Genotoxic Effects of Chlorpyrifos, Cypermethrin, Endosulfan and 2,4-D on Human Peripheral Lymphocytes Cultured from Smokers and Nonsmokers

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ABSTRACT: Pesticides often cause environmental pollution and adverse effects on human health. We have chosen four structurally different pesticides (endosulfan, an organochlorine pesticide; chlorpyrifos, an organophosphate insecticide; cypermethrin, type II pyrethroid insecticide, and 2,4-dichlorophenoxyacetic acid, a chlorinated aromatic hydrocarbon acid pesticide) to examine and compare their effects on DNA damage in acutely cultured human lymphocytes by the comet assay. In addition, possible differences in response between smoking and nonsmoking subjects were also investigated. Venous blood samples were obtained from healthy male nonsmoker ($n = 7$) and smoker ($n = 8$) donors. Primary cultures of lymphocytes were prepared and test groups were treated with three different concentrations (1, 5, and 10 μM) of endosulfan, chlorpyrifos, cypermehrin, and 2,4-D. DNA damage was assessed by alkaline comet assay. We determined an increase in the ratio of DNA migration in human lymphocyte cell cultures as a result of treatment with cypermethrin, 2,4-D and chlorpyrifos at high concentration. Endosulfan had no significant genotoxic effect even at 10 μM concentration. We suggest that chlorpyrifos and cypermethrin are more potentially genotoxic than endosulfan and 2,4-D. Our findings also indicate that the only significant DNA damage between smokers and nonsmokers was observed in the 2,4-D-treated group. © 2010 Wiley Periodicals, Inc. Environ Toxicol 26: 433–442, 2011.

Keywords: genotoxicity; chlorpyrifos; cypermethrin; endosulfan; human lymphocytes; smoking and comet assay

INTRODUCTION

Pesticides have been widely used and often cause environmental pollution. There have been many studies in the literature reporting adverse effects of such chemical compounds on environmental and human health. Exposure to some of these pesticides may lead to alterations in the genetic material thereby causing mutagenicity, carcinogenicity, teratogenicity, and immunotoxicity (Blair and Zahm, 1995; Garry et al., 1996; Meinert et al., 2000; Thrasher et al., 2002; Lafiura et al., 2007).

Endosulfan, a broad-spectrum organochlorine pesticide is classified as hazardous both by World Health Organization (ATSDR, 2002; WHO, 2005) and the US Environmental Protection Agency (US EPA, 2002). It is persistent in the environment and bioaccumulative in organisms because of its lipophilic features (Naqvi and Vaisnavi, 1993; Aggarwal et al., 2008). High levels of endosulfan residues have been detected in several feed such as fish and bovine milk (Kole et al., 2001; Nag and Raikwar, 2008). Endosulfan is largely used worldwide and blamed for various health problems (Venkateswarlu et al., 2000; Delen et al., 2005; Li and

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Macdonald, 2005; Karatas et al., 2006). Neurotoxicity is thought to be the essential cause of acute endosulfan toxicity as evidenced from endosulfan poisoning cases (Paul et al., 1995; Chugh et al., 1998). Subchronic exposure to endosulfan has been shown to cause prominent suppression of humoral and cell-mediated immune responses in rats (Banerjee and Hussain, 1986). Endosulfan-induced apoptosis in human T-cells leukemic cell lines has also been reported (Kannan et al., 2000).

Chlorpyrifos is an organophosphate (OP) insecticide and has been widely used since 1965 (US EPA, 2006a). It has been claimed that chlorpyrifos is nonteratogenic (Deacon et al., 1980), noncarcinogenic (Yano et al., 2000), and does not adversely affect reproductive functions (Breslin et al., 1996). It is well-known that OP pesticides exert their toxic effects mainly through inhibition of acetyl cholinesterase in the nervous system (Al-Saleh, 1994). Chlorpyrifos has been reported to affect chromosomes in mammalian cell cultures (Jamil et al., 2004). However, results of various studies on the genotoxic effects of chlorpyriphos appear to be controversial (Gollapudi et al., 1995; Akcha et al., 2008).

