

Genotoxic Effects of PAH Containing Sludge Extracts in Chinese Hamster Ovary Cell Cultures

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Objective Many studies have been conducted in order to evaluate the genotoxicity of chemicals and waste materials, which utilized *in vivo* test protocols. The use of animals for routine toxicity testing is now questioned by a growing segment of society^[1]. **Methods** Keeping the above fact in mind, we have conducted in the present study the genotoxicity evaluation of oily sludge samples generated from a petroleum refinery and petrochemical industry and ETP sludge from petroleum refinery using DNA damage, chromosomal aberration, p⁵³ protein induction and apoptosis in short term *in vitro* mammalian Chinese Hamster Ovary cell cultures. **Results** It is evident from the results that the oily sludge compounds derived from petroleum refinery and petrochemical industry could cause DNA damage, chromosomal aberration, p⁵³ protein accumulation and apoptotic cell death on exposure to oily sludge extracts in the presence of metabolic activation system (S-9 mix), however, ETP sludge extract could not cause significant genotoxicity in comparison to oily sludge extract and negative control. **Conclusion** The effect may be attributed to polycyclic aromatic hydrocarbons present in the samples as evidenced from GC-MS.

Key words: DNA damage; Chromosomal aberration; p⁵³ protein induction; Apoptosis; Genotoxicity; Polycyclic aromatic hydrocarbons (PAHs); Gas chromatograph mass spectrometer (GC-MS)

INTRODUCTION

For sludge and for any other potentially polluting substances, it is possible to define degrees of noxiousness by either chemico-physical or biotoxicological assays. Recently, especially in the Western Countries, the presence of organic substances such as pesticides, PAHs and PCBs have begun to be a cause of concern, because these substances may enter the food chain^[2] through ground water contamination. At present further studies are necessary in order to elucidate the source and identity of possible genotoxic contaminants in sewage/ industrial sludge and to evaluate their impacts on the environment^[3].

Evaluating the genotoxicity of hazardous waste and environmental samples require the assessment of waste for the presence of possible genotoxicants and carcinogens, and its reactivity with DNA^[4]. The present study has been aimed to investigate the DNA damaging potential of the sludges generated from a petroleum refinery and petrochemical industry

Biographical note of the first author: K. KRISHNAMURTHI, Ph D, 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, bioremediation of contaminated sites, molecular toxicology of contaminants and protective mechanism of natural products.

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through a battery of *in vitro* short term tests involving fluorimetric analysis of DNA unwinding, chromosomal aberration, induction of p^{53} protein and apoptosis in CHO-K1 cells which may be useful tools in the genotoxicity assays to replace the conventional *in vivo* animal tests^[5]. Since the selected samples are industrial waste products, the question of transferability of results from animal experiments to man needs special attention in order to characterize the health risk for employees periodically cleaning the equipment or in case of accident. Therefore the investigation has been focused to use mammalian cell culture system^[6].

MATERIALS AND METHODS

Chemicals and Media

Ham's F-12 culture media for CHO-K1 cells, benzo(a)pyrene and PAH standard mixture were purchased from Sigma, (St. Louis, MO, USA). Trypsin-EDTA, fetal bovine serum (FBS), penicillin and streptomycin were from Gibco Technologies, UK, agarose, ethidium bromide, sodium sulphate, colchicine and D-Glucose from Hi-Media laboratories, India. Tris-Cl, dimethyl sulphoxide (DMSO), sodium hydroxide, potassium chloride were from Sisco Research Laboratories, India, methylene chloride, benzene, methanol and acetic acid were purchased from E-merck India. Lysing solution for p^{53} protein induction and apoptosis were purchased from Amersham International Plc, England, UK. Phenobarbitone and hydrocortisone were kind gift of Prof. I. S. Grover, Punjab University. All other chemicals and solvents were of the highest Analytical Grade available.

The API oily sludge (petroleum refinery) was collected from the API-oil separator unit of a petroleum refinery located at eastern part of India, effluent treatment plant (ETP) sludge was collected from the biological treatment plant of the same refinery and API- oily sludge (petrochemical industry) was from the API-oil separator unit of a petrochemical industry located at western part of India.

Solutions for DNA Strand Break Assay

Solution A: 0.54 mmol/L EDTA in phosphate buffer saline (PBS-EDTA); Solution B: 9 mol/L urea, 10 mmol/L NaOH, 2.5 mmol/L EDTA, 0.1% SDS; Solution C: 0.2 mmol/L NaOH; Solution D: 1 mol/L Glucose, 14 mmol/L mercaptoethanol; Solution E: 0.6 mg of Ethidium bromide was dissolved in 100 mL of 10 mmol/L Tris pH 7.5-8.0.

