flasks with addition of small drop of fetal bovine serum (FBS), and left for 3-4h for tissue attachment on flask surface. After the tissue attachment, cell nutrient medium, e.g. DMEM with 15% FBS, was filled into the flask. It was found that cells started to sprout at 12 d from the tissue explant and propagated for increasing cell density into the

flask. When cells reached in 70-80% confluency, they were detached by adding 1 ml of 0.25% warm trypsin enzyme. After the complete growth, cells were passaged and transferred into the new culture flask for making a pure cell line. Meanwhile, a stock concentration of carbofuran was prepared in a 1% DMSO solution[22]. Malathion was diluted from commercial stock. After growth of the cells, they were exposed to the increasing concentration of carbofuran (0.045mM to 1.08mM) and malathion (5mM to 45mM). Experiments were performed in triplicates for all *in vitro* assays along with controls containing equivalent volume of the solvent run in parallel.

Cell viability and cytotoxicity of fibroblast cells by crystal violet staining and MTT Assay

To carry out these experiments, 1×10^4 numbers of cells were seeded into the 96-well cell culture flask for cell proliferation in CO_2incubator (Thermo Fisher) at 37^0C, 98% humidity and 5% CO_2. When cells reached around 70-80% confluency, these were exposed to increasing concentrations of carbofuran and malathion and incubated for 24h and 48h respectively, along with

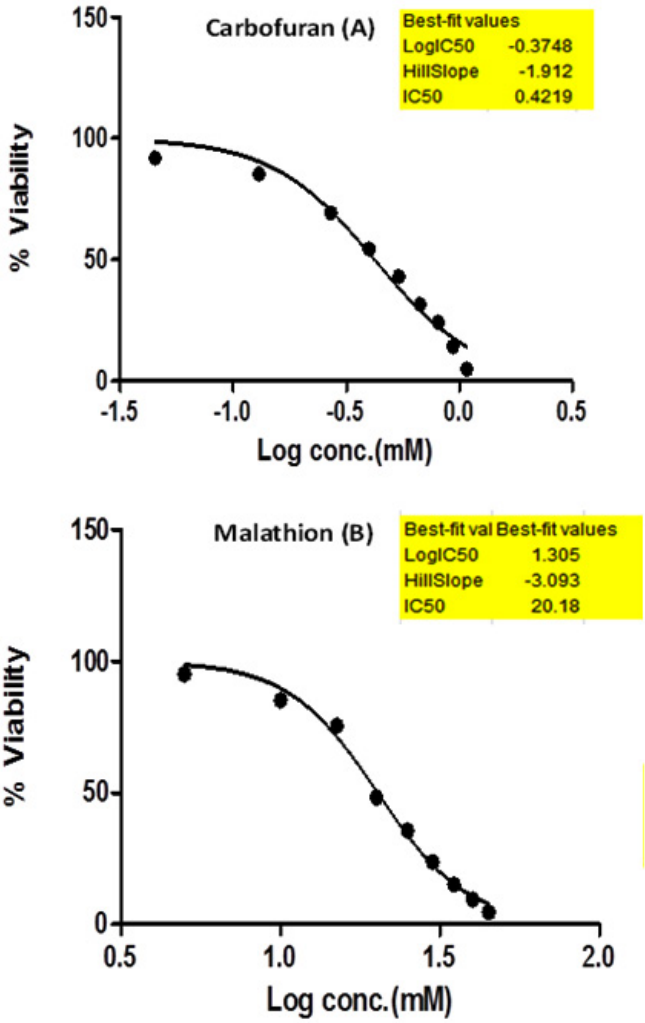

Fig. 1. Showing concentration versus cytotoxicity caused by pesticides on *F. catus* fibroblast cells under *in vitro* condition. ($p<0.05$, One way Anova).
(A) Viability percentage after treatment with carbofuran and (B), malathion pesticides.
Results are expressed as LC_{50}: lethal concentration causing 50% cell death convert in log concentration