Cypermethrin is a type II pyrethroid and more potent insecticide because of the α-cyano group in their structure (Vijverberg et al., 1982; Tabarean and Narahashi, 1998). It is widely used in agriculture, forestry, and household products because of some favorable properties such as wide spectrum, relatively low mammalian toxicity, and rapid biodegradability (Parker et al., 1984; Vijverberg and Van den Bercken, 1990). However, widespread application of pyrethroids has caused to toxic effects in plants, animals, and human beings (US EPA, 2006b). Cypermethrin has been reported to exert carcinogenic activity in both sexes of Swiss albino mice (Shukla et al., 2002). It has been suggested that cypermethrin causes free radical-mediated damage in erythrocytes (Kale et al., 1999) and brain and liver tissues (Giray et al., 2001). The genotoxic and/or cytotoxic potential of cypermethrin has been shown in previous studies (Patel et al., 2006; Suman et al., 2006). It slightly increased the number of micronucleated cells in whole blood lymphocyte cultures in human (Surralles et al., 1995). *In vitro* and *in vivo* studies have shown that cypermethrin induces chromosomal aberrations and micronucleus formation in mouse bone marrow and spleen (Amer and Aboul-ela, 1985; Amer et al., 1993).

The 2,4-dichlorophenoxyacetic acid (2,4-D), a chlorinated aromatic hydrocarbon acid pesticide, has been in extensive use since 1944, in agriculture for broad-leaved weeds, control of woody plants, and reforestation programs (IARC, 1977). It is classified as group II-B carcinogenic agent by IARC (1998). Major target organ systems of 2,4-D toxicity are the central nervous system and the cardiovascular system (Osterloh et al., 1983). Despite the toxicity of 2,4-D, has been a topic of extensive research, mechanism of toxic action caused to carcinogenicity, mutagenicity, or genotoxicity is poorly understood (IARC, 1982; Nesnow et al., 1987). It has been documented that 2,4-D is hepatotoxic: it induces lipid peroxidation and cell death in rat hepatocytes (Palmeira et al., 1995) and causes chromosomal aberrations in human lymphocytes (Korte and Jalal, 1982). In an additional study, it has been reported that 2,4-D or other phenoxy herbicides may cause non-Hodgkin's lymphoma through lymphocyte or immune alteration (Faustini et al., 1996). Blakley et al. (1998) suggested that alterations in lymphocyte proliferation may be a possible mechanism since lymphocytes are one of the targets of 2,4-D. On the other hand, negative findings in rat and hamster models using different techniques such as micronucleus (MN) assay, hypoxanthine-guanine-phospho-ribosyl transferase (HGPRT), and Ames test have also been reported (Charles et al., 1999a,b; Gollapudi et al., 1999).

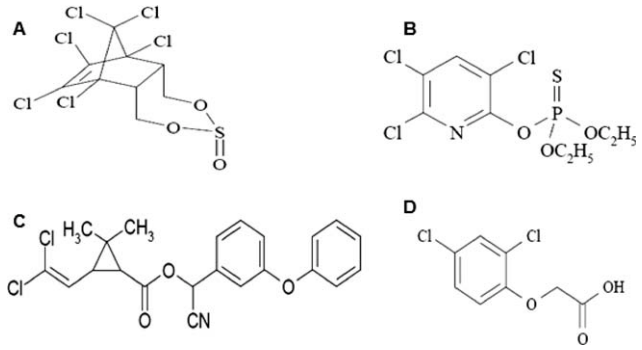

Fig. 1. Chemical structures of endosulfan (A), chlorpyrifos (B), cypermethrin (C), and 2,4-dichlorophenoxyacetic acid (D) used in the present study.

There is evidence for higher chromosomal damage in the somatic cells of smokers than in nonsmokers (IARC, 1986). The result of structural chromosome aberration test (SCA), micronucleus test (MN), and sister chromatid exchanges (SCE) have indicated that scores of SCE, SCA, and MN in lymphocytes of smokers are higher than nonsmokers (Bender et al., 1988; Bonassi et al., 2003). Conversely, some other studies have suggested that smoking has no additional effect on genetic damage (Undeger and Basaran, 2002; Sailaja et al., 2006).

Various techniques (e.g., MN and SCA) have been used to identify substances with genotoxic activity. A more useful method for assessing the level of DNA damage is suggested to be the alkaline single-cell gel electrophoresis, also known as comet assay (Singh et al., 1988; Saleha Banu et al., 2001). This method has previously been used to assess genotoxicity of cigarette smoke on human lymphocytes (Faust et al., 2004; Fracasso et al., 2006; de Assis et al., 2009).

In the present study, four structurally different pesticides (Fig. 1), endosulfan, an organochlorine pesticide; chlorpyrifos, an organophosphate insecticide; cypermethrin, type II pyrethroid insecticide, and 2,4-D, a chlorinated aromatic hydrocarbon acid pesticide) were selected to examine and compare their effects on DNA damage after short-term

exposure of human lymphocytes by the comet assay. In addition, possible differences in response between smoking and nonsmoking subjects were also sought.