Solutions for Chromosomal Aberration

Colchicine: One milligram of colchicine (SRL make) was dissolved in 10 mL of sterile double distilled water; hypotonic solution (0.75 mol/L KCl): Five hundred and sixty milligrams of potassium chloride were dissolved in 100 mL of distilled water and the pH of solution was adjusted to pH 7.2; Conroy's fixative: Methanol: Glacial acetic acid in the proportion of 3:1 (V:V) was prepared freshly and chilled at 4 °C.

Lysing Solution for p^{53} Protein

1% Triton X-100, 1 mL of Nonidet P-40, 0.1% sodium dodecyl sulphate (SDS) and 1 mmol/L EDTA were dissolved in 10 mL of 10X PBS and the volume was adjusted to 100 mL with demonized water. Protease inhibitors such as PMSF 0.2 mmol/L (from 10 mmol/L

stock), 1 $\mu\text{g}/\text{mL}$ aprotinin and 0.5 $\mu\text{g}/\text{mL}$ leupeptine was added before use.

Lysing Solution for Apoptosis

10 mmol/L tris-Cl pH (8.0), 0.5% Triton X-100, 5 mmol/L EDTA, and 0.5% SDS were dissolved in 100 mL of deionized water.

S-9 Liver Fraction

The S-9 liver fraction was prepared from the liver of male albino rats which had been injected with phenobarbital (75 mg/kg body weight) and hydrocortiozone (50 mg/kg body weight) every 8 h for 2 d to induce cytochrome P-450 oxidase and reductase. The liver homogenization and fraction collection was carried out according to the method of Wang *et al.*^[7]

Sludge Extraction and GC- MS Analysis

Weighed amounts of wet sludge samples were heated in a thermostatic oven at 50°C until the samples were completely dried. The sludge samples were weighed and 20 g each of oily sludges and ETP sludge samples were mixed separately with anhydrous sodium sulphate and were then extracted in soxhlet with methylene chloride for 48 cycles about 18 h^[8]. The organic extract was concentrated down to a few milliliters under reduced pressure with a rotovapor (rotary vacuum evaporator) at 50°C and dried by spontaneous evaporation of the solvent at room temperature. The organic percentage of the sludges were calculated using dry sludge weight and weight of organic extract.

The sludge extract was split into two portions: the first was dissolved in 0.1% DMSO and used for genotoxicity assays; the second portion was dissolved in methylene chloride subjected to benzene clean up using activated silica gel column and the elutes were again concentrated to few milliliters using rotovapor and analyzed for presence of PAH by Gas chromatography-Mass spectrometer (GC-MS, Saturn Model-3) with fused silica column (15 m \times 0.3 mm i.d.) and helium as carrier gas. The injector and transfer line temperatures were 50°C and 250°C respectively. Program was: 50°C, hold 1 min, raise to 150°C at 25°C/min, hold 2 min, then to 320°C at 5°C/min. The individual PAH's were identified and quantified by computer search of the National Bureau of Standard Library of Mass Spectra on the basis of retention time/ mass fragmentation.

Cell Culture

Chinese Hamster Ovary cells (CHO-K1) obtained from NCCS, Pune were grown in Ham's F-12 medium containing 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 250 $\mu\text{g}/\text{mL}$ fungizone and 10% fetal bovine serum at 37°C in a humidified 5% CO₂ and 95% O₂ atmosphere. The duration of cell culturing varied between 24 and 48 h. The same type of culture was used for DNA damage, chromosomal aberration, p⁵³ protein induction and apoptotic studies. Viability of the cells were determined by trypan blue exclusion test and viability was always > 90%.

Chemical Treatment

Before testing the below mentioned battery of genotoxicity assays for the wastes with and without S-9 mix, its cytotoxicity was proved to set the experimental conditions by use of trypan blue exclusion staining. The tested concentrations were selected accordingly and

further studies were carried out with S-9 mix.

DNA Unwinding Assay

Chinese hamster ovary cells (containing around $5-7 \times 10^6$ cells) were incubated separately with 25, 50, and 100 μL of oily sludge extract, ETP sludge extract (50, 100, and 200 μL), DMSO (negative control) and benzo(a)pyrene (Sigma, USA) as a positive control at a final concentration of 20 $\mu\text{g}/\text{mL}$ for a period of 3 h in duplicate with S-9 mix and incubated at 37 $^{\circ}\text{C}$ for 2 h. The negative control was prepared with DMSO under the same experimental conditions. The reaction was then arrested by the addition of ice-cold saline (0.9% sodium chloride). The mixture was centrifuged at $400 \times g$ for 10 minutes at 4 $^{\circ}\text{C}$ and the cell pellets were suspended in 1.0 mL of solution A. Aliquots of this suspension was subjected to fluorimetric analysis of DNA unwinding (FADU) as described by Birnboim and Jevcak^[9] with little modifications.

