



Sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary (CHO-K1) cells treated with the insecticide pirimicarb

Sonia Soloneski*, Marcelo L. Larramendy

Laboratorio de Citogenética, Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 No. 3 esq. 120, 1900 La Plata, Argentina

ARTICLE INFO

Article history:

Received 7 May 2009

Received in revised form

11 September 2009

Accepted 14 September 2009

Available online 20 September 2009

Keywords:

Carbamates

Cytotoxicity

Genotoxicity

Mammalian cell lines

Technical formulations

ABSTRACT

Pirimicarb and its formulation Aficida® (50% pirimicarb) effects were studied on CHO-K1 cells employing sister chromatid exchange (SCE), chromosomal aberrations (CA), cell-cycle progression and mitotic index analyses. Continuous treatments were performed within 10–300 µg/ml concentration-range. Pirimicarb, but not Aficida®, induced a concentration-dependent increase of abnormal cells. Pirimicarb induced a greater frequency of chromatid/isochromatid breaks than Aficida® did. Regression analyses showed a concentration-dependent increase in the frequency of chromatid-type breaks for both compounds whereas only the frequency of isochromatid-type breaks did in those pirimicarb-treated cultures. SCEs in pirimicarb- or Aficida®-treated cultures were significantly higher than control values with concentrations of 100–200 µg/ml. Both test compounds induced equivalent frequency of SCEs. A delay in cell-cycle kinetics was observed for pirimicarb and Aficida® within 100–300 and 200–300 µg/ml concentration-range, respectively. An inhibition of MI was observed for both chemicals regardless of tested concentrations. Finally, the CAs appears to be a higher sensitive bioassay to detect DNA damage at lower concentrations of pirimicarb than SCEs does. The results demonstrated that pirimicarb and Aficida® exert geno-cytotoxicity, at least in CHO-K1 cells.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Living species are inevitably exposed to pesticides and they represent both a significant ecological and public health concern due to their toxicity. Furthermore, agrochemicals are ubiquitous in the world because the anthropogenic activities are continuously introducing extensive amounts of them into the environment.

In epidemiological as well as in experimental clastogenesis studies there is an increasing interest in biomonitoring markers for providing a measure of biologically active/passive exposure to genotoxic pollutants. Several studies demonstrated that occupational exposure to some pesticides may be related to several kinds of cancer, including leukemia and other diseases (www.iarc.fr). Different *in vivo* and *in vitro* test systems have been described in mammalian cells to assess genotoxic and cytotoxic damage induced by pollutants [1,2]. The use of *in vitro* cell cultures for genotoxic evaluation is therefore a valuable tool for an early and sensitive detection of xenobiotic exposure [3]. Among them, one of the most used systems for clastogenic and/or aneugenic screening is the cultured mammalian CHO-K1 cells in which sister chromatid exchange (SCE), chromosomal aberrations (CAs), micronuclei, and kinetics

of cell proliferation have been widely employed as cytogenetics end-points [4–9].

Large quantities of carbamates are particularly applied to different environments worldwide. These pesticides have produced both conflicting and inconclusive results in mutagenicity tests varying according to either the end-point or the compound assessed [10–12]. Among them, carcinogenic, teratogenic and neurotoxic properties have been reported for many of these agrochemicals [12–14]. Among carbamates, pirimicarb is a selective insecticide mostly used for aphid control in a broad range of crops, including vegetable, cereal, orchard crops and fruit growing. Its mode of action is by inhibition of acetylcholinesterase activity [12]. While toxicological data for pirimicarb has been well documented in animal experiments, it had not revealed carcinogenic potential in mice or rats [12]. The information accessible on the genotoxic properties of pirimicarb is limited and inconsistent. Only few data are available in the literature about genotoxic studies [12,15]. Pirimicarb has been generally recognized as non-genotoxic in bacteria, yeast and fungi as well as in mammalian cells [16]. It has been reported to be non-mutagenic in *S. typhimurium* when either Ames or Ames reversion mutagenicity (*his*⁻ to *his*⁺) tests for the TA1535, TA1538, TA98 and TA100 strains after S9 metabolic activation has been used [16]. However, a significant positive mutagenic response was observed in mouse lymphoma L5178Y cells [12]. Positive results have been reported in the *w/w*⁺ eye mosaic system with the *D. melanogaster* Oregon-K strain [17]. On the other hand, pirimicarb

* Corresponding author. Fax: +54 221 425 8252.