MATERIALS AND METHODS

Chemicals and Reagents

Lymphorep was purchased from Axis-Shield (Oslo, Norway). Newborn calf serum (FCS) was obtained from Biological Industries (Israel). NaCl, NaOH, dimethyl sulfoxide (DMSO), and HCl were provided by Merck (Dormstadt, Germany). Pesticides, other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Bidistilled water was used in all the experiments. Pesticides were dissolved in DMSO (Oakes and Pollak, 2000) so that the final concentration of DMSO was never greater than 0.1% (v/v).

Subjects

Fifteen healthy male nonsmoker ($n = 7$) and smoker ($n = 8$) donors between 20 and 30 years of age (mean: 27.14 ± 2.85 and 27.57 ± 2.50 years, respectively; Table I) provided blood samples. Subjects had not been exposed to radiation or drugs for at least 6 months prior to the study. This study was approved by the local ethics committee on animal experimentation.

Blood Sampling and Cell Preparation

Whole peripheral blood samples were obtained by venous puncture. The isolation of lymphocytes was performed by centrifugation in a density gradient of lymphorep (15 min, 200 × *g*) and the cells washed with RPMI-1640 medium supplemented with 10% FCS. To determine cell viability 0.4% trypan blue was used and the cells were counted with a hemocytometer. After cells were prepared, test groups were treated with three different concentrations (1, 5, and 10 μM) of endosulfan, chlorpyrifos, cypermehrin, and 2,4-D and the vehicle control group was treated with DMSO for 1 h at 37°C in a Nuaire humidified carbon dioxide incubator (Plymouth, MN). As a positive control, lymphocytes were treated with 50 μM H_2O_2 and incubated for 5 min on ice. After incubation, cell viability was again examined using trypan blue.

Comet Assay

The alkaline comet assay technique was performed as described in our previous study (Sandal et al., 2008). Briefly, microscope slides were precoated with 0.75% (w/v) high melting point agarose at PBS. The lymphocytes were mixed with 100 μL of 0.5% (w/v) low melting point agarose (LMA) at 37°C and the cell suspension was pipetted and layered onto precoated slides. The slides were immediately covered with a large cover slip and kept at 4°C for 5 min to allow the agarose to solidify. After the cover slips were removed, the slides were immersed in ice cold freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium sarcosinate, pH = 10) with 1% Triton X-100 and 10% DMSO for 1 h. Every dose of each compound was repeated eight times (i.e., testing on blood samples of eight different subjects).

Electrophoresis

After lysis, the slides were placed in a horizontal gel electrophoresis tank (Bio-Rad, USA) positioned close to the anode and covered with fresh alkaline electrophoresis buffer

TABLE I. Patients' characteristics

Subjects	Gender	Age (years)	Profession	Smoking (py)	Alcohol (g/day)[a]
1	m	30	Research Assistant	–	–
2	m	29	Research Assistant	10	–
3	m	30	Research Assistant	–	–
4	m	27	Research Assistant	–	–
5	m	29	Security Guard	15	–
6	m	22	Security Guard	–	–
7	m	30	Security Guard	18	–
8	m	29	Security Guard	10	–
9	m	29	Accountant	–	–
10	m	27	Computer Operator	12	–
11	m	25	Programmer	10	–
12	m	26	Clerk	–	–
13	m	26	Clerk	–	–
14	m	30	Driver	16	–
15	m	23	Technician	8	–

m: male.

[a] Alcohol consumption is given in grams per day [g/d] and smoking habit in pack years [py] (1 pack year = 365 days × 20 cigarettes/day; e.g.: 20 py = 1 pack/day for 20 years or = 2 packs/day for 10 years etc.).