Chromosomal Aberration

After cells had been grown for 12 h, the cultured cells were treated with API oily sludge extracts (25, 50, and 100 μL), ETP sludge extract (50, 100, and 200 μL), DMSO (negative control) and benzo(a)pyrene (Sigma, USA) as a positive control at a final concentration of 30 $\mu\text{g}/\text{mL}$ for a period of 3 h in duplicate with S9 mix and further incubated for 48 h at 37 $^{\circ}\text{C}$, 5% CO_2 atmosphere. Three h prior to harvest of cultures were treated with colchicine (1 $\mu\text{g}/\text{mL}$). After 24 h and 48 h treatment, cultures were lysed with hypotonic KCl (0.56%) and fixed with Cornoy's Fixative, slides were prepared and analyzed as per the method of Api and San^[10].

p⁵³ Protein Isolation, Western Blotting and Immunodetection

After cells had been grown for 24 h, the cultured cells were treated with oily sludge extract (25, 50, and 100 μL) and DMSO (negative control) and benzo(a)pyrene (Sigma Corp., USA) as a positive control at a final concentration of 10 $\mu\text{mol}/\text{L}$ for a period of three hours in duplicate with S9 mix and further incubated for 24 h and 48 h at 37 $^{\circ}\text{C}$, 5% CO_2 atmosphere. Thereafter, cells were lysed in PBSTDS buffer pH 7.4 by three cycles of freezing and thawing. The protein samples (20 μg) were fractionated in 10% (w/v) polyacrylamide gel electrophoresis, then transferred to hybond ECL membrane (Amersham International, UK) using electroblotting apparatus. The membranes were incubated with an anti p⁵³ mutant monoclonal antibody Ab-3 (Amersham International, UK) for p⁵³ protein generated by immunizing Balb/c mice with p⁵³ β -galactosidase fusion protein and fused with SP₂ mouse myeloma cells. The treated membranes were thereafter treated with horseradish peroxidase-conjugated with anti-mouse IgG antibody (secondary antibody). Immunodetection was carried out using the ECL Western Blotting analysis system and the bands developed were photographed, which were then compared with negative control.

Apoptosis [DNA Fragmentation Assay]

Cultured cells grown for 24 h described under cell cultures, were exposed to oily sludge extract (25, 50, and 100 μL) and DMSO (negative control) for different time intervals 24 h and 48 h in Ham's F-12 medium in duplicate with S-9 mix in a final volume of 10.0 mL. After treatment, cells were washed with PBS, the cellular DNA was isolated and analyzed according to the method of Martikainen *et al.*^[11]. The cell pellets were resuspended in 1.0 mL

of lysis buffer and digested with proteinase-K (300 $\mu\text{g/mL}$, Sigma Laboratories, USA) for 18 h at 37°C. After digestion, the DNA was sequentially extracted with phenol, phenol:chloroform (1:1) and chloroform and then precipitated with ethanol. The precipitate was dissolved in TE buffer and treated with 100 $\mu\text{g/mL}$ of RNase (Sigma Laboratories, USA) at 50°C for 1 h. DNA was separated electrophoretically on 1.5% agarose gel, stained with ethidium bromide (0.5 $\mu\text{g/mL}$), and the gels were visualized under ultraviolet illumination, then photographed and the bands were compared with control and DNA ladder.

Statistical Analysis

The results were statistically analyzed using ANOVA one way test with “Analyze it Software” and expressed as P values which were considered non-significant when $P>0.05$.

RESULTS

Chemical Characterization

Table 1. and Fig. 1 show the individual polycyclic aromatic hydrocarbons identified and quantified based on retention time and mass spectra using gas chromatograph/mass spectrometer (GC-MS).