control cells without added pesticides. After that cells were subjected to viability determination by adding 0.5% crystal violet solution and measured the optical density (OD) at 570nm using plate reader for percentage cytotoxicity evaluation[6,15,23]. On another 96 well plate having same conditions, a ten µl of 5mg/ml MTT prepared in PBS was added into the carbofuran and malathion treated cells. The plate was further incubated for 3 h for the formation of formazan crystal. After the incubation time, MTT was removed and the formazan crystal was dissolved by adding 100 µl DMSO into the treated and control wells. The OD value was measured at 570nm using Thermo lab systems Multiskan EX model no. 355 microplate reading spectrofluorimeter. Viability was determined by comparing the OD values of controls with the treated cells. Three parallel experiments were carried out and for each experiment triplicate analysis was done.

Chromosomal aberration assay

Cultured fibroblast cells when reached at the density of 1×10^5 were treated with different concentrations of carbofuran (0.045 mM to 1.08mM) and malathion (5mM to 45mM) for toxicity screening along with controls. Chromosomes were arrested during cell division at metaphase stage by adding 1 µl/ml of colchicine prepared in ethanol at a stock concentration of 10 mg/ml. After 45min of incubation at 37^0C the entire culture medium was pipetted out. Cells were detached by adding 1 ml of 0.25% warm trypsin for 2 min followed by hypotonic treatment at 37^0C with 0.56% warm KCl for another 30 min. Cells were found swollen after this treatment. Swollen cells were re-suspended in chilled 5 ml of methanol: acetic acid (3:1 vol/vol) and centrifuged at 1,200 × *g* for 10 min. The process was repeated twice to recover chromosome material as pellet, which was finally suspended in 50 µl methanol-acetic acid mix and dropped on to the clean glass slide[24]. The chromosomes were stained by using giemsa stain solution for 20 min, and thereafter slides were dried and observed under compound microscope (Leica DM 3000) at 100X objective for observing any chromosomal aberration.

Micronuclei (MNi) analysis

The method of Soloneski et al.[18] was modified as follows: at least 500 metaphase-arrested cellswere scored at 20X magnification.