E-mail address: ssoloneski@yahoo.com.ar (S. Soloneski).

did not induce chromosomal alterations in bone marrow cells of Wistar male rats after oral administration [18]. Although no induction of CAs has been reported in *in vitro* human lymphocytes with or without S9 metabolic activation [16], Pilinskaia [19] observed a significant increase of CAs in the peripheral blood lymphocytes from occupational workers after pirimicarb exposure. Furthermore, the induction of single-strand breaks was detected by the alkaline comet assay in human lymphocytes *in vitro* [20].

In agriculture, generally a pesticide is not used as a single active ingredient, but a complex commercial formulation is employed instead. Formulated products, in addition to the active component, contain different solvents and adjuvants, some of which have been reported to induce damage in mammalian cells, among others toxicities [8,21–23]. Hence, additional toxic effects exerted by excipients must be taken into consideration for risk assessment. Accordingly, workers and environment are exposed to the simultaneous action of the active ingredient and a variety of other chemicals contained in the formulated product. Despite the beneficial effects associated with the use of agrochemicals in agriculture and household, many of these products may represent potential hazardous compounds because its extensive use, contamination of food, water and air have become a serious and adverse health problem for humans and the ecosystems (www.epa.gov). So far, available information indicates that 23 formulated products containing pirimicarb as active ingredient has been registered worldwide (www.environmentalchemistry.com). Furthermore, some of these pirimicarb-containing products have been reported to induce toxic effects in the invertebrate *Daphnia magna* [24,25] and in tadpoles of *Rana perezi* [26].

The aim of this work was to investigate the genotoxic effect of the carbamate pesticide pirimicarb and its formulated insecticide product Aficida® (50% pirimicarb). The latter was chosen since it represents one of the carbamates most largely used not only in Argentina in cereal production and for garden insect control but also on a worldwide scale. In this report we employed the CAs, SCEs, cell-cycle progression analysis, and mitotic index (MI) bioassays as different cytogenetic end-points on mammalian Chinese hamster ovary (CHO-K1) cells.

2. Materials and methods

2.1. Chemicals

Pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate, CAS 23103-98-2, purity 99.5%), 5-bromo-2'-deoxyuridine (BrdU, CAS 59-14-3), dimethyl sulfoxide (DMSO, CAS 67-68-5), and colchicine (CAS 64-86-8), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Aficida® (50% pirimicarb, excipients c.s.) was kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina). Bleomycin (BLM) (Blocamycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina).

2.2. Cell cultures and pesticide treatment

Chinese hamster ovary (CHO-K1) cells were grown in Ham's F10 medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum (Gibco), 100 units/ml penicillin (Gibco) and 10 µg/ml streptomycin (Gibco) in the dark at 37 °C in a 5% CO₂ atmosphere. Experiments were set up with cultures in the log phase of growth. Cells were seeded in T25 flasks at a density of 3.5×10^5 cells/flask. Either 12 h or 24 h after plating cells were treated with 10, 50, 100, 200, and 300 µg/ml of the test active ingredient and formulated product. Concentrations of pirimicarb were selected according previous results reported by Undeğer and Başaran [20]. Prior to use, pirimicarb and Aficida® were dissolved