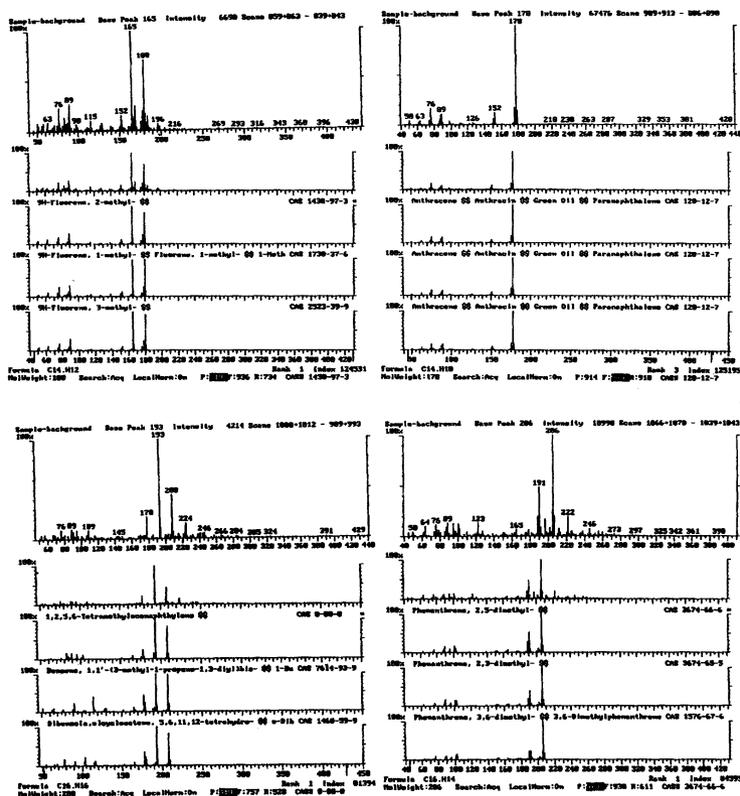


Fig. 1. Reconstructed total ion chromatograph and mass fragmentation pattern (GC-MS) of ETP sludge and oily sludges collected from petroleum refinery and petrochemical industries.

TABLE 1

List of Analytical Proved Compounds and their Quantities
(Identified and Quantified by Gas Chromatography/Mass Spectroscopy)

Petroleum Refinery Oily Sludge Extract		
Sr. No	Compound Name	Quantity (mg/kg)
1.	phenanthrene	27 mg/kg
2.	Fluoranthene	31 mg/kg
3.	Benzo(a)pyrene	19 mg/kg
4.	Chrysene	11 mg/kg
5.	anthracene	30.8 mg/kg
6.	naphthalene, 1,7-dimethyl	Not Quantified
7.	Acenaphthene	NQ
8.	1,3,6-trimethyl naphthalene	NQ
9.	Fluorene	NQ
10.	9H-fluorene, 3-methyl	NQ
11.	Dibenzothiophene	NQ
12.	1-methyl anthracene	NQ
13.	1,2,5,6-tetramethylacenaphthylene	NQ
14.	2,3-dimethyl, phenanthrene	NQ
Petrochemical Oily Sludge Extract		
1.	anthracene	92.8 mg/kg
2.	Fluoranthene	34 mg/kg
3.	benz(a)anthracene	29 mg/kg
4.	benzo(a)pyrene	42 mg/kg
5.	9H-fluorene, 2-methyl	NQ
6.	1,2,5,6-tetramethyl acenaphthylene	NQ
7.	phenanthrene-2,5,-dimethyl	NQ
8.	pyrene-1,3-dimethyl	NQ
Petroleum Refinery ETP Sludge Extract		
1.	1. 2-ethyl-7-methyl benzo (b) thiophene	NQ
2.	1,4-dimethyl naphthalene	NQ

Note. NQ: not quantified.

API Oily Sludge Extract (Petroleum Refinery)

The oily sludge extract caused statistically significant DNA strand break at 25 μL ($P < 0.01$), while 50 μL and 100 μL concentration caused highly significant level of DNA strand break ($P < 0.001$) (Fig. 2). It also caused significant level of chromosomal aberration after 24 h treatment at all the doses (25, 50, and 100 μL) (Table 2). A dose dependent increase in p^{53} protein in CHO-K1 cell cultures could be seen with the sludge extract in 24 h after treatment (Fig. 3). The DNA fragmentation with response to p^{53} protein accumulation was observed with 100 μL concentration 24 h after treatment (Fig. 4), while treatment with 25 μL and 50 μL did not cause DNA fragmentation even after 48 h treatment.

TABLE 2

Chromosomal Aberration Caused by API-Oily Sludge Extract in CHO-K1 Cells (Petroleum Refinery)

Dose	No. of Metaphases	% Aberrated Cells ($\bar{x} \pm s$)	No. of Aberration	Types of Aberration						MI
				CTb	CHb	Ex	Ring	F	me	
DMOS 0.1%	100	2.2 ± 0.837	3	1	ND	ND	ND	ND	2	6.1
Sludge extract										
25 L	100	11.4 ± 2.702*	12	4	2	1	2	1	2	4.1
50 L	100	17.6 ± 5.899**	18	11	2	3	2	ND	ND	4.0
100 L	100	29.4 ± 3.286**	38.0	21	2	5	2	6	1	3.2
Positive control										
B(a)P(30 g/mL)	100	45.8 ± 5.119**	56.0	29	3	9	5	5	5	2.3

Note. The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. ND: Not detected; CTb: Chromatid break; CHb: Chromosomal break; Ex: Exchange; F: Fragments; me: Minutes; MI: Mitotic index; $P < 0.05$ statistically significant; * $P < 0.01$, ** $P < 0.001$.