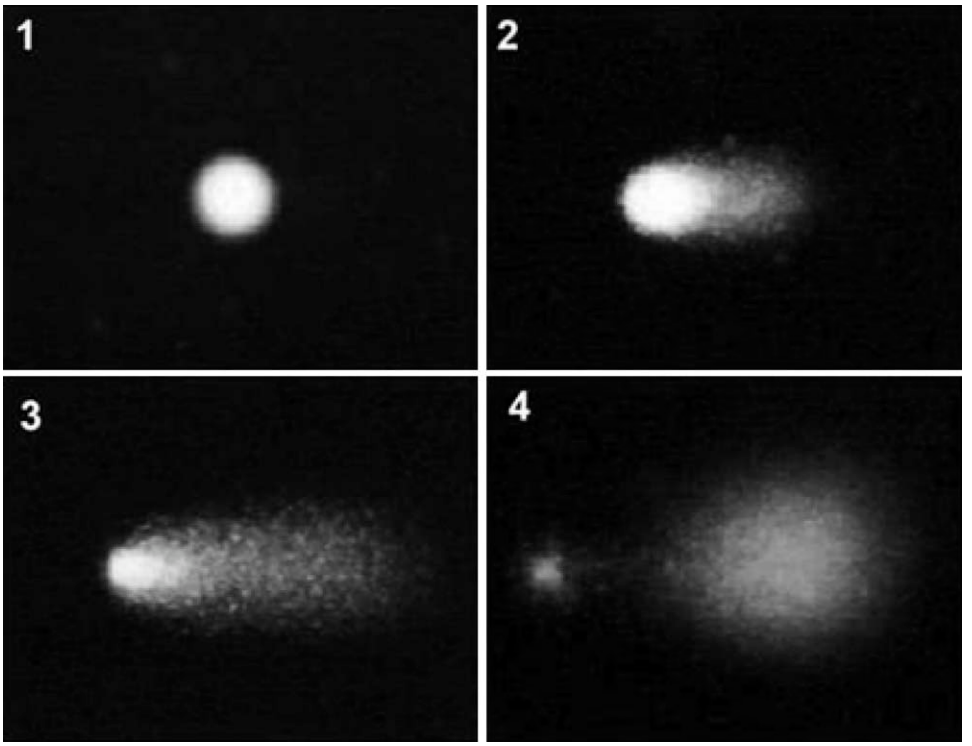

Fig. 2. Undamaged (1) low damaged (2), moderate damaged (3), and high damaged (4).

solution containing 1 mM EDTA, 300 mM NaOH (pH > 13) for 20 min at 4°C. Electrophoresis was conducted at 25 V (0.83 V/cm) and 300 mA for 20 min. After electrophoresis, the slides were washed three times with neutralizing buffer (0.4 M Tris, pH = 7.5) at 4°C for 5 min each. Then the slides were dried and stained with 20 μg mL^{-1} ethidium bromide and covered with a cover slip. The slides were incubated at 4°C in a refrigerator for 20 min before scoring. All of the steps were conducted in the dark to prevent additional DNA damage.

Slide Scoring

Fifty cells per slide and two slides per sample were scored to evaluate DNA damage for each pesticide concentrations. The slides were examined under a fluorescent microscope (Zeiss, Germany) equipped with suitable filters at 200× magnification. The slides were blinded to the scorer. The cells were classified by eye in to four categories on the basis of the extent of DNA migration, undamaged (UD), low damage (LD), moderate damage (MD), and high damage (HD, Fig. 2). Total comet scores (TCSs) were calculated on 100 randomly selected cells as: 1 × UD + 2 × LD + 3 × MD + 4 × HD.

Statistical Analysis

All results are expressed as mean ± SD. The data were statistically analyzed by using One-Way ANOVA, followed by the Tukey HSD test. SPSS 15.0 for Windows was utilized for analysis. Statistical significance was considered at $P < 0.05$.

RESULTS

The cell viability was above 90% in all groups both before and after incubation with pesticides (data not presented).

All results are presented in Table II (nonsmokers) and Table III (smokers). The DMSO control values did not significantly differ between the smokers and nonsmokers. The positive control (H_2O_2) shows a striking increase in DNA damage, especially in the high damage category ($P < 0.001$).

Figure 3(A) shows the effects of cypermethrin in nonsmoking donors, whereas Figure 3(B) illustrates results from smoking persons, assessed at 1 h after treatment. Cypermethrin at a concentration of 10 μM caused significant increases in DNA damage in both smoking and nonsmoking groups compare to control and DMSO ($P < 0.05$). The lower concentration of cypermethrin (1 and 5 μM) had no significant effects in both groups.

The effects of chlorpyrifos on DNA migration in lymphocyte cell cultures of nonsmoking donors are presented in Figure 4(A) while Figure 4(B) shows result of smoking donors. Chlorpyrifos at 1 and 5 μM did not significantly alter DNA damage in lymphocytes of nonsmoking and

TABLE II. Comet assay scores in nonsmokers (mean ± SD)