in DMSO and then diluted in culture medium. Both test compounds were appropriate diluted to reach the required final concentration by adding 100 µl into culture medium. So that, the DMSO concentration was <1% for all treatments in the different experiments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (1 µg/ml BLM, 1 h-pulse treatment) were run simultaneously with pesticide-treated cultures. BLM was selected due to its capacity of introduce both single- and double-strand breaks into DNA and to induce both CAs and SCEs in different *in vitro* mammalian cell systems [27–31]. None of the treatments produced significant pH changes in the culture medium. Afterwards, 10 µg/ml BrdU was incorporated into cultures and then the cells incubated under safety light for an additional 12 h or 24 h period until harvesting. Cultures were duplicated for each experimental point, in at least three independent experiments. The same batches of culture medium, sera and reagents were used throughout the study.

2.2.1. Chromosome preparations

During the last 3 h of culture, the cells were treated with 0.2 µg/ml colchicine. At the end of the colchicine treatment, cells were detached with a rubber-policeman and washed three times in Hank's balanced salt solution (pH 7.0). Afterwards, cells were collected by centrifugation at 1200 rpm 251 g for 10 min, subsequently hypotonically shocked (0.075 M KCl, 37 °C, 20 min), and then fixed three times in methanol:glacial acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique.

2.2.2. Fluorescence-plus-Giemsa (FPG) method for sister chromatid differentiation

Chromosome spreads were stained using the FPG technique for sister chromatid differentiation as previously described elsewhere [5]. Slides were coded and scored blind by one researcher.

2.2.3. Cell-cycle kinetics, proliferative rate index and mitotic index

A minimum of 100 metaphasic cells per experimental point from each experiment were scored to determine the percentage of cells that had undergone one (M_1) and two (M_2) mitoses. The proliferative rate index (PRI) was calculated for each experimental point according to the formula $PRI = [(\%M_1) + 2(\%M_2)]/100$, which indicated the average number of times the cells had divided in the medium since the addition of BrdU until harvesting, originally proposed by Lamberti et al. [32]. The mitotic index (MI) was determined by scoring 2000 cells from each experimental point and expressed as number of mitoses among 1000 nuclei. Changes in the MI were expressed as a factor (f) of the mean MI from treated cultures (MI_t) over the mean MI from controls (MI_c) ($f = MI_t/MI_c$) [33].

2.2.4. Sister chromatid exchange analysis

For the SCE assay, a total of 25 well-spread diploid M_2 cells metaphases were scored per experimental point from each experiment. Data were expressed as the mean number of SCEs/cell \pm S.E. from 75 pooled cells scored per test compound concentration.

2.2.5. Chromosomal aberration analysis

For the CAs assay, a total of 100 well-spread diploid M_1 metaphases cells from those cultures harvested 12 h after test compound treatment were analyzed per experimental point from each experiment after slides processed with FPG technique. Structural chromosomal aberrations were scored and the number of each type of aberration, number of aberrations per cell and the percentage of cells with aberrations were scored following to the recommendations of the OECD Guide [3]. Marker chromosomes and achromatic lesions smaller than the width of a chromatid and continuous with

the chromosome axis, considered chromatid or isochromatid gaps, respectively, were not included in the scoring. Radial figures and ring and/or dicentric chromosomes were scored as chromatid or isochromatid exchanges, respectively [34]. Data were expressed as the total mean number of CAs/experimental point \pm S.E. of the mean from 300 cells scored in three independent experiments.

2.3. Statistical analysis

Due to the sample size, a normal distribution of statistics prior analyses was assumed. The one-tailed Student's *t*-test was used to compare pooled data of three independent experiments as mean values of SCE/cell and CAs/cell data between treated and control groups. A χ^2 -test was employed for cell-cycle progression and MI data. SCEs, frequencies of M_1 and M_2 cells, PRI and MI data were evaluated by regression analysis. The level of significance chosen was 0.05 unless otherwise indicated.