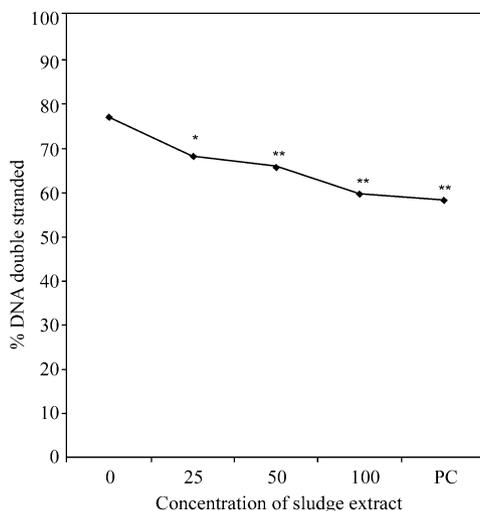


FIG. 2. DNA Strand break induced by API-oily sludge extract in CHO-K1 cells (petroleum refinery). The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. * $P < 0.01$, ** $P < 0.001$.

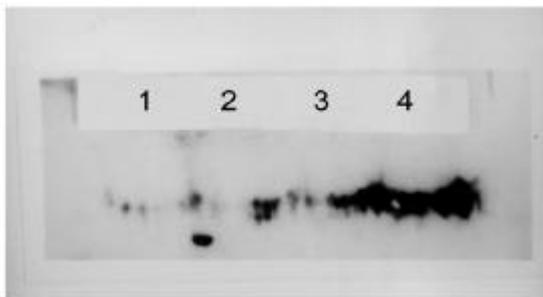


FIG. 3. Immunoblot analysis showing p⁵³ protein induction in CHO-K1 cell culture exposed to API-oily sludge extract (petroleum refinery) Lane 1: negative control; Lane 2: oily sludge extract 25 µL; Lane 3: oily sludge extract 50 µL; Lane 4: oily sludge extract 100 µL.

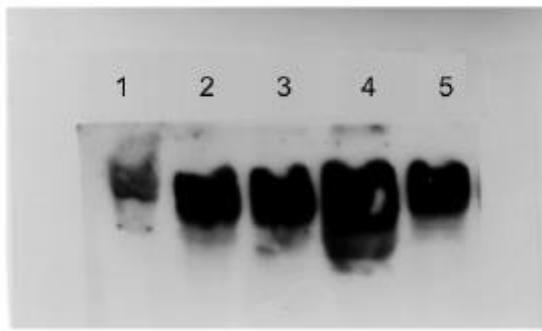


FIG. 4. DNA fragmentation in genomic DNA of CHO-K1 cell culture exposed to API-oily sludge extract (petroleum refinery) Lane1: molecular weight marker; Lane 2: oily sludge extract 25 μL ; Lane 3: oily sludge extract 50 μL ; Lane 4: oily sludge extract 100 μL ; Lane 5: negative control; Lane 6: positive control [benzo (a) pyrene, 10 $\mu\text{g}/\text{mL}$].

API Oily Sludge Extract (Petrochemical Industry)

Fig. 5 show the statistically significant level ($P < 0.002$) of DNA strand break caused by the sludge extract at 10 μL concentration, while highly significant level of ($P < 0.001$) DNA unwinding was observed at 25 μL , 50 μL , and 100 μL doses of the extract compared with negative control. The statistically significant level of chromosomal aberration was observed ($P < 0.001$) with sludge extract at all the concentrations tested (25 μL , 50 μL , and 100 μL) (Table 3). The dose dependent increase in p^{53} protein accumulation was also observed with the oily sludge extract in 24 h after treatment (Fig. 6). The sludge extract could also cause dose dependent increase in apoptosis after 24 h treatment (Fig. 7).

