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Genotoxicity of Pesticide Waste Contaminated Soil and Its Leachate

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Objective Improper land disposal of hazardous waste can result in leaching of hazardous constituents which may contaminate ground and surface water leading to adverse impact on human health and environment consequences. The present study utilized mammalian cell culture for the genotoxicity assessment of waste and its leachate. **Methods** Genotoxic potential and chemical analysis of pesticide derived tarry waste contaminated soil extract and its leachate was assessed using *in vitro* human lymphocyte cultures and GC-MS. **Results** The investigation revealed that the soil extract could cause significant to highly significant genotoxicity in the form of DNA strand break at 25 μL (P<0.01), 50 μL, 100 μL and 200 μL (P<0.001) and chromosomal aberration at 25 μL (P<0.01) and 50 μL and 100 μL (P<0.001). The leachate could cause significant DNA strand break and chromosomal aberration only at 100 μL and 200 μL (P<0.01) dose levels. **Conclusion** The genotoxicity observed is attributed to carbaril and tetra methyl naphthyl carbamate, the major ingredients of the extracts, as revealed by GC-MS.

Key words: Genotoxicity; Soil extract; Leachate; DNA strand break; Chromosomal aberration; Gas chromatography mass spectrometery (GC-MS); Fluorimetric analysis of DNA unwinding assay (FADU)

INTRODUCTION

Land disposal has been the predominant alternative for hazardous waste disposal[1] because it is less expensive as compared to incineration, neutralization, stabilization and other land-based options. However, improper land disposal results in the release of leachates from hazardous wastes which often contain toxic chemicals that may contaminate ground and surface water leading to adverse impact on human health and environment. Hazard assessment of leachates is traditionally based on the evaluation of potential toxic compounds present in the complex matrices. The leachates contain a broad range of toxic, carcinogenic and mutagenic chemicals[2]. Although regulatory limits have been established for some of the chemicals present in the leachates that have toxic effect, many leached chemicals have

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Biographical note of the first author: Dr. S. D. SIVANESAN is a scientist, having an experience of more than 11 years in research and development, currently is engaged on genotoxic and carcinogenic risk assessment of hazardous waste and toxic chemicals, protective mechanism of natural products, molecular toxicology of contaminants, bioremediation of contaminated sites and biological degradation of recalcitrant chemicals and wastes.

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neither been studied nor regulated. As chemical analyses of the waste do not reveal the complex interaction phenomena, a bioassay in combination with chemical analysis approach would integrate the hazard impact evaluation of all the compounds and their interactions such as synergism and antagonism and enable the evaluation of toxicity of a waste with complex matrix[3].

The present study was directed towards investigation of the genotoxic potential of soil contaminated with pesticide wastes and its leachate collected from an industrial site using human leukocyte cell cultures.

MATERIALS AND METHODS

Chemicals and Media

Dulbecco's modified Eagles medium (DMEM) (Sigma, USA), phytohaemagglutinin (PHA) and fetal bovine serum (FBS) were procured from GIBCO, UK. All other chemicals and solvents were of chromatographic and analytical grade.

Sample Preparation and Extraction

Pesticide derived tarry waste contaminated soil samples were collected from five locations at a depth of 180 cm below the surface from a dumpsite near carbaryl manufacturing industry located at Central India and were composited. The tarry waste was generated following a fire in carbaril storage tank of the industry.

Extraction

Ten grams of composited contaminated waste was extracted with 500 mL of hexane using soxhlet extraction method[4]. The extract was subjected to florisil clean-up and sequentially eluted with 50 mL N-hexane and 50 mL dichloromethane (DCM). Hexane and DCM clean-up fractions were evaporated to dryness using a rotary vacuum evaporator and then dissolved in dichloromethane for chemical analysis. Both hexane and DCM fractions were pooled together, evaporated and replaced with dimethyl sulfoxide (DMSO) used for genotoxicity study.

Leachate Preparation and Extraction

Ten grams of composited contaminated waste was leached with extraction fluid (5.7 mL of glacial acetic acid made up to 1000 mL, the pH of the fluid being 2.10)[5]. Leachates were extracted using liquid-liquid extraction with hexane: acetone (1:1). The extract was subjected to filtration through the anhydrous sodium sulphate column to remove the moisture. The resulting extract was evaporated to dryness using a rotary vacuum evaporator, one part of the extract was dissolved in methylene chloride and subjected to chemical analysis. The other portion of the extract was dissolved in DMSO, and used for genotoxicity study.

