Cytotechnology (2017) 69:865–874
DOI 10.1007/s10616-017-0099-y

ORIGINAL ARTICLE

Mutagenic and genotoxic effects of Anilofos with micronucleus, chromosome aberrations, sister chromatid exchanges and Ames test

Dilek Akyil · Muhsin Konuk · Yasin Eren · Recep Liman · Esra Sağlam

Received: 8 April 2016 / Accepted: 10 April 2017 / Published online: 12 June 2017

Abstract We aimed to evaluate the mutagenic effect of Anilofos, organophosphate pesticide, by using Ames/*Salmonella*/microsome test. Its cytotoxic and genotoxic effects were also determined by chromosome aberration (CA), sister chromatid exchange (SCE) and micronucleus (MN) test in human peripheral blood lymphocytes. In the Ames test, five different concentrations of Anilofos were examined on TA97, TA98, TA100 and TA102 strains in the absence and presence of S9 fraction. According to the results all concentrations of this pesticide have not shown any mutagenic activity on TA97, TA100 and TA102 strains in the absence and presence of S9 fraction. But, 10, 100 and 1000 μg/plate concentrations of Anilofos were determined to be mutagenic on TA98 strain without S9 fraction. Lymphocytes were treated with various concentrations (25, 50, 100 and 200 μg/ml) of Anilofos for 24 and 48 h. The results of the assays showed that Anilofos did not induce SCE frequency, replication index and MN formation at all concentrations for both treatment periods. Anilofos significantly increased CA frequency at 100 and 200 μg/ml concentrations at 24 h treatment periods and at 50, 100 and 200 μg/ml concentrations in 48 h treatment periods. Additionally, it was determined that this pesticide decreased mitotic index and nuclear division index significantly. It was concluded that Anilofos has genotoxic and cytotoxic effects in human peripheral lymphocytes.

Keywords Anilofos · Ames test · Chromosome aberration · Sister chromatid exchange · Micronucleus

D. Akyil (✉)
Molecular Biology and Genetic Department, Faculty of Sciences and Literatures, Afyon Kocatepe University, 03200 Afyonkarahisar, Turkey
e-mail: dilekakyil9@gmail.com

M. Konuk · E. Sağlam
Molecular Biology and Genetics Department, Faculty of Engineering and Natural Sciences, Üsküdar University, Altunizade, 34662 Istanbul, Turkey

Y. Eren
Science Education Department, Education Faculty, Suleyman Demirel University, 32200 Isparta, Turkey

R. Liman
Molecular Biology and Genetics Department, Faculty of Sciences and Literatures, Usak University, 64200 Uşak, Turkey

Introduction

Nowadays, chemical substance are used intensively in agriculture in order to increase crop yield. One of these chemical substances is pesticides. Pesticides are very useful substances when used carefully and in appropriate doses for the purpose. However, as a result of false and random use these substances can damage human health and as well can adversely affect other organisms via environmental pollution. In developing

countries, pesticide poisoning causes more deaths than infectious diseases (Eddleston 2000).

OPs (organophosphorus pesticides) are the most important class of pesticides which have been widely used in houses, industry and generally in agriculture (Zhang et al. 2002; Ballesteros and Parrado 2004). It is reported that OPs are worth nearly 40% of the global market and that they are anticipated to maintain dominance for some time into the future (Singh and Walker 2006). Depending on the wide-range of application, OP pesticides may enter the environment and reach high concentrations (Fleischli et al. 2004) and can affect non-target species. In addition, pesticide self-poisoning is an important public health problem (Jeyaratnam 1990; Eddleston and Phillips 2004), and is killing at least 250,000–370,000 people every year (Gunnell et al. 2007). Exposure to even small amounts of an OP compound can be fatal; death is generally caused by respiratory failure (Jokanovic 2009). OPs act as acetylcholinesterase (AChE) inhibitors (Eddleston 2000) which results in the accumulation of acetylcholine at cholinergic receptor sites, producing continuous stimulation of cholinergic fibers throughout the central and peripheral nervous systems (Jokanovic 2009).

Anilofos is also an OP which has an important role to control weeds and marsh grasses in rice fields. Hazarika and Sarkar (2001a) investigated subacute toxicity of Anilofos and they reported that Anilofos inhibited chloinesterase activities of plasma, erythrocyte, blood, liver and brain. Total protein was reduced in plasma and liver. Results indicate moderate toxic potential of Anilofos in mammals. Therefore, the aim of this study was to determine whether Anilofos represents any genotoxic risks by employing CA, SCE, MN in human peripheral lymphocytes in vitro and mutagenic risk by Ames test.