TABLE 3

Chromosomal Aberration Induced by API-Oily Sludge From a Petrochemical Industry in CHO-K1 Cells

Doses	No. of Metaphases	% Aberrated Cells ($\bar{x} \pm s$)	No. of Aberration	Types of Aberration						MI
				CTb	CHb	Ex	Ring	F	me	
DMOS 0.1%	100	2.6 ± 1.140	3	1	ND	1	ND	ND	1	6.2
Sludge extract 25 L	100	$10.6 \pm 3.647^*$	16.2	8	1	2	ND	2	3	4.6
50 L	100	$23.0 \pm 4.848^{**}$	28.0	14	2	3	2	4	3	3.5
100 L	100	$33.6 \pm 6.58^{**}$	42.6	16	7	4	5	8	3	2.6
Positive control B(a)P(30 g/mL)	100	$45.8 \pm 5.119^{**}$	56.0	29	3	9	5	5	5	2.3

Note. The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. ND: Not detected; CTb: Chromatid break; CHb: Chromosomal break; Ex: Exchange; F: Fragments; me: Minutes; MI: Mitotic index; $P < 0.05$ statistically significant; $^{**}P < 0.001$.

ETP Sludge (Petrol Refinery)

ETP sludge extract at 200 μL dose could cause significant DNA strand break ($P < 0.01$) (Fig. 8) while the lower doses (50 μL and 100 μL) could not cause significant DNA damage in Chinese hamster ovary cells. The sludge extract could only cause statistically insignificant

level of ($P>0.05$) (Table 4) chromosomal aberration. Moreover, the sludge extract could not induce p^{53} protein accumulation or apoptosis at all the concentrations tested.

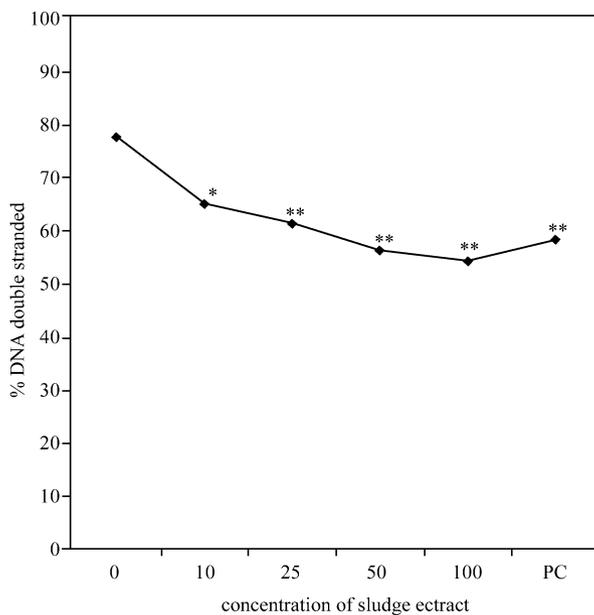


FIG. 5. DNA Strand break induced by API-oily sludge extract in CHO-K1 cells (petrochemical industry). The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. * $P < 0.002$, ** $P < 0.001$.

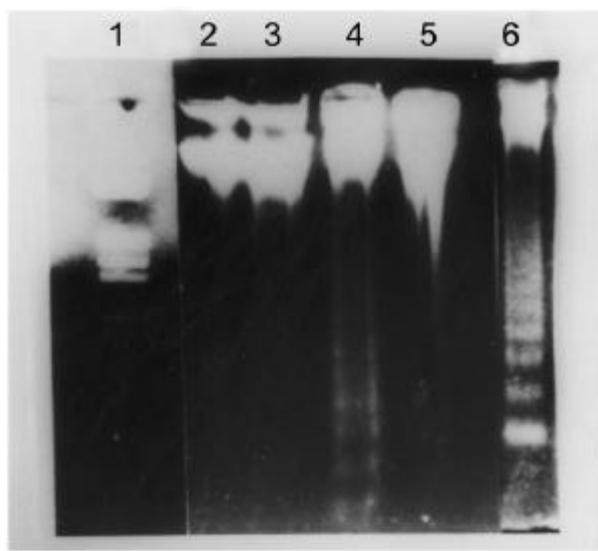


FIG. 6. Immunoblot analysis showing p^{53} protein induction in CHO-K1 cell culture exposed to API-oily sludge extract (petrochemical industry) Lane 1: negative control; Lane 2: oily sludge extract 10 μ L; Lane 3: oily sludge extract 25 μ L; Lane 4: oily sludge extract 50 μ L; Lane 5: benzo(a)pyrene 10 μ mol/L.

