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Evaluation of the cytogenetic damage induced by the organophosphorous insecticide acephate

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Abstract The organophosphorous insecticide acephate was tested for its ability to induce in vitro cytogenetic effect in human peripheral lymphocytes by using the chromosomal aberrations (CAs), sister chromatid exchange (SCE) and micronuclei (MN) assay. The level of nuclear DNA damage of acephate was evaluated by using the comet assay. Concentrations of 12.5, 25, 50, 100 and 200 μg mL^{-1} of acephate were used. All concentrations of acephate induced significant increase in the frequency of CAs and in the formation of MN dose dependently ($r = 0.92$ at 24 h, $r = 0.95$ at 48 h for CAs, $r = 0.87$ for MN). A significant increase was observed in induction of SCE at 50, 100 and 200 μg mL^{-1} concentrations during 24 h treatment and at all concentrations (except 12.5 μg mL^{-1}) during 48 h treatment period in a dose-dependent manner ($r = 0.84$ at 24 h, $r = 0.88$ at 48 h). Acephate did not affect the replicative index and cytokinesis-block proliferation index (CBPI). However, it significantly decreased the mitotic index at all three highest concentrations (50, 100, 200 μg mL^{-1}) for 24 h treatment and at all concentrations (except 12.5 μg mL^{-1}) for 48 h treatment, dose-dependently ($r = 0.94$ at 24 h, $r = 0.92$ at 48 h). A significant increase in mean comet tail length was observed at 100 and 200 μg mL^{-1} concentrations compared with negative control in a concentration-dependent manner ($r = 0.94$). The mean comet tail intensity was significantly increased at only 200 μg mL^{-1} concentration. The present results indicate that acephate is a clastogenic, cytotoxic agent and it causes DNA damage at high concentrations in human lymphocytes in culture.

Keywords Acephate · DNA damage · Cytogenetic effect · Human lymphocytes

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Introduction

Pesticides are substances used to prevent, destroy, repel or mitigate any pest ranging from insect, animals and weeds to microorganisms (Donaldson et al. 2002). Among the pesticides, organophosphorous insecticides are the most widely used synthetic chemicals for the control of agricultural and domestic insects. The use of this class of chemicals has resulted in worldwide increases in food and fiber production,

the control of major disease carrying vectors and structure damaging insect pest (Chen et al. 1999; Rahman et al. 2002). However, in addition to their intended effects, they are sometimes found to affect non-target organisms, including humans (Chantelli-Forti et al. 1993; Chaudhuri et al. 1999; Karabay and Oğuz 2005).

Acephate is widely used organophosphorous insecticide with a total annual use of 4–5 million pounds of active ingredient throughout the world. It is a systemic insecticide with contact and stomach action (Tomlin 2003). The toxicity of acephate is attributed to its bioactivation to methamidophos, which acts not only as an acetylcholinesterase inhibitor, but also as a delayed neurotoxic agent (Mahajna et al. 1997). Although the toxicity of organophosphorous compounds is primarily through blockade of neural transmission via inhibition of acetylcholinesterase, studies have revealed histopathological alterations in the renal proximal tubules suggesting a role for additional mechanisms in renal toxicity (Poovala et al. 1998).

The genotoxicity of acephate has been studied in a variety of assays. The genotoxic activity of acephate revealed highly contradictory results. It has mutagenic activity and has been shown to produce gene mutation, chromosomal alteration, micronuclei (MN) and dominant lethality in prokaryotes and eukaryotes, and in mammalian cells including human and monkey lymphocytes, mouse lymphoma cells, CHO cells, and murine bone marrow cells (Simmon et al. 1978; Waters et al. 1982; Jones et al. 1984; Carver et al. 1985; Behera and Bhunya 1989; Jena and Bhunya 1994; Hour et al. 1998). In addition, Das et al. (2006) reported that acephate induced apoptosis and necrosis in cultured human peripheral blood lymphocytes in in vitro conditions using the DNA diffusion assay. However, some studies indicated that acephate is not genotoxic in in vivo SCE assay in mammalian cells, mutation test in *Escherichia coli* and *Bacillus subtilis* (Carver et al. 1985; Garrett et al. 1986; Wildemauve et al. 1983).