Materials and methods

Materials

Test organisms and chemicals

The test substance Anilofos was obtained from Fluka (CAS No. 64249-01-0- Sigma-Aldrich (St. Louis, MO, USA). Histidine deficient (his-) tester strains of *Salmonella typhimurium* TA97, TA98, TA100 and TA102 were obtained from Nuran Diril Hacettepe University, Turkey. Nutrient broth no. 2 (Oxoid), 2-aminoanthracene, β-nicotinamide-adenine dinucleotide phosphate, glucose-6-phosphate, mitomycin-C were obtained from Sigma-Aldrich. 5-Bromodeoxyuridine (CAS No. 59-14-3), colchicine (CAS No. 9754), cytochalasin B (CAS No. 14930-96-2), chromosome medium B (Biochrom cat. no. F5023) and DMSO (CAS no. 67-68-5) were obtained from Sigma-Aldrich.

Methods

Ames/Salmonella/microsome assay

Ames test was applied as a standard plate incorporation assay with *S. typhimurium* strains TA97, TA98, TA100 and TA102 in absence and presence of metabolic activation (Maron and Ames 1983). Prior to use in the experiment, each strain was checked for the presence of strain-specific marker as described by Maron and Ames (1983). For each tester strain, a specific positive control was always used in the experiment. While 4-nitro-o-phenylenediamine (NPD) for TA97 and TA98, mitomycin-C (MMC) for TA102, sodium azide (SA) for TA100 were used as positive controls without metabolic activation, 2-aminofluorene (AF), sodium azide (SA), 2-aminoanthracene (2AA) was used as positive controls with metabolic activation, respectively. All positive controls were obtained from Sigma-Aldrich. Before the test, a thawed bacterial stock suspension ($1–2 \times 10^9$ cells/ml) was inoculated in 20 ml nutrient broth No. 2 and grown overnight at 37 °C in an incubator shaker at 140 rpm. 0.1 ml of different concentrations of Anilofos (0.1, 1, 10, 100, 1000 µg/plate), 0.5 ml S9 mix or 0.5 ml PBS, and 2 ml top agar (kept at 45 °C) were mixed and poured directly onto the minimal glucose agar plates. The plates were incubated at 37 °C for 72 h. Revertants on each plate were counted. The positive and negative controls (DMSO) also were calculated from the three independent tests. All experiments were performed twice and each concentration was evaluated with three replicate plates.

CA and SCE assay

Whole blood samples were obtained by venipuncture in heparinized tubes, from four healthy donors

(nonsmokers, not exposed to any drug therapy, aged 22–30 years) under sterile conditions and were added to 2.5 ml chromosome medium B supplemented with 10 μg/ml bromodeoxyuridine. Lymphocytes were incubated in the dark at 37 °C for 72 h. Four different concentrations (25, 50, 100, 200 μg/ml) of test substance were added to the culture for 24 and 48 h. A positive control (0.25 μg/ml mitomycin-C) and a negative control (untreated cultures) were also used in parallel 0.06 μg/ml colchicine was added in all tubes during the last 2 h. At the end of the incubation, the cells were harvested by centrifugation (2000 rpm, 5 min) and treated with hypotonic solution for 5 min at 37 °C. Then, fixed once with fixative (methanol:-glacial acetic acid (3:1) for 20 min at room temperature. The fixative periods were repeated three times. Then, the slides were prepared by dropping and were air dried and stained with Giemsa for chromosome aberrations. For SCE, slides were stained according to FPG (fluorescence plus giemsa) technique. All chemicals for both experiment were obtained from Sigma-Aldrich.

Micronucleus assay

For MN, the blood from 4 healthy donors was added to 2.5 ml chromosome medium B and incubated at 37 °C for 68 h and cytochalasin B (final concentration 6 μg/ml) was added into the medium to arrest cytokinesis 44 h after the initiation. Different concentrations of Anilofos (25, 50, 100, 200 μg/ml) were added 24 and 48 h after initiation of culture. A positive and a negative control were also used like for CA and SCE. At the end of the incubation period, the cells were treated with hypotonic solution and fixed once with fixative (methanol:glacial acetic acid, 0.9% NaCl 5:1:6) for 20 min. Fixation was repeated twice with methanol/glacial acetic acid (5:1). The slides were prepared and 5% Giemsa was used for staining the slides for 14 min.

Microscopic evaluation

Chromosomal aberrations were counted from 100 well spread metaphases for each donor (totally 400 metaphases per concentration). 3000 cells were scored to obtain the MI. For SCE, a total of 25 well-spread cells under second mitosis were scored from each concentration. In addition, 100 cells from each donor, totalling 400 cells, were counted for RI. The replication index was calculated as follows: $RI = (M1 + 2M2 + 3M3/N)$. M1, M2, and M3 are the fraction of cells undergoing the first, second and third mitosis during the 72 h cell culture period and N is the total number of metaphase scored.