GC-MS Analysis

Chemical analyses of pesticide waste and its leachate extracts were carried out by gas chromatography-mass spectrometer (GC-MS, Saturn Model Varian Associates, Walnut Creek, CA) with DB-5, MS column (M/s. J & W Scientific, USA 25 m ×0.25 mm id ×0.25 μ) and helium gas as carrier. The injector and transfer line temperatures were 250℃. Ion trap

temperature was 210℃. The oven program was 50℃ hold 1 min, raised to 150℃ at 25℃/min hold 2 min, and then raised to 320℃ at 5℃/min. The pesticides and PAHs were identified by computer search of the National Institute of Science & Technology (NIST-1998) Library of Mass Spectra on the basis of retention time and mass fragmentation pattern.

Cell Culture

Human venous blood was collected from healthy non-smoking laboratory volunteers (aged 25-30 years) for the whole blood cell culture (chromosomal aberration study) and peripheral blood polymorphonuclear leukocytes (PMNs) (for DNA strand break study). Viability of the cells was determined by trypan blue exclusion test and viability was always >90%.

Fluorimetric Analysis of DNA Unwinding

Human peripheral blood mononuclear cells were suspended in DMEM with the cell density of 5 to 10×10^6/mL. About 1 mL of cells was treated with 25 μL, 50 μL, 100 μL and 200 μL of waste extract and 50 μL, 100 μL, 200 μL of leachate extract and incubated with a positive control (H_2O_2 150 μmol/L final concentration) and a negative control (0.1% DMSO) for 1 h at 37℃ in duplicate without serum. After the treatment, the reaction was arrested with 0.9% ice-cold saline and centrifuged at 400×g for 10 min at 4℃ and subjected to fluorimetric analysis of DNA unwinding assay according to the method of Birnboim and Jevcak (1981)[6] with minor modifications by Krishnamurthi *et al.* (2003)[7].

Chromosomal Aberration Assay

About 0.5 mL of human venous blood was added to 3.5 mL of lymphocyte culture (Dulbecco's minimal eagle's medium, Sigma) medium supplemented with 20% of fetal bovine serum and phytohaemagglutinin (50 μg/mL) and incubated at 37℃ in a humidified atmosphere containing 95% air and 5% CO_2. After cells were grown for 24 h, the cultured cells were treated with soil extract (25 μL, 50 μL and 100 μL), leachate extract (50 μL, 100 μL, 200 μL), 0.1% DMSO (negative control) and 0.1 μg/mL mitomycin-C (positive control) and then cultured for a further period of 24 h in triplicate under identical conditions. The cultures were treated with colchicine (0.2 μg/mL) for two hours before the culture was harvested. The cultures were harvested and slides were prepared for chromosomal aberration study according to the method of Api and San[8]. Hundred well spread metaphases per culture were investigated for aberrations including breakage type chromatid aberrations (chromatid breaks and chromatid fragments), exchange type aberrations (intrachange & interchange between chromatids) and breakage type chromosome aberrations (chromosome breaks and chromosome fragments).

Statistical Analysis

The results were statistically analyzed using ANOVA one way test with "Analyze it Software" and expressed as *P* values which were not considered significant when $P>0.05$.

RESULTS

GC-MS

The gas chromatography & mass spectrometric analysis of the contaminated soil samples

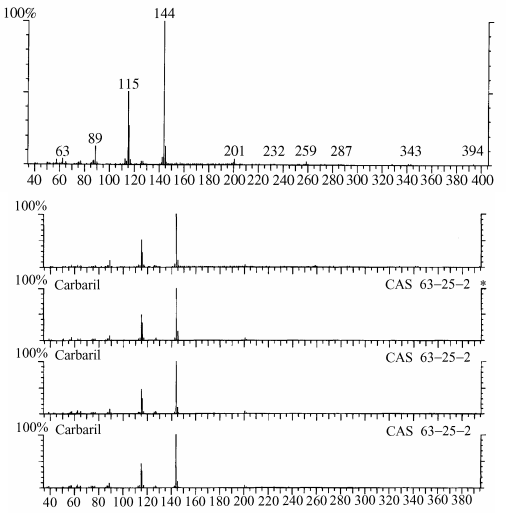

FIG. 1A. Gas chromatograph and mass spectrometric analysis contaminated soil (methylene chloride fraction).