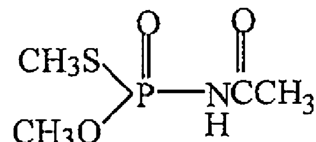
Taking into account the above cited contradictory results, the present study was planned to provide further additional genotoxicity data for acephate by using four mostly used and not studied cytogenetic endpoints, namely; the chromosomal aberrations (CAs), sister chromatid exchanges (SCE) and MN assay and single cell gel electrophoresis or comet assay in human lymphocytes in vitro.

Materials and methods

Chemicals

In this study, human peripheral lymphocytes were used as the test material. Acephate is obtained from Dr. Ehrenstorfer GmbH (CAS No: 30560-19-1) (pure). The other chemicals cytochalasin-B (CAS no. 14930-96-2), mitomycin C (CAS no. 50-07-7), bromodeoxyuridine (CAS no. 59-14-3), NaCl (CAS No. 7647-14-5) were obtained from Sigma. DMSO (CAS no. 67-68-5), NaOH (CAS no. 1310-73-2), Tris (CAS no. 77-86-1), EDTA (CAS no. 6381-92-6), Triton X-100 (CAS no. 9002-93-1), Low Melting Agarose (CAS no. 9012-36-6), Normal Melting Agarose (CAS no. 9012-36-6), EtBr (CAS no. 1239-45-8) were obtained from Applichem.

The chemical structure of acephate is as following:



IUPAC name: *O,S*-dimethyl acetylphosphoramidothiolate, Molecular formula: C_4H_{10} NO_3PS, Molecular weight: 183.16 gr/mol (pure %99.0).

Lymphocyte cultures

Peripheral venous blood was collected from two healthy non smoking donors (aged 25 years). Peripheral lymphocytes were cultured in chromosome medium B (containing fetal bovine serum, heparin, antibiotics and phytohaemagglutinin) supplemented with 10 μg mL^{-1} bromodeoxyuridine. Because bromodeoxyuridine is a thymidine analogue and it is incorporated for two cell cycle, the sister chromatids, which are different, namely bifilarly and unifilirily incorporated BrdU (BB-TB) can be distinguished by using Giemsa staining (Zakharov and Egolina 1972).

Chromosomal aberrations and sister-chromatid exchange assay

The cultures were incubated at 37°C for 72 h. Test substance was added after 24 h and 48 h of culture initiation. Human lymphocytes were treated with different concentrations (12.5, 25, 50, 100 and 200 μg mL^{-1}) of acephate. Negative and positive control

(mitomycin-C = MMC, 0.20 μg mL^{-1}) were also included. Colchicine (0.06 mg/mL) was present for the last 2 h of the culture. The cells were collected by centrifugation (216*g*, 10 min), treated with hypotonic solution (KCL, 0.075 M) for 30 min at 37°C and fixed in cold methanol:acetic acid (3:1) for 20 min at room temperature. The treatment with fixative was repeated 3 times. Finally, metaphase spreads were prepared by dropping the concentrated cell suspension onto slides.

For chromosome aberrations, air dried slides were stained with 5% Giemsa (pH. 6.8) prepared in Sorensen buffer solution for 20 min and mounted in depex. To observe SCEs, slides were stained in FPG technique according to Speit and Houpter's (1985) method.

One hundred metaphases were analysed for the CA assay per donor (totaly 200 metaphases per concentration). Gaps were not identified as CAs. In addition, 1,000 cells were analysed to obtain the mitotic index (MI). For the SCE assay, 50 second-division metaphases (25 metaphases from each donor) were analysed for each concentration. In SCE assay, a total of 200 cells (100 cells from each donor) were scored for replication index (RI), calculated according to the following formula; $RI = M_1 + 2M_2 + 3M_3/N$, where M_1, M_2 and M_3 represent the number of cells undergoing first, second and third mitosis, N total number of metaphase scored (Lin et al. 1987; Palus et al. 2003).