For MN, 2000 binucleated cells with well-preserved cytoplasm were counted from each donor (8000 binucleated cells per concentration) for each experiment. In addition, in total 2000 viable cells were scored to determine the frequency of cells with 1, 2, 3, or 4 nuclei and the NDI for cytotoxicity of Anilofos was calculated using the following formula: $NDI = [(1 \times M1) + (2 \times M2) + (3 \times M3) + (4 \times M4)]/N$; where M1–M4 represent the number of cells with one to four nuclei and N is the total number of intact cells scored (Fenech 2000).

Statistical analysis

The results of Ames test were analyzed statistically by using SPSS for Windows, and for this, Mann–Whitney U test was used. The results of CA, SCE, MN, RI, MI, NDI and other nuclear anomalies were analyzed using the Student t test.

Results

In *Salmonella* mutagenicity assay, five different concentrations of Anilofos were tested by Ames test on TA97, TA98, TA100 and TA102 strains with and without S9 metabolic activation. This pesticide did not increase the number of revertants of TA97, TA100 and TA102 with and without S9 fraction. But, 10, 100 and 1000 μg/plate concentrations of Anilofos were observed to be mutagenic on TA98 strain without S9 fraction. The Ames test results are shown in Table 1.

For the in vitro CA, MN and SCE assay, the cultures were treated with four different concentrations of the pesticide (25, 50, 100 and 200 μg/ml) for 24 and 48 h. Anilofos did not induce SCE (Table 2) and MN frequency (Table 3) at all treatment periods and concentrations. Anilofos induced CA frequency in 100 and 200 μg/ml concentrations for 24 h treatment period and 50, 100 and 200 μg/ml concentrations for 48 h treatment period when compared with the negative control. One type of numerical (polyploidy) aberration and six types of structural aberrations

Table 1 Mutagenicity analysis of Anilofos using *S. typhimurium* assay with TA97, TA98, TA100 and TA102 strain with or without metabolic activation

Test substance	Dose (μg/plak)	No of His + revertants/plate, mean ± SD							
		TA97		TA98		TA100		TA102	
		S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)	S9(−)	S9(+)
Anilofos	1000	82.16 ± 8.9*	117.16 ± 12.8	**47.33 ± 5.7*[m]**	21.33 ± 4.5*	127.16 ± 3.7*	106.83 ± 10*	278.16 ± 20*	314.33 ± 8.6*
	100	91.66 ± 5.6*	107 ± 10.8*	**47.66 ± 5.4*[m]**	24.33 ± 5.3	98 ± 4.9*	115.33 ± 7.7*	332.16 ± 11.9*	315 ± 7.4*
	10	93.16 ± 11.6*	109 ± 10.6*	**47.33 ± 7*[m]**	21.33 ± 1.9*	105 ± 7.3	132.33 ± 18.8*	296.33 ± 13.1*	304.83 ± 5.8*
	1	93.66 ± 6.2*	119.16 ± 8.6*	40 ± 6.3*	19.33 ± 3.2*	98.66 ± 7.11*	144.33 ± 5.5*	337.5 ± 16.5*	325.66 ± 16*
	0.1	104.66 ± 7.2*	110 ± 11.2*	45 ± 5.9*	19.66 ± 2.3*	107.5 ± 11.2	149.5 ± 6*	325.5 ± 11.4*	295.33 ± 6*
Control		91.66 ± 5.7*	124.66 ± 5.9*	32.16 ± 3.1	29.5 ± 1.5*	118.66 ± 12,8*	149.33 ± 6,2	349 ± 8*	337.33 ± 28.7
DMSO		87.5 ± 4.9	115.33 ± 9.9	32.33 ± 6.5	24.5 ± 2.7	106.5 ± 4.8	156.5 ± 13.4	313 ± 25.91	342 ± 9.4
SA	10					2743 ± 202.8*			
2AF	200		1365.33 ± 100.8*		971.16 ± 32.2*				
NPD	2	1193.33 ± 99.5*		1495 ± 178.4*					
2AA	5						2128 ± 310.5*		2207 ± 197*
MMC	0.5							1739.83 ± 110.9*	

Bold values are statistically significant

m mutagen, *SD* standard deviation, *SA* sodium azide, *2AF* 2-aminofluorene, *NPD* 4-nitro-o-phenylenediamine, *2AA* 2-aminoanthracene, *MMC* mitomycin-C

* Statistically significant at $P < 0.05$ (Mann–Whitney *U* test)

Table 2 Sister chromatid exchange and replication index in cultured human lymphocytes treated with Anilofos