	UD	LD	MD	HD	TCS
Control	95.57 ± 0.78	5.43 ± 0.72	3.86 ± 1.82	1.71 ± 0.81	106.00 ± 1.20
DMSO	96.00 ± 0.49	4.86 ± 0.74	3.86 ± 1.26	1.14 ± 0.74	105.86 ± 1.14
H_2O_2	50.57 ± 3.26	43.14 ± 4.27	39.86 ± 2.83	58.29 ± 6.81	191.86 ± 6.16**, aa
Endosulfan (1 μM)	94.29 ± 1.41	8.29 ± 1.77	3.86 ± 1.70	1.14 ± 1.14	107.57 ± 2.25
Endosulfan (5 μM)	90.14 ± 1.06	15.14 ± 1.99	4.71 ± 1.44	2.86 ± 1.14	112.86 ± 1.74
Endosulfan (10 μM)	90.71 ± 1.61	13.43 ± 2.61	5.57 ± 1.02	2.86 ± 1.68	112.57 ± 2.29
Cypermethrin (1 μM)	93.86 ± 1.64	10.00 ± 2.47	2.57 ± 1.38	1.14 ± 0.74	107.57 ± 2.30
Cypermethrin (5 μM)	94.57 ± 1.23	7.43 ± 0.95	4.29 ± 2.25	1.14 ± 0.74	107.43 ± 2.29
Cypermethrin (10 μM)	88.29 ± 1.81	16.86 ± 2.86	6.86 ± 1.42	4.00 ± 1.23	116.00 ± 2.59*, a
2,4-D (1 μM)	94.86 ± 1.44	8.29 ± 1.71	2.57 ± 1.66	0.57 ± 0.57	106.29 ± 2.22
2,4-D (5 μM)	91.71 ± 1.90	12.00 ± 2.23	5.14 ± 2.68	2.29 ± 1.48	111.14 ± 3.33
2,4-D (10 μM)	90.43 ± 2.28	15.43 ± 3.54	5.57 ± 2.21	–	111.43 ± 2.89
Chlorpyrifos (1 μM)	94.29 ± 1.60	7.43 ± 2.26	3.86 ± 1.42	2.86 ± 2.26	108.43 ± 2.73
Chlorpyrifos (5 μM)	93.14 ± 1.44	9.43 ± 1.49	3.86 ± 1.82	3.43 ± 1.62	109.86 ± 2.55
Chlorpyrifos (10 μM)	87.43 ± 2.55	18.29 ± 4.20	7.29 ± 1.44	4.00 ± 0.87	117.00 ± 3.17*, a

Effects of various pesticides on DNA damage in nonsmoking human lymphocyte cell cultures. Undamaged (UD), low damaged (LD), moderate damaged (MD), and high damaged (HD, Fig. 1). Total comet scores (TCS).
*$P < 0.001$ and **$P < 0.05$ compared to the control group values; [a]$P < 0.001$ and [aa]$P < 0.05$ compared to the vehicle (DMSO) group values, One-Way ANOVA followed by the Tukey HSD test.

smoking persons, but at 10 μM chlorpyrifos caused significant increases in both groups ($P < 0.05$).

Figure 5 shows effects of 2,4-D on smoking human lymphocyte cells. While 10 μM dose of 2,4-D caused genotoxic effects on human lymphocytes ($P < 0.05$), 1 and 5 μM 2,4-D caused no significant change. All concentrations of 2,4-D (1, 5, and 10 μM) had no significant effects on lymphocytes of nonsmoking donors.

No significant alteration of DNA fragmentation was seen in human lymphocytes treated with endosulfan at 1, 5, and 10 μM concentrations.

DISCUSSION

In the present study, genotoxic properties of four different group of pesticides have been comparatively tested for the first time by using comet assay. We have determined an increase in the ratio of DNA migration in human lymphocyte cell cultures as a result of treatment with cypermethrin, 2,4-D, and chlorpyrifos. The lower doses (1 and 5 μM) of all pesticides tested did not cause any significant change in both smokers and nonsmokers. Endosulfan had no significant genotoxic effect even at 10 μM concentration.

TABLE III. Comet assay scores in smokers (mean ± SD)

	UD	LD	MD	HD	TCS
Control	97.00 ± 0.49	3.71 ± 0.68	2.57 ± 1.02	1.14 ± 0.74	103.14 ± 0.96
DMSO	97.86 ± 0.63	2.86 ± 0.96	0.86 ± 0.55	1.71 ± 0.81	103.71 ± 0.81
H_2O_2	50.57 ± 3.26	43.14 ± 4.27	39.86 ± 2.83	58.29 ± 6.81	191.86 ± 6.16**, aa
Endosulfan (1 μM)	97.29 ± 0.64	2.00 ± 0.62	3.00 ± 1.13	2.86 ± 1.14	104.71 ± 1.39
Endosulfan (5 μM)	96.29 ± 0.52	3.14 ± 0.40	2.14 ± 1.08	3.90 ± 1.48	104.71 ± 1.27
Endosulfan (10 μM)	93.43 ± 0.48	3.14 ± 0.96	5.57 ± 1.21	12.57 ± 2.68	109.29 ± 0.92
Cypermethrin (1 μM)	96.43 ± 0.43	3.43 ± 0.95	2.14 ± 0.86	4.57 ± 1.36	106.57 ± 1.25
Cypermethrin (5 μM)	96.86 ± 0.34	2.29 ± 0.68	2.57 ± 0.43	4.57 ± 1.36	106.29 ± 0.81
Cypermethrin (10 μM)	92.29 ± 0.78	6.00 ± 0.82	3.43 ± 1.21	14.29 ± 2.11	116.00 ± 1.90*,a
2,4-D (1 μM)	97.29 ± 0.68	2.00 ± 0.44	1.71 ± 0.89	4.57 ± 2.21	105.57 ± 1.74
2,4-D (5 μM)	96.00 ± 0.79	4.00 ± 0.87	1.71 ± 0.61	5.71 ± 2.88	107.43 ± 2.02
2,4-D (10 μM)	92.71 ± 0.61	4.57 ± 1.36	6.86 ± 1.42	10.86 ± 2.72	115.00 ± 1.59*,a
Chlorpyrifos (1 μM)	97.43 ± 0.57	2.28 ± 0.29	2.14 ± 1.08	2.86 ± 1.44	104.71 ± 0.99
Chlorpyrifos (5 μM)	97.29 ± 0.61	3.14 ± 0.74	0.86 ± 0.55	3.43 ± 1.62	107.29 ± 1.27
Chlorpyrifos (10 μM)	95.43 ± 0.57	3.43 ± 0.95	3.00 ± 0.65	7.43 ± 0.57	114.71 ± 1.49*,a