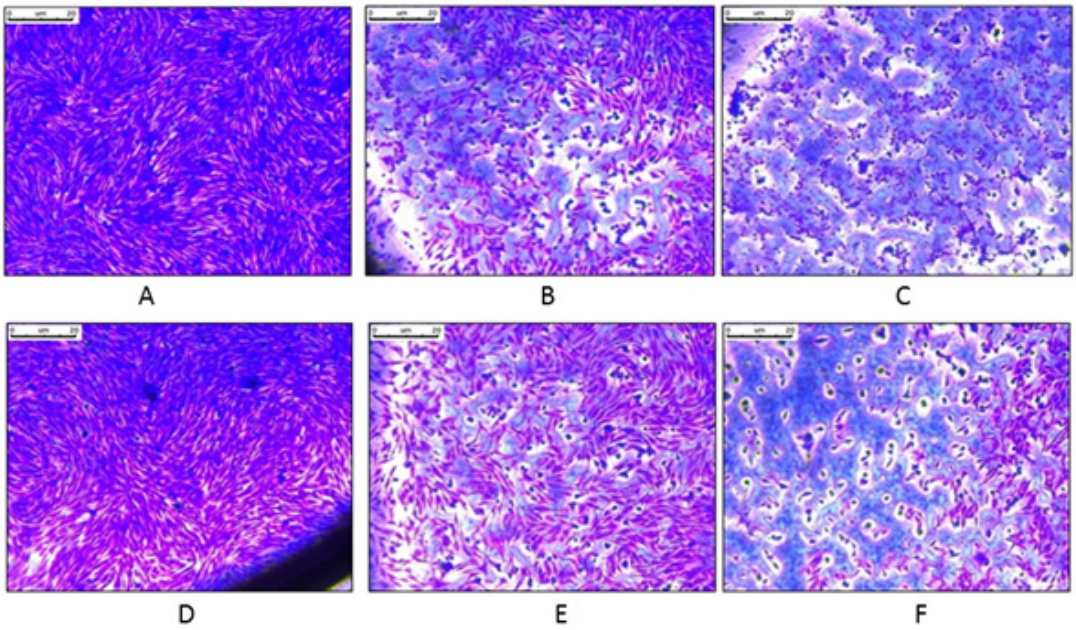

Fig. 2. Showing crystal violet stained cells of carbofuran and malathion treated *F. catus* fibroblast cells.
(A) Control cells, (B) Cell toxicity at LC_{50} concentration (0.42 mM) of carbofuran and (C) Cell toxicity at 1.08mM concentration of carbofuran; (D) Control cells, (E) Cell toxicity at LC_{50}concentration (20mM) of malathion and (F) Cell toxicity at 45mM concentration of malathion

MNi were counted in untreated controls (solvent alone) and pesticide-treated cells according to the OECD Test Guideline #473 at concentrations which resulted 55±5% cytotoxicity in the above MTT assay.The criteria employed in identifying MNi were: approximate diameter smaller than the main nuclei, same staining intensity as or lighter than that of the main nuclei, and MNi boundary distinguishable from main nuclei boundary.

Single cell gel electrophoresis (Comet assay)

DNA damage caused by the pesticides, carbofuran and malathion, was observed using Single Cell Gel Electrophoresis (SCGE) as described by Dhawan et al.[25] with slight

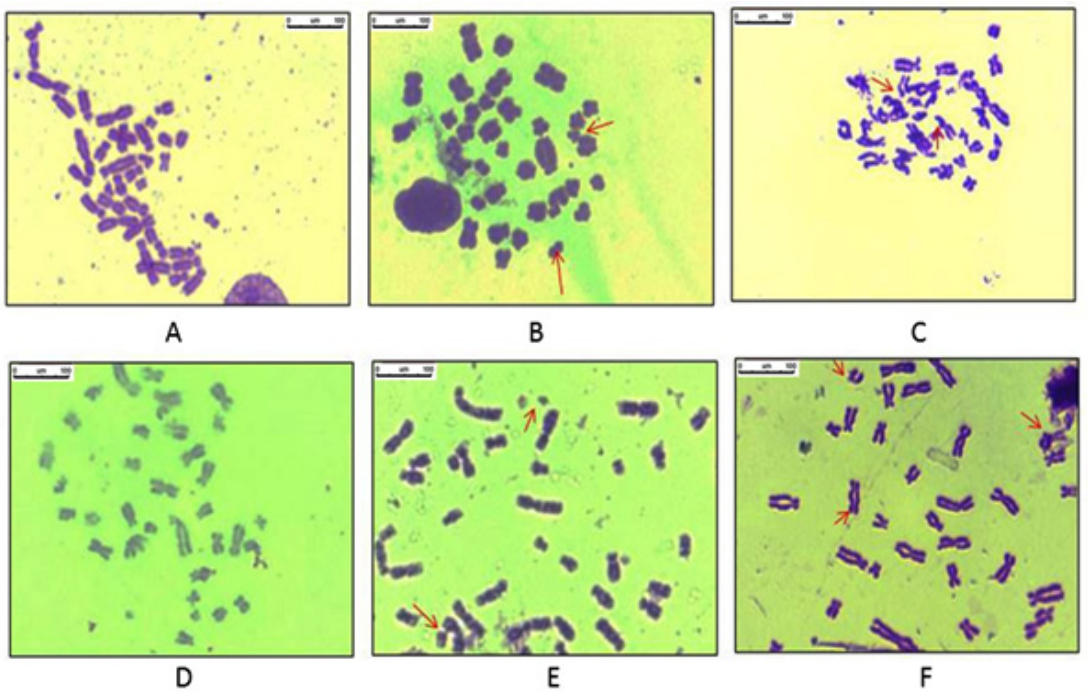

Fig.3. Showing chromosomal aberrations in *F. catus* fibroblast cells caused by treatments with (A-C) carbofuran and (D-F), malathion.
(A) Control metaphase plate without chromosomal aberrations, (B) Arrow in metaphase plate showing chromatid break and fragment and (C) Arrow in metaphase plate showing chromatid breaks and arm deletion; (D) Control metaphase plate without chromosomal aberration, (E) Arrow in metaphase plate showing chromatid break and satellite associations and (F) Arrow in metaphase plate showing chromatid break, satellite associations, and a gap