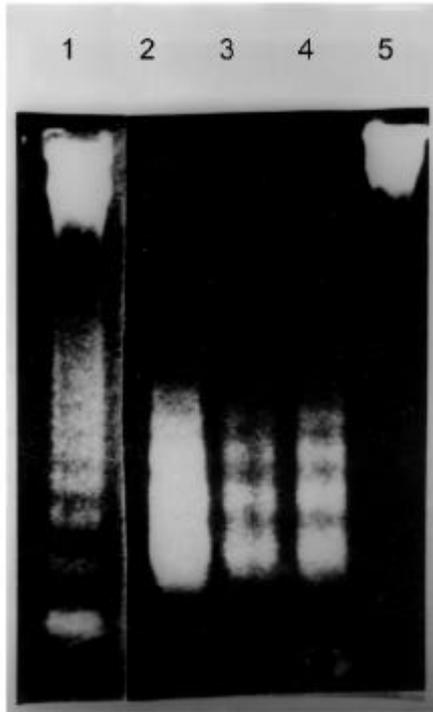


FIG. 7. DNA fragmentation in genomic DNA of CHO-K1 cell culture exposed to API-oily sludge extract (petrochemical industry) Lane1: positive control [benzo(a)pyrene, 10 $\mu\text{g}/\text{mL}$]; Lane 2: oily sludge extract 10 μL ; Lane 3: oily sludge extract 25 μL ; Lane 4: oily sludge extract 50 μL ; Lane 5: negative control.

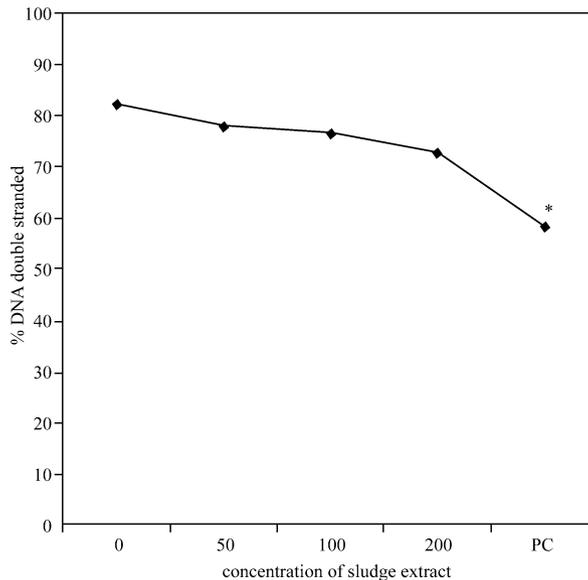


FIG. 8. DNA Strand break induced by ETP sludge extract in CHO-K1 cells. The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. * $P < 0.01$, ** $P < 0.001$.

TABLE 4

Chromosomal Aberration Caused by ETP Sludge Extract in CHO-K1 Cells (Petroleum Refinery)

Doses	No. of Metaphases	% Aberrated Cells ($\bar{x} \pm s$)	No. of Aberration	Types of Aberration						MI
				CTb	CHb	Ex	Ring	F	me	
DMOS 0.1%	100	3.2 \pm 0.837	3	2	ND	ND	ND	ND	1	6.0
ETP Sludge extract 50 L	100	4.6 \pm 1.817*	5	2	ND	1	ND	ND	2	5.4
ETP Sludge extract 100 L	100	5.0 \pm 2.345*	5	2	ND	2	ND	1	2	5.2
ETP Sludge extract 200 L	100	4.6 \pm 1.517**	5	2	ND	1	NA	1	2	5.0
Positive control B(a)P(30 g/mL)	100	45.8 \pm 5.119**	56.0	29	3	9	5	5	5	2.3

Note. The results are average of five sets of experiments. Values marked with asterisks are significantly differing from negative control. ND: Not detected; CTb: Chromatid break; CHb: Chromosomal break; Ex: Exchange; F: Fragments; me: Minutes; MI: Mitotic index; $P < 0.05$ statistically significant; * $P > 0.05$; ** $P < 0.001$.

DISCUSSIONS

The oily sludge originating from petroleum refineries and petrochemical industries showed the presence of polycyclic aromatic hydrocarbons such as benzo(a)pyrene, benzo(a)anthracene, fluorene, phenanthrene, chrysene etc.^[12]. GC-MS analysis carried out in this study showed the presence of polycyclic aromatic hydrocarbons, which agrees with the earlier observation that polycyclic aromatic hydrocarbons were present in the oily sludge from petrochemical industries. Further, the studies carried out by International Agency for Research on Cancer (IARC)^[13] Radian Corp^[14] have detected the PAH compounds such as anthracene, pyrene, chrysene, benzo(a)pyrene, fluoranthene, the range of PAH levels being $< 0.0011 - 0.29 \mu\text{g}/\text{m}^3$ in refineries.