Effects of various pesticides on DNA damage in smoking human lymphocyte cell cultures. Undamaged (UD), low damaged (LD), moderate damaged (MD), and high damaged (HD, Fig. 1). Total comet scores (TCS).
*$P < 0.001$ and **$P < 0.05$ compared to the control group values; [a]$P < 0.001$ and [aa]$P < 0.05$ compared to the vehicle (DMSO) group values, One-way ANOVA followed by the Tukey HSD test.

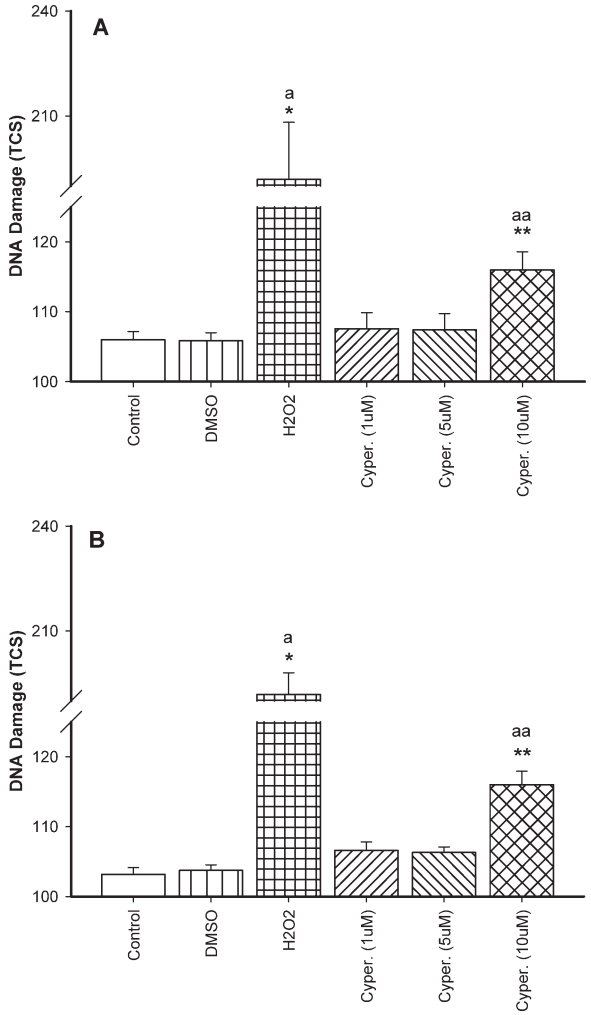

Fig. 3. The DNA damage in nonsmoking (A) and smoking (B) human lymphocyte cell cultures after 1 h from treatment with different concentrations of cypermethrin (One-Way ANOVA, post hoc Tukey HSD test; mean ± SD). $^{*}P < 0.001$, $^{**}P < 0.05$ compared to control group values; $^{a}P < 0.001$ and $^{aa}P < 0.05$ compared to the vehicle (DMSO) group values, One-Way ANOVA followed by the Tukey HSD test.

Results of several studies on the genotoxicity of endosulfan have demonstrated inconsistent results (Chaudhuri et al., 1999; Falck et al., 1999). Das et al. (2007) reported that 0.5–4.0 μM concentrations of endosulfan caused significant DNA damage as indicated by visible tail lengths. In the present study, we have shown that endosulfan had no significant effect on DNA damage in lymphocyte cell cultures obtained from smoker and nonsmoker subjects. Discrepancy between our findings and those reported by others (Akay et al., 1999; Jamil et al., 2004) may be attributed to selected cell types and concentrations of endosulfan.