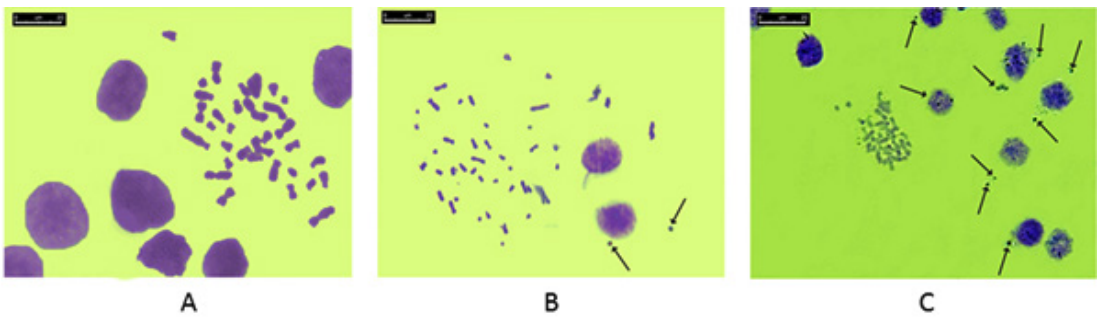

Fig. 4. Showing micronuclei (MNi) formation in (A) untreated control, and upon exposure to (B) carbofuran at 0.46mM and (C), malathion at 22mM (doses equivalent to LD_{55}, as per OECD guideline). Arrow indicates MNi formation around main nuclei without overlapping boundaries.

modification. For this experiment, cat fibroblast cells (CFC) were incubated for 24h and 48h with various concentrations of the two pesticides. Positive controls consisted of cells treated with1µM concentration of cyclophosphamide[16]. Negative controls were set up by incubating fibroblasts with the solvent DMSO at a final concentration of 1%. All the 24 wells having fibroblast cells treated with pesticides along with control samples were mixed with 20µl of 0.5% low melting agarose (LMA), and layered on the surface of clean glass slides which was previously coated by 1% normal melting agarose (NMA). Coverslip was put on the glass surface and placed on the ice packs for fixation. After that the slides having cells were equilibrated for 2h at 4^0C into the cold cell lysis buffer comprising of 2.5 M NaCl, 100 mM Na-EDTA and 10 mM Tris base, pH 10.0. After this, the slides were left into the alkaline electrophoresis solution buffer (1mMN a-EDTA and 300mM NaOH, pH ≥13) and electrophoresed for 20min at 24 volts (~0.74 v/cm) and current 300 mA.The slide was removed off the tank and drop-wise coated with neutralization buffer (0.4 M Tris-HCl pH 7.5) and allowed to stand for at least 5 min. After draining the liquid, the process was repeated two more times and finally absolute methanol was poured for dehydration. Slides were than air-dried by placing them into the hot air oven at 50^0C for 30 min and stored in a dry area. As per the requirements, slides were rehydrated by adding chilled distilled water for 30min and werestained up to 5min with 0.5ml of 1× EtBr (10 mg/ml). The whole process of comet assay was performed under yellow/ dimmed light to prevent any DNA damage owing to fluorescent white light. The analysis was performed under 100X objective by using fluorescent compound microscope (Leica DM 3000). At least 100 cells were screened per slide, and per treatment at least three slides were visualized. In the first set of analysis comet tail length was measured using micrometer fitted with the microscope.