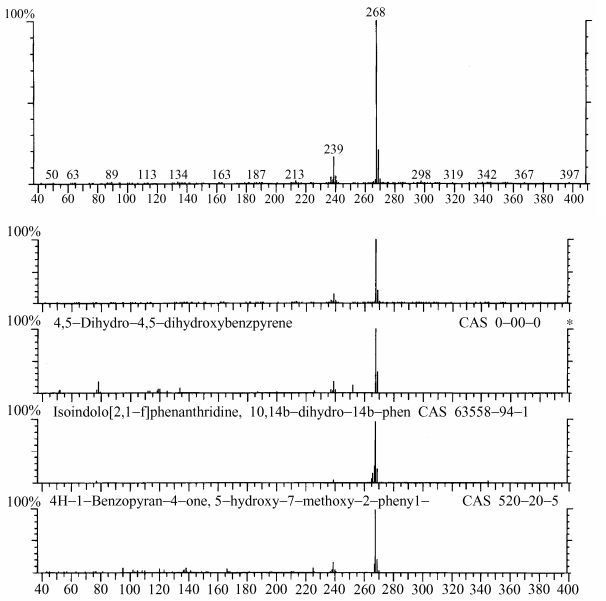

FIG. 1B. Gas chromatograph and mass spectrometric analysis of contaminated soil (hexane fraction).

are shown in Figs. 1A and 1B. The methylene chloride clean-up fraction of soil sample contained tetrahydronaphthyl methyl carbamate and carbaril, while the hexane clean-up fraction of soils samples contained delta-lindane, benz[a]anthracene, 8-propyl and 4,5-dihydroxy-benzpyrene. The gas chromatography & mass spectrometric analysis of leachates contained carbaril and tetrahydro naphthyl methyl carbamate (data not shown). Out of these chemicals identified, only carbaril (46 mg/g in soil and 23 mg/L in leachate) and tetrahydronaphthyl methyl carbamate (2.85 mg/g of dry wt in soil and 1.25 mg/L in leachate) could be quantified.

DNA Unwinding Study

DNA strand break caused by the extracts of pesticide contaminated soil and leachate was analyzed by fluorimetric analysis of DNA unwinding. The results expressed as the percentage of DNA strand break in treated and control cells are shown in Figs. 2 and 3. The control cells treated with 0.1% DMSO showed about 29% of DNA strand breaks. The soil extract treated cells showed an average of 43% DNA strand breaks for 25 μL (P<0.01), 55% for 50 μL (P<0.001), 64% for 100 μL (P<0.001) and 69% for 200 μL (P<0.001). The leachate extract treated cells showed an average of 30% DNA strand breaks for 50 μL (P>0.05), 44% for 100 μL (P<0.01) and 45% for 200 μL (P<0.01), 0.1% DMSO caused 22% and H_2O_2 caused 65% DNA strand breaks respectively.

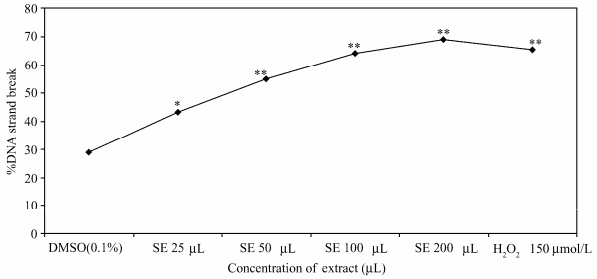

FIG. 2. DNA strand breaks induced by soil extract in human polymorphonuclear leukocytes. Note: The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. $^{*}P<0.01$;$^{**}P<0.001$.

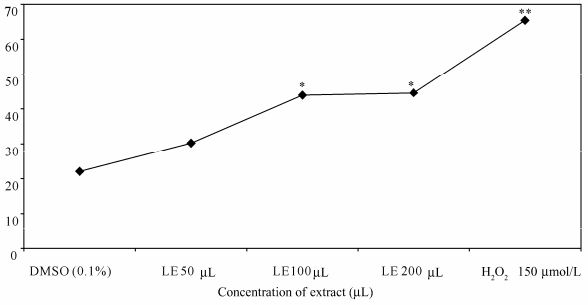

FIG. 3. DNA strand breaks induced by leachate extract in human polymorphonuclear leukocytes. Note: The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. $^{*}P<0.01$;$^{**}P<0.001$.