Micronucleus test

The lymphocyte cultures were incubated at 37 °C for 72 h. 44 h after the initiation of the cultures, cytochalasin-B was added at a final concentration of 5.2 μg mL^{-1}. Acephate in five different concentrations (12.5, 25, 50, 100 and 200 μg mL^{-1}) was added 24 h after phytohaemagglutinin (PHA) stimulation. Cultures were harvested at 72nd h after the initiation of culture. The cells were harvested, treated with hypotonic solution (0.075 M KCl for 5 min) and fixed with methanol:glacial acetic acid (3:1 v/v) supplemented with formaldehyde according the Palus et al. (2003). The slides were air-dried and stained with 5% Giemsa. MN were scored from 1,000 binucleated cells per donor (totaly 2,000 binucleated cells per concentration). 500 lymphocytes per donor (totaly 1,000 lymphocytes) were scored to evaluate the percentage of cells with 1–4 nuclei. Cytokinesis-block proliferation index (CBPI) was calculated according to Surrales et al. (1995) as follows; $(1 \times N_1) + (2 \times N_2) + (3 \times (N_3 + N_4))/N$ where N_1–N_4 represent the number of cells with 1–4 nuclei, respectively, and *N* is the total number of cells scored.

Lymphocyte isolation and comet assay

The primary DNA damaging effects caused by the acephate were determined using comet assay according to the Singh et al. (1988) with some modifications. Blood samples were obtained from two healthy non-smoking donors (aged 25 years). Peripheral blood was obtained with heparinized syringe immediately before the performance of the test. Lymphocytes were isolated by Biocoll separating solution. To detect viability of cells, trypan blue exclusion test was used. Cell viability was >98%. Isolated human lymphocytes were incubated with 12.5, 25, 50, 100 and 200 μg mL^{-1} concentrations of acephate for 1 h at 37 °C. Negative and positive control (MMC, 0.30 μg mL^{-1}) were also included. After the incubation, lymphocytes were centrifuged at 1,348*g* for 5 min, and then supernatant was removed and re-suspended in PBS. Treated cells were suspended in low melting point agarose (0.65%) and 75 μL of suspension was quickly layered over slides which precoated with normal melting point agarose (0.65%), immediately covered with a cover slip and the slides were placed on +4 °C for 10–15 min. After solidification, the coverslip was gently removed and immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH = 10 in which 10% DMSO and 1% Triton X-100 were added) at 4 °C for 1 h. The slides were removed and placed on a horizontal gel electrophoresis platform covered with electrophoresis buffer (300 mM NaOH, 1 mM EDTA pH > 13). The slides were left in the solution for 20 min to allow the unwinding of the DNA. The DNA was electrophoresed (25 V, 300 mA) for 20 min. After electrophoresis, the slides were removed and rinsed with neutralization buffer (0.4 M Tris, pH = 7.5). Each slide was stained with 50 μL of 20 μg mL^{-1} ethidium bromide.

Image analysis and comet scoring

The slides were examined using fluorescent microscope (Olympus) equipped with an excitation filter 546 nm and a barrier filter of 590 nm at 400×

magnification. Two slides were prepared for each concentration of acephate. The tail length and tail intensity (%) of one hundred comets on each slide (totally 200 comets per concentration) were determined using specialized image analyzes system ("Comet Assay IV", Perceptive Instruments Ltd., UK).

Statistical analysis

For the statistical analysis of the results, *z*-test for percentage of abnormal cell, CA/cell, RI, CBPI, MI, MN and *t*-test for SCEs were used. Students *t*-test were also performed to determine the possible DNA damage induced by acephate. Concentration-response relationships were determined from the correlation and regression coefficients for the percentage of abnormal cell, CA/cell, SCE, MN, mean comet tail length and mean comet tail intensity.