In the other set, image analysis was carried out according to Collin's method modified by Driessens et al.[26]. Briefly, DNA damage was

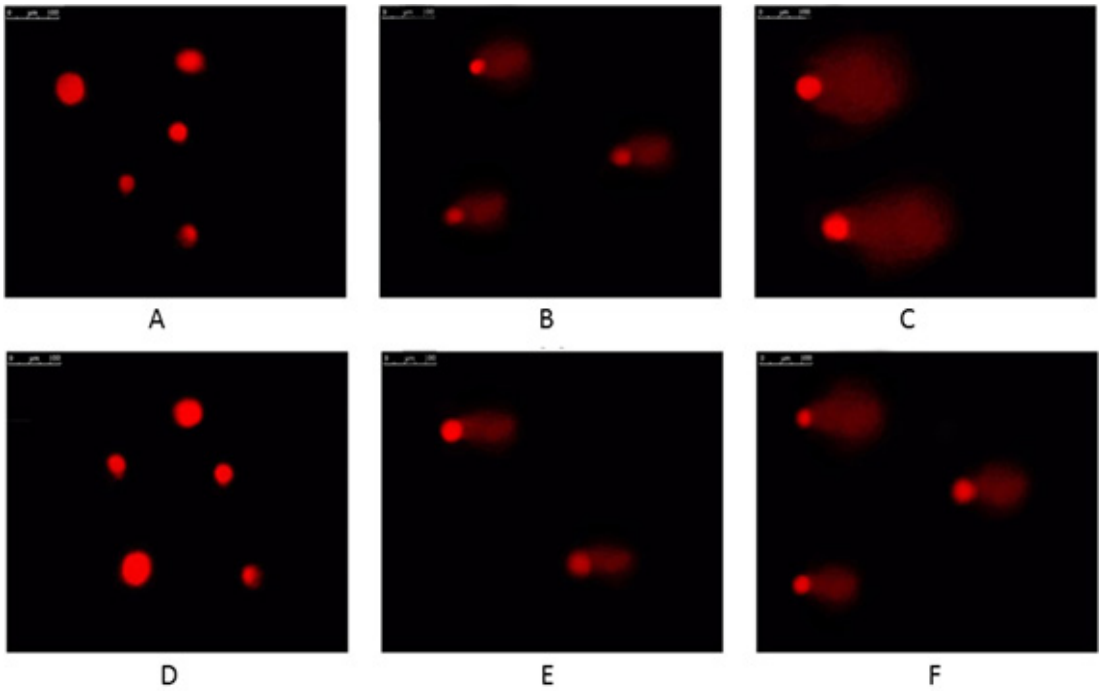

Fig. 5. Comet assay showing DNA damage in *F. catus* fibroblast cells caused by treatments with (A-C) carbofuran and (D-F), malathion. (A) Control cells, (B) Cells showing DNA damage as comets at LC_{50} concentration and (C) DNA damage at higher (1.08 mM) concentration; (D) Control cells, (E) Cells showing DNA damage at LC_{50} concentration and (F) DNA damage at higher (45 mM) concentration

Table 1. Estimation of percentage viability by crystal violet stain in cat fibroblast cells after exposure to carbofuran and malathion pesticides

Carbofuran Conc.(mM)	Treatments (24h)					
	Control (Untreated) (A)		0.42 (LC_{50}) (B)		1.08 (>LC_{50}) (C)	
	Mean OD±SE	%Viability	Mean OD±SE	%Viability	Mean OD±SE	%Viability
	0.96±0.020	100%	0.45±0.030	49%	0.01±0.010	1%
Malathion Conc. (mM)	Treatments (48h)					
	Control (untreated) (D)		20 (LC_{50}) (E)		45 (>LC_{50}) (F)	
	Mean OD±SE	%Viability	Mean OD±SE	%Viability	Mean OD±SE	%Viability
	0.91±0.031	100%	0.50±0.020	55%	0.10±0.010	10%