Treatment			M1	M2	M3	RI ± SD	SCE/cell ± SD
Time (h)	Test substance	Dose (μg/ml)					
24	Control	–	255	75	74	1.58 ± 0.1	3.38 ± 0.68
	DMSO	9 μl	286	63	51	1.41 ± 0.15	3.08 ± 0.68
	MMC	0.25	312	40	48	1.33 ± 0.08	9.39 ± 0.89***
	Anilofos	25	310	41	49	1.34 ± 0.07	3.12 ± 0.51
		50	295	58	47	1.37 ± 0.1	3.42 ± 0.41
		100	264	70	36	1.35 ± 0.07	3.54 ± 0.23
		200	309	59	34	1.32 ± 0.04	3.93 ± 0.66
48	Control	–	277	64	59	1.45 ± 0.21	3.36 ± 0.41
	DMSO	9 μl	267	53	80	1.53 ± 0.27	3.03 ± 0.24
	MMC	0.25	331	35	34	1.25 ± 0.06	11.34 ± 1.35***
	Anilofos	25	249	68	83	1.58 ± 0.04	2.62 ± 0.17
		50	247	66	87	1.55 ± 0.03	2.67 ± 0.28
		100	256	59	85	1.57 ± 0.07	2.77 ± 0.37
		200	307	36	57	1.37 ± 0.04	3.23 ± 0.28

Data are expressed as the mean ± SD

* Significantly different from the negative control $P \leq 0.05$

** Significantly different from the negative control $P \leq 0.01$

*** Significantly different from the negative control $P \leq 0.001$

Table 3 The frequency of micronucleus and nuclear division index in cultured human lymphocytes treated with Anilofos

Treatment			Micronucleated binuclear cells (%) ± SD	Nuclear division index ± SD
Time (h)	Test Substance	Dose (μg/ml)		
24	Control	–	0.5 ± 0.15	1.8 ± 0.31
	DMSO	9 μl	0.58 ± 0.21	1.73 ± 0.14
	MMC	0.25	3.46 ± 0.2***	1.51 ± 0.13
	Anilofos	25	0.65 ± 0.21	1.75 ± 0.47
		50	0.65 ± 0.19	1.73 ± 0.29
		100	0.65 ± 0.18	1.63 ± 0.12
		200	0.66 ± 0.2	1.28 ± 0.19**
48	Control	–	0.98 ± 0.3	1.89 ± 0.21
	DMSO	9 μl	0.66 ± 0.18	1.75 ± 0.12
	MMC	0.25	7.47 ± 1.21***	1.27 ± 0.19***
	Anilofos	25	0.72 ± 0.15	1.73 ± 0.13
		50	0.61 ± 0.14	1.63 ± 0.12
		100	0.53 ± 0.07	1.55 ± 0.16
		200	0.52 ± 0.06	1.4 ± 0.14**

Data are expressed as the mean ± SD

* Significantly different from the negative control $P \leq 0.05$

** Significantly different from the negative control $P \leq 0.01$

*** Significantly different from the negative control $P \leq 0.001$

(chromatid and chromosome breaks, chromatid exchanges, fragments, sister chromatid union and dicentric) were observed in chromosome aberration test. Chromatid breaks were the mostly observed abnormality in all experimental groups (Table 4).

Anilofos did not decrease the RI at all concentrations and treatment periods. But cytotoxic effects were observed for this pesticide due to decreasing MI. NDI decreased only at high concentration (200 μg/ml) for both treatment periods significantly.

Table 4 The structural chromosome aberrations and mitotic index in cultured human lymphocytes treated with Anilofos

Treatment			CA/cell ± SD	Abnormal cell ± SD (%)	Aberrations						MI ± SD
Time (h)	Test Substance	Dose (μg/ml)			ctb	csb	f	dc	cte	p	
24	Control	–	0.05 ± 0.01*	6.5 ± 1.73	3	1	1				6.78 ± 0.93
	DMSO	9 μl	0.07 ± 0.01	12.5 ± 8.89	3	1	3				6.17 ± 0.68
	MMC	0.25	0.51 ± 0.01***	39.5 ± 12.77*	20	6	14	5	3	3	3.85 ± 0.499***
	Anilofos	25	0.06 ± 0.01	5.13 ± 0.63	1	1	3	1			5.77 ± 0.39
		50	0.06 ± 0.01	6 ± 2.16	2	1	2	1			5.40 ± 0.59
		100	0.16 ± 0.01***	9.75 ± 3.20	3	1	5	1		1	4.43 ± 0.33***
		200	0.12 ± 0.01***	10.63 ± 1.49	8	4	2			2	3.89 ± 0.46***
48	Control	–	0.06 ± 0.01	6.25 ± 1,5	2	1	2	1			6.75 ± 0.26*
	DMSO	9 μl	0.05 ± 0.01	7.5 ± 3	2	1	2				6.31 ± 0.16
	MMC	0.25	0.75 ± 0.03***	51 ± 6.68***	36	18	15	3		3	4.33 ± 0.15***
	Anilofos	25	0.07 ± 0.02	4.5 ± 1.29	3	1	2	1			5.35 ± 0.17***
		50	0.08 ± 0.02*	4.55 ± 0.84	2	3	2	1			4.3 ± 0.14***
		100	0.15 ± 0.01***	8.5 ± 3.42	3	2	5	1			4.06 ± 0.33***
		200	0.19 ± 0.02***	17 ± 3.74	9	5	6	1		1	3.43 ± 0.35***