TABLE 1

Frequency of Chromosomal Aberrations in Human Leukocyte Culture Exposed to Soil Extract

Dose	Treatment/ Harvesting	No. of Metaphases	% Aberrated Cells $\overline{x} \pm s$	No. of Aberrations	Types of Aberrations						MI
					Ctb	Chb	E	R	M	F	
DMSO 0.1%	2/22	100	2.0 ± 1.000	3	1	ND	ND	ND	1	1	5.8
Soil Extract 25 μL	2/22	100	7.8 ± 2.588*	2	19	ND	ND	ND	1	2	3.2
Soil Extract 50 μL	2/22	100	11.4 ± 4.159**	41	20	ND	ND	ND	ND	2	2.7
Soil Extract 100 μL	2/22	100	18.6 ± 3.847**	48	24	3	1	2	17	11	2.1
Mitomycin C 0.1 mg/mL	1/23	100	19 ± 3.241**	22	16	1	1	ND	2	2	4.3

Note. The results are average of five sets experiments. Values marked with asterisks are significantly differing from negative control. ND: not detected; ctb: chromatid break; chb: chromosomal break; E: exchange; R: ring; M: minute; F: frgment; $^{*}P<0.01$; $^{**}P<0.001$.

TABLE 2

Frequency of Human Chromosomal Aberrations in Human Leukocyte Culture Exposed to Leachate Abstract

Dose	Treatment/ Harvesting	No of Metaphases	% Aberrated Cells $\bar{x} \pm s$	No. of Aberrations	Types of Aberrations						MI
					Ctb	Chb	E	R	M	F	
DMSO 0.1%	2/46	100	1.8 ± 0.84	2	ND	ND	ND	ND	1	1	6.2
Leachate 50 µL	2/46	100	5.0 ± 2.12	5	2	ND	ND	ND	1	2	4.2
Leachate 100 µL	2/46	100	$5.8 \pm 2.77^{*}$	8	6	ND	ND	ND	ND	2	4.3
Leachate 200 µL	2/46	100	$6.8 \pm 2.49^{*}$	11	9	ND	ND	ND	1	1	4.2
Mitomycin C	1/46	100	$19 \pm 3.24^{**}$	22	16	1	1	ND	2	2	4.3

Note. The results are average of five sets experiments. Values marked with asterisks are significantly differing from negative control. ND: not detected; ctb: chromatid break; chb: chromosomal break; E: exchange; R: ring; M: minute; F: frgment; $^{*}P<0.01$; $^{**}P<0.001$.

Chromosomal Aberration

The frequency of chromosomal aberrations in human leukocyte cultures exposed to the soil and leachate extracts are shown in Tables 1 and 2. The type of aberrations induced by contaminated soil extracts and leachate was of chromatid break, chromosomal break and chromatid gap. Statistical analysis showed that the soil extract caused a significant level of clastogenicity at 25 μL ($P<0.01$), 50 μL ($P<0.001$) and 100 μL ($P<0.001$) when compared with the negative control. The leachate also caused a significant level of clastogenicity at 100 μL ($P<0.01$) and 200 μL ($P<0.01$) treatments.

DISCUSSION

In the present investigation, the genotoxicity of pesticide derived tarry waste contaminated soil and its leachate extract were studied. The DNA damage and chromosomal aberration caused by the contaminated soil extract were statistically significant at doses ranging between 25 μL and 100 μL, whereas the leachate extract at higher doses (100 and 200 μL) caused significant genotoxicity (Figs. 2 and 3, Tables 1 and 2). The less toxic effect of leachate could be due to non leachability of some of the identified and non-identified compounds in the contaminated soil sample. Thus the human leukocytes exposed to the contaminated soil extract containing carbaril, delta lindane, tetra naphthyl methyl carbamate and polycyclic aromatic hydrocarbons such as benz[a]anthracene-8-propyl and 4,5-dihydro, 4,5-dihydroxy benzpyrene, could induce DNA strand break and chromosomal aberration. The results of the present study agree with the earlier reports that DNA damage could be induced by carbaril in SV 40 transformed human cell culture[9]. The DNA adduct formation was reported in human peripheral lymphocytes by benzo[a]anthracene without metabolic activation[10]. Carbaril has also been shown to affect cell division and chromosomal aberration in rats, it could also induce sister chromatid exchange in V-79 Chinese hamster cells[11]. Benz[a]anthracene was found to induce chromosomal aberration in Chinese hamster bone marrow cells[12]. It has been reported that benzo[a]pyrene metabolites could react with several nucleophilic sites in DNA[13].

With the available literature and results obtained in the present investigation, it may be concluded that carbaril, terahydro naphthyl methyl carbamate, delta lindane, benzo[a]-anthracene and 4,5-dihydroxy, 4,5-dihydroxy benzpyrene, a metabolite of benzo[a]pyrene in the contaminated soil (as revealed by GC-MS), may be responsible for the observed genotoxicity. Though the leachate showed DNA damage and chromosomal aberration in human leukocytes, significant damages could be observed at higher concentrations only. This may be due to the non-leachability of some of the identified and non-identified compounds in the contaminated soil samples.

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