Mean and standard error value of cell viability of control (A, D), carbofuran (B, C) and malathion (E, F) treated cells (*cf.* Fig. 2). Average OD comparison of non-stimulated cells (control; untreated) viable and attached with stimulated (treated; weakly attached or lost from cell population) generating reduced amount of crystal violet stain at LC_{50} treated or highly concentration exposed cells (B, C & E, F) that undergo cell death lose their adherence and are subsequently lost from the population of cells by reducing the amount of crystal violet staining in a culture. The amount of crystal violet staining in the assay is directly proportional to the cell biomass.

categorized into four classes (*refer*, Supplementary Fig. 1): class 1 (no tail or halo around the nucleus), and class 2-4 (corresponding to gradual increase in DNA damage). At least 100 randomly cells per slide and three slides per treatment were measured. The scores were expressed per 100 comets in arbitrary units in a range of 100 (all comets in class 1) and 400 (all in class 4).

Statistical analysis

Statistical significance between control and pesticide treated cells were calculated by One way Anova with post hoc Tukey HSD test[16].The concentration required to reduce the cell viability by 50% was determined by Graph pad Prism Software v. 5[27,28].Data on statistical analysis were presented as mean ± standard deviations (SDs). $p<0.05$ values were considered as being statistically significant.

RESULTS

The cytotoxicity percentage graph based on MTT test is presented in Fig. 1. Cell viability percent decreased with the increase in the concentrations of both carbofuran and malathion. The LC_{50} values for carbofuran and malathion in CFC were calculated to be 0.42 mM and 20mM respectively, which caused 50% cytotoxicity calculated by Graph pad Prism Software v. 5. The cytotoxicity test as against increasing doses of the pesticides was also performed using crystal violet staining. This experiment shows a clear dose-dependent cytotoxic effect of the pesticides on CFC as shown in Fig. 2; Table 1.

For cytogenic analysis, the metaphase-arrested cells were screened to check the frequency of chromosomal aberration. The data presented in Table 2 clearly indicates that the frequency kept on increasing with concentration of the pesticides. Metaphase plates with chromosomal aberrations are presented in Fig. 3a–f. At LC_{50} concentrations (0.42 mM carbofuran and 20mM malathion) the mean percentage of satellite associations, and mean percentage of breaks, gaps and fragments were found to be 1.2-1.5-fold higher upon treatment with carbofuran than malathion. At higher concentrations (1.08mM carbofuran and 45mM malathion) too, the trend was almost the same.

Our next objective was to observe, to what extent the chromosomes micronucleated at

aberration stages. As per the OECD guideline the doses chosen were slightly higher than the LC_{50} ($\approx LC_{55}$). Fig. 4a-c shows the presence of MNi under different treatments. Upon enumeration, significant increase in micronucleated cells under malathion treatment (55.0±2/500 *versus* 5.0±1/500 in DMSO control, $p<0.01$; ANOVA) was found in CFC. There was marginal yet significant increase in MNi recorded under carbofuran (12.0±2/500 *versus* 6.5±1/500 in solvent control,$p<0.05$). The MNi were quite discrete from the main nuclei in the carbofuran treated cells whereas they were closely attached in case of malathion.

The extent of DNA damage was monitored in the comet assays by treating the CFC with both the pesticides keeping individual control sets. The images are presented in Fig. 5a-f.The values of average tail length and comet scores are tabulated in Table 3. It can be seen that in control sets there was no apparent DNA damage as intact nuclei were visualized both without any treatment and with DMSO solvent (controls). At LC_{50} concentrations, 0.32±0.015 and 0.22±0.010 µm length of comet tails was recorded respectively with carbofuran and malathion. At these strengths, the comet scores were only marginally higher than respective controls (115%). At higher concentrations (1.08mM for carbofuran and 45mM for malathion) much lengthier comet tails were recorded which accounted for 0.88±0.020 µm and 0.70±0.022 µm. Accordingly, the comet scores also went up (1.32- to 1.52 fold) relative to solvent controls. The maximum comet scores and tail lengths were in a range of values corresponding to those of the positive controls.