Results

Acephate increased the number of CAs at all treatment concentrations in both treatment times in a concentration-dependent manner compared with the negative control ($r = 0.92$ at 24 h, $r = 0.95$ at 48 h) (Table 1). It induced five types structural chromosomal aberration: chromatid and chromosome breaks, sister-chromatid union, chromatid exchange and fragment. The chromatid and chromosome breaks were more frequent than the other types of aberrations in total at 24 h and 48 h treatments. Acephate also induced numerical aberration (polyploidy) (Table 1).

Significant increase was observed for the number of MN in binucleated cells concentration dependently ($r = 0.87$) (Table 2). It caused one, two and four micronucleated binucleate cells. On the other hand, CBPI value was not affected by the acephate treatments.

Acephate significantly increased the frequency of SCEs at 50, 100 and 200 μg mL^{-1} concentrations during 24 h treatment and at all concentrations (except 12.5 μg mL^{-1}) during 48 h treatment period. The increase of SCEs was concentration-dependent at both treatment period ($r = 0.84$ at 24 h, $r = 0.88$ at 48 h). However, it did not effect the replicative index (RI) (Table 3).

A significant decrease was observed in the mitotic index in concentrations of 50, 100, 200 μg/mL at

Table 1 Chromosome aberrations induced by acephate in cultured human lymphocytes

Test substance	Treatment		Aberrations							Abnormal cell ± SE (%)	CA/Cell ± SE
	Period (h)	Dose (μg ml^{-1})	ctb	csb	f	scu	dic	cte	p		
Control	24	0.00	2	–	–	–	–	–	–	1.00 ± 0.703	0.010 ± 0.007
MMC	24	0.20	23	9	1	6	–	6	–	22.50 ± 2.953	0.255 ± 0.031
Acephate	24	12.5	6	1	1	–	–	–	1	4.50 ± 1.466*	0.045 ± 0.015*
		25	10	1	–	–	–	–	–	5.50 ± 1.612*	0.055 ± 0.016*
		50	13	2	1	–	–	–	–	8.00 ± 1.918*	0.080 ± 0.019*
		100	15	1	–	1	–	1	1	9.50 ± 2.073*	0.095 ± 0.021*
		200	17	4	–	1	–	–	–	11.00 ± 2.212*	0.110 ± 0.022*
Control	48	0.00	3	–	–	–	–	–	–	1.50 ± 0.860	0.015 ± 0.008
MMC	48	0.20	45	13	13	4	2	19	–	35.50 ± 3.384	0.470 ± 0.353
Acephate	48	12.5	9	–	1	–	–	–	–	4.50 ± 1.466*	0.045 ± 0.014*
		25	10	–	1	–	–	–	–	5.50 ± 1.612*	0.055 ± 0.016*
		50	12	–	–	–	–	–	–	6.00 ± 1.679*	0.060 ± 0.017*
		100	18	3	–	–	–	–	–	10.00 ± 2.121*	0.105 ± 0.022*
		200	21	3	–	2	–	–	–	11.50 ± 2.212*	0.135 ± 0.024*

200 metaphases were scored for each treatment

ctb chromatid break, *csb* chromosome break, *f* fragment, *scu* sister chromatid union, *dic* dicentric, *cte* chromatid exchange, *p* polyploidy

* Significantly different from the control $p < 0.001$ (*z*-test)

Table 2 Micronuclei induced by acephate in cultured human lymphocytes

Test substance	Treatment		Binucleated cells (BN) scored	Distribution of BN cells according to the No. of MN				MN (%)	Cytokinesis-block proliferation index (CBPI)
	Period (h)	Dose (μg mL^{-1})		(1)	(2)	(3)	(4)		
Control	48	0.00	2,000	–	–	–	–	0.00 ± 0.00	2.02 ± 0.314
MMC	48	0.20	2,000	38	2	–	–	2.00 ± 0.31	0.71 ± 0.188
Acephate	48	12.5	2,000	10	–	–	–	0.50 ± 0.16*	1.99 ± 0.312
		25	2,000	13	–	–	–	0.65 ± 0.18*	1.91 ± 0.306
		50	2,000	15	1	–	1	1.05 ± 0.23**	1.74 ± 0.292
		100	2,000	20	3	–	–	1.30 ± 0.25**	1.87 ± 0.302
		200	2,000	22	3	–	–	1.40 ± 0.26**	1.91 ± 0.306