Patel et al. (2006) have demonstrated that cypermethrin induces systemic genotoxicity in mammals as it causes DNA damage in vital organs like brain, liver, and kidney, apart from that in the hematopoietic system. It has been reported that cypermethrin induced a clear significant positive dose-dependent increase in DNA damage in the rat liver cells (El-Khatib et al., 2006). Undeger and Basaran (2002) observed DNA damage in lymphocytes of workers occupationally exposed to cypermethrin. Later, the same group confirmed that cypermethrin and permethrin significantly increased DNA damage in human lymphocytes (Undeger and Basaran, 2005). Cypermethrin induced DNA damage in Chinese hamster ovary cells a dose-dependent manner (Patel et al. 2007) and increased frequency of sister chromatic exchange in mouse bone marrow cells (Giri et al., 2003). Our findings show that cypermethrin causes

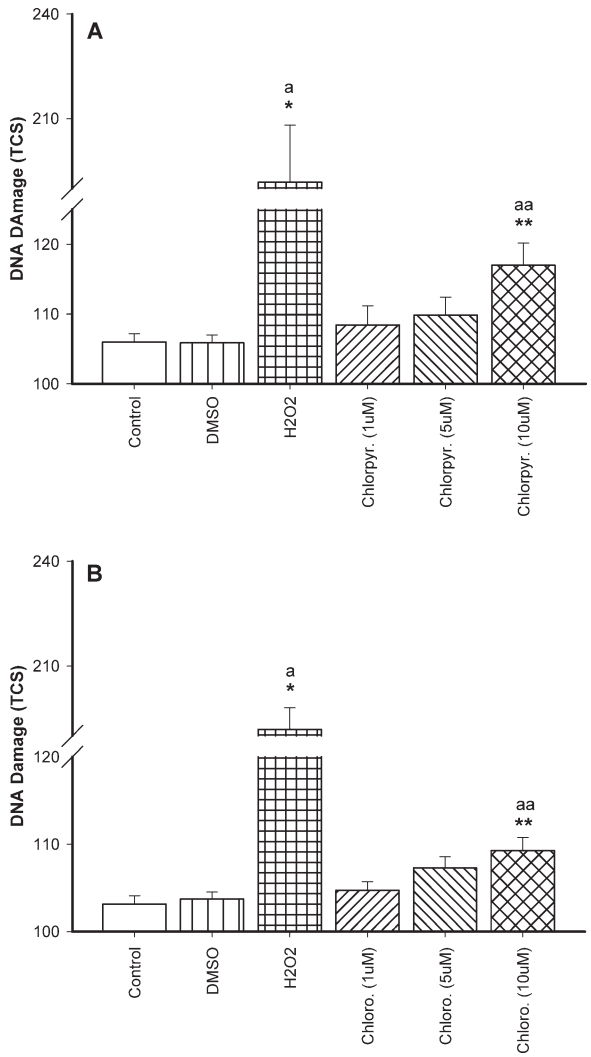

Fig. 4. The DNA damage in nonsmoking (A) and smoking (B) human lymphocyte cell cultures after 1 h from treatment with different concentrations of chlorpyrifos (One-Way ANOVA, post hoc Tukey HSD test; mean ± SD). $^{*}P < 0.001$, $^{**}P < 0.05$ compared to control group values; $^{a}P < 0.001$ and $^{aa}P < 0.05$ compared to the vehicle (DMSO) group values, One-Way ANOVA followed by the Tukey HSD test.

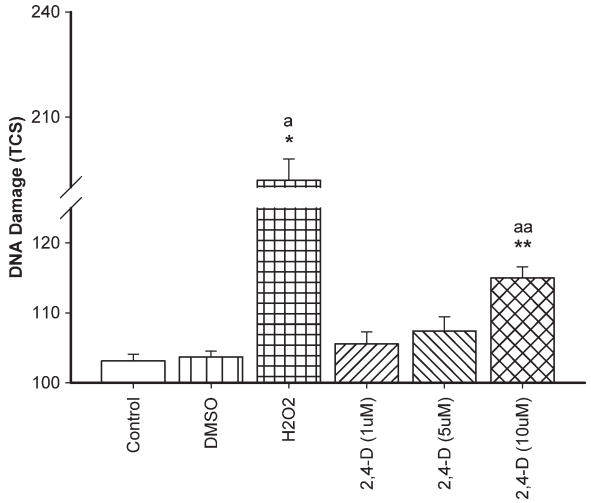

Fig. 5. The DNA damage in smoking human lymphocyte cell cultures after 1 h from treatment with different concentrations of 2,4-D (One-Way ANOVA, post hoc Tukey HSD test; mean ± SD). $^{*}P < 0.001$, $^{**}P < 0.05$ compared to control group values; $^{a}P < 0.001$ and $^{aa}P < 0.05$ compared to the vehicle (DMSO) group values, One-Way ANOVA followed by the Tukey HSD test.