DISCUSSION

The present study was undertaken to evaluate the *in vitro* cytotoxicity and genotoxicity in cat fibroblasts. It was found that exposure of carbofuran and malathion, in a concentration-dependent manner, significantly reduced the viability of fibroblast cells ($p<0.05$). The calculated LC_{50} values of carbofuran and malathion were 0.42mM and 20 mM, respectively. Previous studies on malathion in human liver carcinoma cells revealed an estimated LC_{50} of 15 mM[6].More recently it was shown that genotoxic effects, e.g. comet tail, of malathion-treated human lymphocytes began to appear at concentrations much below the putative LC_{50}values[19]. Therefore, though the lethal doses of malathion for feline and human model cells are analogous, perhaps at chronic doses human cells are more prone to be adversely affected than CFC. There are also cell-specific differences with regard to carbofuran exposure. Our LC_{50} value with CFC was found to

Table 2. The frequency of chromosomal aberrations in CFC treated with increasing concentrations of carbofuran and malathion pesticides

Used chemicals	No. of cell scored	Breaks	Gaps	Satellite association	Fragments
Carbofuran*					
Control untreated	100	ND	1±0.020	3±0.023	ND
0.045mM conc.	100	4±0.012	3±0.012	6±0.011	1±0.010
0.42 mM conc.	100	7±0.023	5±0.018	12±0.020	3±0.018
1.08mM conc.	100	12±0.020	9±0.022	16±0.022	5±0.022
Malathion*					
Control untreated	100	ND	1±0.010	1±0.010	ND
5mM conc.	100	2±0.018	2±0.015	4±0.020	1±0.018
20mM conc.	100	5±0.020	4±0.022	8±0.023	2±0.020
45mM conc.	100	8±0.022	8±0.020	12±0.022	4±0.022

*$p<0.05$ (ANOVA) found significant when compared to the control for all experiments

ND: Not detectable.

Each experiment was carried out in triplicate manner for checking the chromosomal aberrations, and 100 metaphases were screened for each set.Chromosomal aberration found to be dose dependent with increasing breaks, gaps, satellite association, fragments and micronuclei formation as compared with untreated (control).

Table 3. Comet assay assessment of DNA damage (mean ± SD) of comet tail length (in µm) and visual examination of DNA damage classes (1-4) in CFC treated with carbofuran and malathion pesticides respectively for 24 h and 48 h

Treatments (mM)		Average tail length in µm (Mean ± SD)		Number of nuclei per comet class†and total scores (Mean ± SD)									
				Carbofuran				Malathion					
Carbofuran	Malathion	Carbofuran	Malathion	1†	2	3	4	Score	1†	2	3	4	Score
Control (Untreated)		0.01±0.010	0.01±0.010	63	37	0	0	137±2.50	64	36	0	0	136±2.50
0.045	5	0.05±0.020	0.02±0.020	62	37	1	0	139±5.03	60	39	1	0	141±2.51
0.13	10	0.12±0.010*	0.08±0.018*	55	42	2	1	144±4.50	57	37	3	1	143±4.04
0.27	15	0.20±0.015*	0.10±0.022*	52	45	2	1	152±2.51	55	40	3	1	148±3.60
0.42	20	0.32±0.015*	0.22±0.010*	44	49	4	3	166±3.05#	50	41	3	6	165±4.58#
0.54	25	0.45±0.015*	0.28±0.010*	46	41	8	5	172±5.77#	48	42	5	5	167±3.05#
0.67	30	0.54±0.017*	0.39±0.015*	41	43	10	5	176±2.10#	44	43	8	5	174±5.56#
0.81	35	0.66±0.019*	0.47±0.028*	40	44	6	9	188±5.29#	40	46	7	6	177±3.21#
0.94	40	0.78±0.020*	0.58±0.025*	34	46	7	13	199±5.00#	36	51	6	7	184±4.93#
1.08	45	0.88±0.020*	0.70±0.022*	31	37	16	17	221±3.51#	41	36	15	8	190±5.50#
1 µM cyclophosphamide (positive control)		0.98±0.010**	24	33	23	20	236±2.00#						
1% DMSO (negative control)		0.02±0.020	58	40	2	0	144±4.61						