Data are expressed as the mean ± SD

ctb chromatid break, *cab* chromosome break, *f* fragment, *dc* dicentric chromosome, *cte* chromatid exchange, *p* poliploidi

* Significantly different from the negative control $P \leq 0.05$

** Significantly different from the negative control $P \leq 0.01$

*** Significantly different from the negative control $P \leq 0.001$

Discussion

Organophosphorus pesticides are widely used in agriculture and increased economic and social profits significantly (Sparling and Fellers 2007). However, the unconscious usage of pesticides during decades caused serious hazards on environmental and public health (Mann et al. 2009; Burridge et al. 2010). Anilofos is an important organophosphorus herbicide which is used as AchE inhibitor. This substance is a pre- or post-emergent selective herbicide and widely used to control weeds and sedges in rice fields (Hazarika and Sarkar 2001a). This substance is readily absorbed through the mucosal membrane of the digestive tract by the blood and other body tissues and can spread through the respiratory system.

In vitro genotoxicity and mutagenicity test systems determine different chemical substances that define genetic damage directly or indirectly by various mechanisms and they are used as an early surrogate for potential carcinogenicity prediction. These test systems are divided into two groups, including bacterial and cytogenetic methods and they must be used in combination to obtain more reliable results (Leme and Marin-Morales 2009).

One of these test systems is the Ames test which is used to evaluate the mutagenic activity of chemicals; it is a short-term bacterial reverse mutation assay (Mortelmans and Zeiger 2000; Konuk et al. 2008; Akyıl et al. 2012; Arriaga-Alba et al. 2013; Escobar et al. 2013). In this assay, five different concentrations of Anilofos were tested on TA97, TA98, TA100 and TA102 strains in absence and presence S9 metabolic activation. All concentrations of this pesticide have not shown mutagenic activity on TA97, TA100 and TA102 strains with and without S9 fraction. However, 10, 100 and 1000 μg/plate concentrations of Anilofos were observed to be mutagenic on TA98 strain without S9 fraction. So this pesticide was able to cause frameshift mutation in a G–C sequence. However, when S9 fraction was added for TA98, the mutagenic activity was removed. S9 fraction consists of different

cofactors and different enzyme systems. Hence it may be thought to reduce Anilofos toxicity with the interaction between this enzyme system and pesticide.

A biologically active chemical can transformed inactive metabolite after biotransformation. Additionally an inactive chemical can be transformed to an active metabolite (Paolini and Forti 1997). We think that the presence of the liver enzyme in eukaryotes can remove the mutagenic activity of the tested substances. Therefore, S9 mix is an important parameter for this assay.

Thiono OPs cannot act as direct inhibitors of AChE and thioesters of these compounds (P=S) gain inhibitory properties after conversion into oxo (P=O) form. Therefore, the chemicals which contain thioster bonds must turn into oxo form for activation (Maroni et al. 2000). Anilofos is a thiono organophosphate and is expected to be metabolically activated to the corresponding oxon. In this study, this chemical has P=S bond. So we think that Anilofos may have induced frame shift mutations due to turning into oxo form in TA98 without S9 fraction.

There is more information on the mutagenic studies of organophosphate pesticides by usig Ames test (Aufderheide and Gressmann 2007; Coral et al. 2009; Wu et al. 2012) and in all these studies, pesticides have been found mutagenic. Furthermore, some other investigators have not been determined a mutagenic activity (Gollapudi et al. 1995). Structure of DNA is basically similar in all organisms. But secondary factors are important criteria for assessing the toxicity on DNA. At the same time, the size of the target DNA and the DNA repair mechanism is quite important in the emergence of the genotoxic hazard. All these parameters also vary from organism to organism (Brusick 1988). So, a chemical must also investigate with multiple test systems for support to experiment results.