The observation made in the present study on genotoxicity of the sludge extracts is mainly due to the presence of polycyclic aromatic hydrocarbons and has been supported by the literature reports that anthracene, chrysene, phenanthrene, pyrene and benz(a)-anthracene, benzo(a)pyrene which are the main PAH compounds present in the oily sludge could individually cause DNA damage in *E. Coli* PQ 37, Mouse lymphoma L5178 Y cells with and without metabolic activation^[15,16]. Benz(a)anthracene could also induce DNA adduct in human peripheral blood lymphocytes without metabolic activation^[17], sister chromatid exchange in Chinese hamster bone marrow cells^[18] and benz(a)anthracene could induce sister chromatid exchange in Chinese hamster ovary cells, rat liver epithelial ARL 18 and Chinese hamster V79 cells^[19-21]. Fluoranthene has been reported to cause SCE in CHO-K1 cells without metabolic activation^[22-24].

Phenanthrene induced mutagenicity in human lymphoblastoid TK6 cells with S-9^[25,26] and chromosomal aberration in Chinese hamster 79-4 cells without S-9^[27]. Benzo(a)pyrene could induce mutagenicity in mouse Balb/C-3T3 cells^[28], in Chinese hamster lung cells V79^[29] MN, SCE in human hepatoma cells^[30] SCE in rat pleural mesothelioma cells^[31] SCE in Chinese hamster V 79 cells^[21] Chinese hamster epithelial liver cells^[32] in Chinese hamster

ovary cells^[22,33] and in human epithelial EUE cells^[34] without metabolic activation. Benzo(a)pyrene could induce mutagenicity in Chinese hamster V79 cells^[35-37] and in mouse lymphoma L5178 cells with metabolic activation^[38]. Benzo(a)pyrene could induce mutagenicity with and without metabolic activation in Chinese hamster ovary^[39] in human lymphoblastoid cells K-6^[40], Chinese hamster lung cells^[41] and also DNA adduct formation in hamster trachea epithelial cells^[42]. Fluoranthene and pyrene have been reported to induce SCE in Chinese hamster ovary (CHO-K1) cells^[43,18,20-22,24]. Pyrene also induced chromosomal aberration in Chinese hamster V79-4 cells without metabolic activation^[27].

The present study has utilized p⁵³ protein induction and apoptosis parameters in addition to the DNA strand break assay and chromosomal aberration test. The tumor suppressor gene p⁵³ plays an important role in guarding genomic integrity. In response to environmental stress, the induced gene product of p⁵³ protein functions as a transcription factor in transactivating genes involved in arresting the cell cycle, as well as a facilitator of DNA repair. Sterum *et al.*^[44] have stated that p⁵³ induction is found to be time dependent and the accumulation occurs after 24 h of the onset of treatment. In the present investigation, the induction of p⁵³ protein was observed in 24 h after onset of the treatment. This observation is supported by that of Vaziri and Faller^[45] who reported that benzo(a)pyrene induced elevated level of p⁵³ protein and growth arrest in Murine Swiss 3T3 cells.

Fernandez and Haridon^[46] studied the genotoxicity of benzo(a)pyrene and an oil refinery effluent in the larvae and embryos of newt and observed the genotoxicity in the effluent and in B(a)P, whose findings suggested that metabolic activation was not necessary for hydrocarbons to induce toxic effects. However, in this study we have used metabolic activation system (S-9 fraction). In addition, the p⁵³ protein can be localized in the cytoplasm as well as in the nucleus, especially when there is active production of p⁵³ protein going on, as in MCF-7 cells after serum stimulation^[47]. Benzo(a)pyrene is a classical complete carcinogen, which can act both as an initiator and promoter in the carcinogenic process for PAH carcinogenesis, and the consequences of DNA binding of activated PAH metabolites are of great interest. Nevertheless, p⁵³ mutation alone was designated to act as a molecular “dosimetry” of genotoxic carcinogen exposure, providing information about the molecular epidemiology of human exposure to carcinogens^[4,48]. Exposure to chemical carcinogens is a crucial step in the development of many human malignancies and further, a more thorough knowledge of the putative protective role of p⁵³ protein is needed^[49].

Further, Shaw^[50] has observed that, although a number of end points such as micronucleus, chromosomal aberration and sister chromatid exchange have been identified and are being used as a single assay to express genotoxicity of chemicals and wastes, all end points could not be simultaneously induced by one compound under a given experimental condition. Therefore, the present investigation and literature surveyed strongly suggest that for genotoxicity and carcinogenicity evaluation of chemicals and environmental complex mixtures, instead of single assay system, a battery of tests should be used.

The outcome of this research strongly suggests that the oily sludges generated from petroleum refineries and petrochemical industries are required to be carefully handled after complying with all safety requirements as these sludges contain genotoxic polycyclic aromatic hydrocarbons (priority pollutants as identified by USEPA). Further studies are in progress as to characterize the genotoxicity of single compounds (present in the waste) alone and in combination using the same test systems to identify the causative agents of the waste mixture and to analyze possible interactions (synergistic/ antagonistic effects) between individual components.

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