*$p<0.01$,significant comet length difference when compared with the control (untreated) and negative control (cells treated with 1% DMSO)

**pÃ0.05, non-significant difference when compared to pesticide-treated samples at higher concentrations

Values were highly significant compared to untreated control and negative control (1% DMSO) whereas the positive control showed a non-significant result when compared to samples treated with higher concentrations of both the pesticides.

be *ca*. 20-times higher than human lymphocytes, which was recorded to be ≤ 18 μM[29]. This vast difference in cell-specific cytotoxicity clearly indicates that, feline fibroblast cells seemingly are highly resistant to carbofuran. Therefore, use of lab based mammalian cell lines may not give accurate data that is applicable to feline species.

Genotoxicity assessment of carbofuran and malathion at the tested concentrations revealed that these pesticides could cause significant chromosomal aberrations in CFC *in vitro*. The extent of chromosome aberration increased upon increasing the doses of carbofuran and malathion. The mean values of both the toxicants clearly indicate that malathion exerted lesser degree of aberrations compare to carbofuran. Micronuclei frequency was much greater under malathion, though. At the moment the micronucleation seems to be the consequence of clastogenic effect of malathion and nucleation of broken chromosome, as also could be found with other OP compounds[30]. The LC_{50} concentrations of both the pesticides were able to cause significantly higher DNA damage relative to the untreated cells. As also could be inferred from alkaline comet images, there was double stranded DNA breaks which trailed as tails. Most of the investigations pointed out excess reactive oxygen species as the main cause of oxidative damage of DNA[31].

Past investigation on the genotoxic and/or mutagenic and carcinogenic manifestations of malathion and some other OP pesticides under *in vivo* or *in vitro* conditions have led to controversial outcome[9,19]. In this regard, the present work should be evaluated under the guidelines set by *Genotoxicity Expert Panel* for another OP herbicide, glyphosate[32]. The evaluations recommended for such pesticides must consider "weights" of the genotoxicity assays. In this context, oxidative DNA, micronuclei *in vitro*, chromosomal aberration *in vitro* exhibit "moderate weights" whereas comet *in vitro* was placed under "low weight". Consequently, the interpretations were not robust as several of the influenced physiological changes, *viz*. DNA damage, ROS production etc. were reversible and could have not been passed on to the next generation. Besides, the parameters were not examined in conjunction in a particular cell line. In this work, other than ROS, we examined all the "low to moderate weight" parameters and found these were dose-dependent. Hence, it can be undoubtedly said that malathion can promulgate genotoxic effect under *in vitro* state. Moreover, by following the same guidelines, it is reasonable to assume that the genotoxic effects of malathion are secondary to toxicity rather than it is target oriented (DNA alteration), because the applied doses were well in excess of the physiological limits set for testing genotoxicity. However, it is noteworthy to mention that malathion was shown to over-express oncogenes in human lymphocytes[33] which could explain the carcinogenic attributes.

It is possible that malathion in conjunction with some other pesticide(s) could be toxic even at lower doses. It has been shown that combination of OP pesticides brings about synergistic effects on viability of human lymphocytes[16]. Pesticide mixing is a common practice in agriculture. Thus we propose that OP pesticides, if are mixed as a formulation, even at environmental chronic state of exposure can be harmful because of their synergistic effects and/or carcinogenic manifestation.

CONCLUSIONS

Carbofuran and malathion exert cytotoxic and genotoxic effects which may be the primary cause of impairment of cellular functions in skin fibroblast cells of domestic cat. Furthermore, the effect of malathion seems to be more of a secondary response brought out at unusually high dose. This study opens up new avenues for forecasting the environmental exposure and standardizing the dose of antidotes against OP pesticides in feline animals.

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