There are a few studies on the effects of genotoxic activity of Anilofos in the literature. Hazarika and Sarkar (2001a) investigated the toxicity of Anilofos in rats and they showed that this substance has moderate toxicity in mammals. In another study, Aggarwal et al. (2007) investigated the embryo–fetal toxicity of Anilofos in groundwater which contains arsenic. In this study Anilofos and sodium arsenite were applied to pregnant rats both individually and in combination by gavage. There were no significant effects to neither adults nor youngs when arsenic was only applied. But when Anilofos was applied alone, it caused weight loss of the mother, and anorexia and reductions in body weight of the young animals. These results showed that Anilofos induced very important changes in the embryo–fetal development both alone and when combined with arsenic. Hazarika et al. (2003) studied toxicity of anilofos and malathion and their combination. They indicated that anilofos may enhance oxidative damage to rat brain. Effects of isoproturon pretreatment (675 mg/kg/day for 3 consecutive days) on the toxic actions of anilofos administered orally as a single dose (850 mg/kg) were evaluated by determining some biochemical attributes in blood (erythrocyte/plasma), brain and liver of rats by Hazarika and Sarkar (2001b) and they found that isoproturon pretreatment did not alter the toxicity of anilofos, and that the GSH-GST metabolic pathway may not have a significant implication in the detoxification of anilofos and the production of a reactive oxygen species may be a factor in mediating anilofos toxicity.

In our study four different concentrations (25, 50, 100, and 200 μg/ml) and six different parameters (SCE, CA, MN, MI, NDI and RI) were used to evaluated the genotoxic effects of Anilofos for 24 and 48 h on human peripheral lymphocytes in vitro. According to the results, it was determined that Anilofos did not increase SCE and MN frequency at all concentrations of both treatment periods in comparison to untreated control lymphocytes.

Sister chromatid exchange is a well-known method for determining the genotoxic potential of different chemicals at the chromatid level. Pommier et al. (1985) reported that topoisomerase-II enzymes are effective in the formation of sister-chromatid exchange. DNA topoisomerase II enzyme compose a complex connecting the DNA. Agents which affect DNA prevent the formation of this complex. Several observations suggest that chemical substances disrupt the structure of topoisomerase II enzyme. These chemicals eliminate the catalytic effect of enzyme or cause DNA double- strand breaks. According to this hypothesis SCE is an event due to the S-phase. As a result of our work we can state that Anilofos neither induced SCE, nor interacted with the cellular DNA replication and caused no DNA damages.

There are no studies on the effects of genotoxic activity of Anilofos in the literature on human peripheral blood lymphocytes. However most of the organophosphorus pesticides also did not increase the

frequency of SCE and MN formation in vivo and in vitro, paralleling our study (Gollapudi et al. 1995; Yuzbasıoglu et al. 2006; Ali et al. 2008; Satar et al. 2009; Revankar and Shyam 2009). In contrast to these studies some of organophosphorus pesticides have been found to increase frequency of SCE and MN formation (Balaji and Sasikala 1993; Yuzbasıoglu et al. 2006; Martínez-Valenzuela et al. 2009). Perhaps different results at frequency of SCE might be related to the positions of functional groups in the main chemical.

MN assay detects both clastogenicity and aneugenicity irreversible DNA damage caused by chromosome loss and/or breakage during mitosis. Micronuclei are indirect indicators of numerical and structural chromosomal aberrations (Albertini et al. 2000).

Albertini et al. (2000) observed that, an increase of MN frequency indicates that this substance has clastogenic potential. In this study, it was observed that Anilofos did not increase MN frequency at all concentrations of both treatment period compared to untreated control lymphocytes and values were generally close to the values of the control group. In this case, it can be thought Anilofos is not clastogenic in MN assay.

According to the CA results, it was determined that Anilofos increased CA frequency at 100 and 200 μg/ml concentrations in the 24 h treatment periods and 50, 100, and 200 μg/ml concentrations in the 48 h treatment periods significantly. In addition, Anilofos caused structural CAs instead of numerical CA. Furthermore, it was obtained that the 48 h treatment period caused more aberrations than the 24 h period. Consequently, after application of Anilofos, clastogenic effects can change depending on the concentration and duration of treatment. This also showed that Anilofos is a clastogen and can lead to formation of CA by breaking the phosphodiester backbone of DNA. So, results demonstrated that Anilofos most probably has a clastogenic effect and based on this we can say that the clastogenic effect was increased in a dose and time dependent manner.

Chromatid-type CAs (CTAs) and chromosome-type CAs (CSAs) differ mechanistically from each other. CSAs are stimulated by ionizing radiation and other S-independent clastogens while CTAs are induced by S-dependent agents such as many chemical substances in peripheral lymphocytes (Norppa et al., 2006). As a result of this study chromatid-type abnormalities were more common than the chromosome type ones. These results demonstrated that Anilofos had a significant clastogenic effect. But, it had no aneugenic effect.