DNA damage in human lymphocytes and thus appear to be consistent with earlier human and experimental studies. We have also determined that there was no significant difference in terms of cypermethrin-induced DNA damage between smokers and nonsmokers.

Recent studies have demonstrated that OP pesticides at sublethal acute doses both affect several biochemical pathways and exert genotoxic effects (Al-Saleh, 1994; Masoud et al., 2003). Navarro et al. (2001) reported that exposure to chlorpyrifos during developmental period at which this OP pesticide is known to produce lasting changes in neural function, elicits corresponding, long-term deficits in immune system. The use of this insecticide continues to increase both in domestic and agricultural application, a reflection of the safety of this agent relative to the other related compounds (Roy et al., 1998). Gurunathan et al. (1998) indicated that spraying of chloropyriphos in the indoor environment may pose considerable risk to public health. Genotoxicity was also observed for chlorpyriphos-ethyl in phytoplanktons (Akcha et al., 2008). Das et al. (2006) have revealed that OP pesticides such as chlorpyrifos induced apoptosis and necrosis in cultured human peripheral blood lymphocytes in *in vitro* conditions at low cytotoxic doses. The present study has shown that chloropyriphos produce genotoxic effects on human lymphocyte cells at the highest concentration (10 μM) used. Smoking habit did not seem to significantly affect direction and intensity of the genotoxicity.

Despite its decades of usage, there is relatively little data regarding 2,4-D's effects on human health and environmental risk (IARC, 1991). Mustonen et al. (1986) have reported no significant change in the number of aberrations in human lymphocyte cultures treated with pure 2,4-D, whereas the commercial 2,4-D formulation (in different concentration), significantly increased the number of chromosomal aberrations *in vitro*. While some workers (Andersen et al., 1972; Zetterberg et al., 1977) did not observe any mutagenic activity of 2,4-D, others have suggested a relationship between 2,4-D and development of soft tissue sarcoma as well as malignant lymphomas (Bond et al., 1989). Immunosuppressive effects have been reported in rats treated with subtoxic doses of this herbicide (Imel'baeva et al., 2000). Application of a commercial 2,4-D in cultured human lymphocytes caused an increase in rate of SCE (Turkula and Jalal, 1985). In the present study, the highest concentration of 2,4-D (10 μM) significantly increased DNA damage in smokers' lymphocyte cell cultures while it did not cause any significant change in those collected from nonsmokers. This appears to be an important observation, however, lack of relevant literature makes it difficult to compare and discuss. Mechanism of 2,4-D induced genotoxicity and its correlation with smoking habit should be further investigated.

Cigarette smoke contains a large number of substances that are hazardous to human health. Smoking habits cause DNA damage in human lymphotyctes (de Assis et al., 2009). Impact of smoking habit on mutagenic effects of pesticide exposure has long been questioned. In a study conducted on cotton field workers who were exposed to several persistent pesticides; total frequency of chromosomal aberrations in smokers was more than those determined in the unexposed population ($n = 20$) (Rupa et al., 1989). It was reported that these subjects were exposed to dichloro diphenyl trichloroethane, benzene hexa chloride, endosulfan, malathion, methyl parathion, monocrotophos, quinolphos, dimethoate, phosphomidon, cypermethrin, and fenvelrate in the cotton fields. However, others have suggested that smoking has no additional effect on genetic damage (Undeger and Basaran, 2002; Sailaja et al., 2006). In our study, we did not determine additional genotoxic effects of the pesticides tested (except 2,4-D) on human lymphocytes collected from smoking subjects. This discrepancy may be attributed to long exposure period of some of these pesticides in smoker workers (Rupa et al., 1989) compared to very short incubation period *in vitro* in our study. Exposure to a mixture of chemicals in the aforementioned study may also be another factor increasing the chromosomal aberrations in smokers.

In conclusion, results of the present study suggest that OP (chlorpyrifos) and type II pyrethroid (cypermethrin) compounds are more potentially genotoxic than organochlorinated (endosulfan) and chlorinated aromatic hydrocarbon acid (2,4-D) pesticides at the same concentration. These effects of cypermethrin and chlorpyrifos were observed at the highest concentrations tested. Our findings also suggest that smoking habit of the subject from whom

primary lymphocyte cultures were prepared does not influence the direction and level of DNA damage in terms of exposure to cypermethrin and chlorpyrifos. The only different result between smokers and nonsmokers was found in the 2,4-D-treated group.

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