* Significantly different from the control $p < 0.01$ (z-test)

** Significantly different from the control $p < 0.001$ (z-test)

Table 3 Frequency of SCE, RI and MI induced by acephate in cultured human lymphocytes

Test substance	Treatment		Min–max SCE	SCE/cell ± SE	M_1	M_2	M_3	RI ± SE	MI ± SE (%)
	Period (h)	Dose (μg mL^{-1})							
Control	24	0.00	1–8	4.36 ± 0.26	33	50	117	2.42 ± 0.05	11.00 ± 0.41
MMC	24	0.2	20–56	38.30 ± 1.30	82	68	50	0.74 ± 0.09	5.00 ± 0.49
Acephate	24	12.5	6–13	4.64 ± 0.27	41	41	118	1.77 ± 0.07	10.35 ± 0.68
		25	4–19	5.60 ± 0.32	40	48	112	2.36 ± 0.05	9.75 ± 0.66
		50	5–17	10.38 ± 0.56†	32	42	126	2.47 ± 0.04	8.50 ± 0.62**
		100	6–18	11.60 ± 0.50†	34	43	123	2.44 ± 0.04	7.65 ± 0.59***
		200	8–19	12.00 ± 0.39†	21	33	146	2.62 ± 0.05	6.55 ± 0.55***
Control	48	0.00	2–7	3.64 ± 0.24	18	64	128	2.48 ± 0.05	11.93 ± 0.72
MMC	48	0.2	25–83	54.32 ± 2.36	104	50	46	1.71 ± 0.06	4.00 ± 0.44
Acephate	48	12.5	2–19	4.90 ± 0.29	45	52	103	2.30 ± 0.05	10.35 ± 0.68
		25	2–13	7.42 ± 0.37†	31	85	84	2.26 ± 0.05	9.50 ± 0.65*
		50	7–14	9.74 ± 0.34†	35	77	89	2.80 ± 0.06	8.00 ± 0.61***
		100	6–19	11.20 ± 0.60†	25	47	128	2.51 ± 0.03	7.15 ± 0.58***
		200	8–22	12.58 ± 0.44†	32	49	119	2.68 ± 0.04	6.15 ± 0.54***

50 metaphases were scored for each concentration in SCE

200 metaphases were scored for each treatment in RI

2,000 metaphases were scored for each treatment in MI

† Significantly different from the control $p < 0.05$ (t-test)

* Significantly different from the control $p < 0.05$ (z-test)

** Significantly different from the control $p < 0.01$ (z-test)

*** Significantly different from the control $p < 0.001$ (z-test)

24 h treatment and in concentrations of 25, 50, 100, 200 μg/mL at 48 h treatment. Significant concentration-response correlation was observed in MI at both treatment period ($r = 0.94$ at 24 h, $r = 0.92$ at 48 h) (Table 3).

Table 4 shows the comet assay endpoint values for the control and treatment groups. A significant increase in mean comet tail length was observed at 100 and 200 μg mL^{-1} concentrations compared with negative control in a concentration-dependent manner

Table 4 DNA damaging activity of acephate in isolated human lymphocytes

Test substance	Concentration ($\mu g\ mL^{-1}$)	Mean tail length (μm)	Mean tail intensity (%)
Control	0.00	50.83 ± 0.90	5.81 ± 0.67
Acephate	12,50	50.33 ± 0.81	7.46 ± 1.04
	25,00	50.03 ± 0.90	6.37 ± 0.84
	50,00	49.72 ± 0.69	6.30 ± 0.74
	100,00	55.12 ± 1.29*	6.24 ± 0.67
	200,00	57.45 ± 1.56*	11.30 ± 1.48*
MMC	0.30	68.65 ± 1.97	14.45 ± 1.64

200 comets were scored for each treatment

* Significant from the control $p < 0.005$ (t test)

($r = 0.94$). The mean comet tail intensity was significantly increased at only 200 $\mu g\ mL^{-1}$ concentration.