Organophosphorus pesticides were studied to determinate their genotoxic activity with chromosome aberration test and a lot of them have been found to induce frequency of CA (Balaji and Sasikala 1993; Webster et al. 2002; Adhikari and Grover 2006) and some of them have not been shown to increase CA (Maroni et al. 2000; Adhikari and Grover 2006). These divergent results can be due to a lot of different factors, including contact time of organophosphorus pesticides, the dose of the substance, direct or indirect metabolisation of pesticides, etc.

The results of this study showed that Anilofos caused damage in chromosomes of cultured human peripheral lymphocytes and rapidly reduced the MI and NDI by killing cytogenetically damaged cells. However, significant reduction was not observed for the replication index. It can be concluded that Anilofos has a cytotoxic effect based on the decrease of MI and NDI and may pose a genotoxic risk for environment and humans. Briefly, it is necessary to be attentive when using it in food industries, beverage industry, pharmaceutical industry, cosmetics and perfumery.

Acknowledgements The authors wish to thank to Dr H Shazly (Swansea-UK) for editing the language of the paper.

References

Adhikari N, Grover IS (2006) Genotoxic effects of some systemic pesticides: in vivo chromosomal aberrations in bone marrow cells in rats. Environ Mol Mutagen 12:235–242

Aggarwal M, Wangikar PB, Sarkar SN, Rao GS, Kumar D, Dwivedi P, Malik JK (2007) Effects of low-level arsenic exposure on the developmental toxicity of anilofos in rats. J App Toxicol 27:255–261

Akyıl D, Oktay S, Liman R, Eren Y, Konuk M (2012) Genotoxic and mutagenic effects of aqueous extract from aerial parts of *Achillea teretifolia*. Turk J Biol 36:441–448

Albertini RJ, Anderson D, Douglas GR, Hagmar L, Hemminki K, Merlo F, Natarajan AT, Norppa H, Shuker DEG, Tice R, Waters MD, Aitio A (2000) IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mutat Res 463:111–172

Ali D, Nagpure NS, Kumar S, Kumar R, Kushwaha B (2008) Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Chemosphere 71:1823–1831

Arriaga-Alba M, Ruiz-Perez NJ, Sanchez-Navarrete J, Angel BL, Flores-Lozada J, Blasco JL (2013) Antimutagenic evaluation of vitamins B1, B6 and B12 in vitro and in vivo, with the Ames test. Food Chem Toxicol 53:228–234

Aufderheide M, Gressmann H (2007) A modified Ames assay reveals the mutagenicity of native cigarette mainstream smoke and its gas vapour phase. Exp Toxicol Pathol 58:383–392

Balaji M, Sasikala K (1993) Cytogenetic effect of Malathion in in vitro culture of human peripheral blood. Mutat Res 301:13–17

Ballesteros E, Parrado MJ (2004) Continuous solid-phase extraction and gas chromatographic determination of organophosphorus pesticides in natural and drinking waters. J Chromatogr A 1029:267–273

Brusick D (1988) Evolution of testing strategies for genetic toxicity. Mutat Res 205:69–78

Burridge L, Weis JS, Cabello F, Pizarro J, Bostick K (2010) Chemical use in salmon aquaculture: a review of current practices and possible environmental effects. Aquaculture 306:7–23

Coral MN, Uçman S, Yıldız H, Öztaş H, Dalkılıç S (2009) Potential neoplastic effects of parathion-methyl on rat liver. J Environ Sci 21:696–699

Eddleston M (2000) Patterns and problems of deliberate self-poisoning in the developing world. QJM 93:715–731

Eddleston M, Phillips MR (2004) Self poisoning with pesticides. BMJ 328:42–44

Escobar PA, Kemper RA, Tarca J, Nicolette J, Kenyon M, Glowienke S, Sawant SG, Christensen J, Johnson TE, McKnight C, Ward G, Galloway SM, Custer L, Gocke E, O'Donovan MR, Braun K, Snyder RD, Mahadevan B (2013) Bacterial mutagenicity screening in the pharmaceutical industry. Mutat Res 752:99–118

Fenech M (2000) The in vitro micronucleus technique. Mutat Res 455:81–95

Fleischli MA, Franson JC, Thomas NJ, Finley DL, Riley W (2004) Avian mortality events in the United States caused by anticholinesterase pesticides: a retrospective summary of National Wildlife Health Center records from 1980 to 2000. Arch Environ Contam Toxicol 46:542–550

Gollapudi BB, Mendrala AL, Linscombe VA (1995) Evaluation of the genetic toxicity of the organophosphate insecticide Chlorpyrifos. Mutat Res Genet Toxicol 342:25–36

Gunnell D, Eddleston M, Phillips MR, Konradsen F (2007) The global distribution of fatal pesticide self-poisoning: systematic review. BMC Public Health 7:357

Hazarika A, Sarkar SN (2001a) Subacute toxicity of anilofos, a new organophosphorus herbicide, in male rats: effect on some physical attributes and acetylcholinesterase activity. Indian J Exp Biol 39:1107–1112

Hazarika A, Sarkar SN (2001b) Effect of isoproturon pretreatment on the biochemical toxicodynamics of anilofos in male rats. Toxicol 165:87–95

Hazarika A, Sarkar SN, Kataria M, Hajare S, Malik JK (2003) Influence of malathion pretreatment on the toxicity of anilofos in male rats: a biochemical interaction study. Toxicol 185:1–8

Jeyaratnam J (1990) Acute pesticide poisoning: a major global health problem. World Health Stat Q 43:139–144

Jokanovic M (2009) Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. Toxicol Lett 190:107–115

Konuk M, Barış A, Liman R, Akyıl D (2008) A study on mutagenicity of different types of pesticides by using of Ames/*Salmonella*/microsome test system. Fresen Environ Bull 17:463–466

Leme DM, Marin-Morales MA (2009) *Allium cepa* test in environmental monitoring: a review on its application. Mutat Res 682:71–81

Mann RM, Hyne RV, Choung CB, Wilson SP (2009) Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ Poll 157:2903–2927

Maron DH, Ames BN (1983) Revised methods for the Samonella typhimurium mutagenicity test. Mutat Res 113:173–215

Maroni M, Colosio C, Feridi A, Fait A (2000) Organophosphorus pesticides. Toxicol 143:9–37

Martínez-Valenzuela C, Gómez-Arroyo S, Villalobos-Pietrini R, Waliszewski S, Calderón-Segura ME, Félix-Gastélum R, Álvarez-Torres A (2009) Genotoxic biomonitoring of agricultural workers exposed to pesticides in the north of Sinaloa State, Mexico. Environ Int 35:1155–1159

Mortelmans K, Zeiger E (2000) The Ames *Salmonella*/microsome mutagenicity assay. Mutat Res 455:29–60

Norppa H, Bonassi S, Hansteen IL, Hagmar L, Strömberg U, Rössner P, Boffetta P, Lindholm C, Gundy S, Lazutka J, Cebulska-Wasilewska A, Fabiánová E, Srám RJ, Kunudsen LE, Barale R, Fucic A (2006) Chromosomal aberrations and SCE as biomarkers of cancer risk. Mutat Res 600:37–45

Paolini M, Forti GC (1997) On the metabolizing systems for short-term genotoxicity assays, a review. Mutat Res 387:17–34

Pommier Y, Zwelling LA, Kao-Shan C, Whang-Peng J, Bradley MO (1985) Correlations between intercalator-induced DNA strand breaks and sister chromatid exchanges, mutations, and cytotoxicity in Chinese hamster cells. Cancer Res 45:3143–3149

Revankar PR, Shyam SK (2009) Genotoxic effects of Monocrotophos, an organophosphorous pesticide, on an estuarine bivalve, *Meretrix ovum*. Food Chem Toxicol 47:1618–1623

Satar S, Kayraldız A, Rencüzoğulları E, Karakoç E, Sebe A, Avcı A, Yeşilağaç H, Topaktaş T (2009) The genotoxicity and cytotoxicity among patients diagnosed with organophosphate poisoning. Bratisl Lek Listy 110: 476–479

Singh B, Walker A (2006) Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev 30:428–471

Sparling DW, Fellers G (2007) Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. Environ Poll 147:535–539

Webster LR, McKenzie GH, Moriarty HT (2002) Organophosphate-based pesticides and genetic damage implicatedin bladder cancer. Cancer Genet Cytogenet 133:112–117

Wu JC, Chye SM, Shih MK, Chen CH, Yang HL, Chen SC (2012) Genotoxicity of Dicrotophos, an organophosphorus

pesticide, assessed with different assays in vitro. Environ Toxicol 27:307–315

Yuzbasıoglu D, Çelik M, Yılmaz S, Ünal F, Aksoy H (2006) Clastogenicity of the fungicide Afugan in cultured human lymphocytes. Mutat Res 604:53–59

Zhang Z, Hong H, Wang X, Lin J, Chen W, Xu L (2002) Determination and load of organophosphorus and organochlorine pesticides at water from jiulong river estuary, China. Mar Pollut Bull 45:397–402