Discussion

Chromosomal aberrations, SCE and micronucleus analysis of human lymphocytes as well as single cell gel electrophoresis (SCGE) or "comet" assay are used as the most rapid, sensitive and useful assays to detect the potential genotoxicity of chemicals (Rahman et al. 2002; Çelik et al. 2004; Yüzbaşıoğlu et al. 2006, 2008; Yılmaz et al. 2008). They have been considered to be the markers of early biological effects of carcinogen exposure (Liou et al. 2002). So, this study was planned to find genotoxic potential of acephate in human peripheral lymphocytes since such assays have not been carried out previously.

In this study, acephate significantly increased the number CAs, SCE and micronucleus and induced DNA damage in human lymphocytes. In the study carried out by Behera and Bhunya (1989), commercial form of acephate (asataf) induced significant enhancement in the percentage of chromosome aberrations in all concentrations (150, 200, 250 mg/kg and 5 days × 50 mg/kg), routes and times, even with sub-acute concentrations in mouse bone marrow cells. A significant difference in the occurrence of MN was found only at the highest concentration (250 mg/kg). On the other hand, Carver et al. (1985) showed that acephate did not induce CAs, SCE or MN in mouse bone marrow cells. After the treatment with acephate, Rahman et al. (2002) observed a significant increase in mean comet tail length indicating DNA damage in vivo in mouse lymphocytes in comparison to control. The damage was concentration related. Acephate induced apoptosis and necrosis in cultured human peripheral blood lymphocytes in in vitro conditions at low cytotoxic doses (Das et al. 2006). Methamidophos which is the active metabolite of acephate induced numerical and structural chromosome aberrations, increased micronucleus frequency in rat bone marrow cells (Karabay and Oğuz 2005). It also significantly induced DNA damage in erythrocytes of black porgy (Rixian et al. 2005).

In this study, acephate also decreased the mitotic index. The decrease of the MI could be due to blocking of G_2, which prevents the cell from entering mitosis (Van't Hof 1968) or it may be caused by a decrease in the ATP level and the stress from the energy-production centre (Epel 1963; Jain and Andsorbhoy 1988). Inhibition of certain cell cyle-specific proteins/enzymes remains as a possible pesticide target that inhibits DNA synthesis or may inhibit spindle production, assembly or orientation (Hidalgo et al. 1989).

Results obtained in this and in the other studies mentioned above showed that acephate is DNA damaging chemical. Acephate is a pentavalent phosphorous chemical. Its major reactions are phosphorylation and alkylation. The phosphorylation reaction is responsible for inhibition of acetylcholinesterase which may be one of the factors for mutation (Behera and Bhunya 1989; Ando and Wakamatsu 1982). Wild (1975) reported that the phosphorous moiety in organophosphorous pesticides is a good substrate for nucleophilic attack. This may cause phosphorylation of DNA. Alkylating property of phosphorous esters is the other cause of mutations as well (Eto and Ohkawa 1970; Wild 1975). Because several phosphorotioate compounds can lead to mutagenesis (Breau et al. 1985). Poovala et al. (1998) published that acephate significantly increased the cellular reactive oxygen species production. Reactive oxygen species are highly active and toxic biological

materials. They can lead to formation of DNA single and/or double strand breaks (Saleha et al. 2001).

The ability of acephate in induction of mutations might be due to phosphorylation and/or alkylation properties or production of toxic reactive oxygen species in cellular level. Another mechanism of action of acephate in DNA damaging might be due to bioactivating to methamidophos in blood. Singh (1984) reported that blood may contain certain enzymes capable of metabolizing acephate to methamidophos which highly active acetylcholinesterase inhibitor.

From the present study, it can be concluded that acephate induce chromosomal alterations and DNA damage in human lymphocytes. In this and other studies of acephate show that there are more positive results than negative concerning genotoxicity. Therefore, acephate can be considered genotoxic and may carry a risk to human health and it should be used more carefully.

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