3. Results

No differences of CAs, SCEs, cell-cycle progression, PRI, MI, and mitotic proliferative factor values were observed between untreated and negative controls (DMSO-treated cells) ($P > 0.05$). Then, all control data presented in the throughout results correspond to the values obtained for DMSO-treated CHO-K1 cells.

Table 1 summarizes the results of the analysis of pesticide-induced CAs in CHO-K1 cells. The frequencies of CAs in the BLM (positive control) treatment cells were significantly increased in regard to the control cultures ($P < 0.001$). No equivalent induction of CAs was observed after treatment with pirimicarb and Aficida®. In 10 $\mu\text{g/ml}$ pirimicarb treatments, approximately 13 mitotic cells were found to carry at least one CA, reaching the frequency of abnormal cells values as high as 45.67 ± 1.44 in those 300 $\mu\text{g/ml}$ treated cultures. In treatments with the formulated product, only 27.67 ± 0.77 of aberrant mitotic cells were found with the maximal concentration employed. Statistical analysis showed that there is a significant difference in the extent of aberrations between control and treated cells, since both test compounds were able to induce an increase in the number of both chromatid- and isochromatid breaks in the 10–300 $\mu\text{g/ml}$ concentration-range ($P < 0.05$ – $P < 0.001$). Exposure of CHO-K1 cells to pirimicarb resulted in a greater induction of chromatid and isochromatid breaks than Aficida® treatment. However, chromatid- and isochromatid-

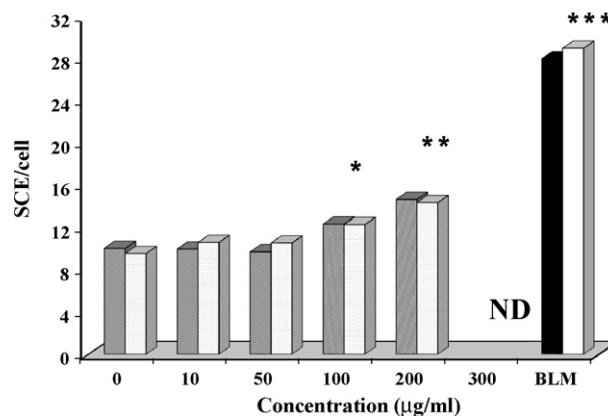


Fig. 1. Effect of the *N,N*-dimethylcarbamate pirimicarb (stripped bars) and its commercial formulation Aficida® (dotted bars) on SCE frequency from CHO-K1 cells. Cultures were harvested at 24 h from pesticide treatment and the proportion SCEs were determined in 75 M_2 mitoses for each experimental point. For each insecticide, pool data from three independent experiments are reported as mean SCE values \pm S.E. (y-axis) and plotted against the insecticide concentration (0–300 $\mu\text{g/ml}$ concentration-range; x-axis). A 1 $\mu\text{g/ml}$ BLM (Bleomycin) 1 h-pulse-treated cells were used as positive control for pirimicarb (black bar) and Aficida® (empty bar), respectively. ND, not determined. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

type exchanges were rarely observed for either pirimicarb or Aficida® ($P > 0.05$) (Table 1). Overall, pirimicarb induced a significant concentration-dependent increase in the number of abnormal cells ($r = 0.93$, $P < 0.01$) but not Aficida® ($r = 0.65$, $P > 0.05$). A regression analyses showed a concentration-dependent increase in the frequency of chromatid-type breaks when pirimicarb ($r = 0.94$, $P < 0.01$) or Aficida® ($r = 0.92$, $P < 0.01$) were titrated into cultures while only the frequency of isochromatid-type breaks did in those pirimicarb-treated cultures ($r = 0.96$, $P < 0.01$) but not in those Aficida®-treated cells ($r = 0.80$, $P > 0.05$) (Table 1).

Fig. 1 shows the results of SCEs analysis in CHO-K1 cells treated during 24 h with 10, 50, 100, 200 and 300 $\mu\text{g/ml}$ of pirimicarb and Aficida® and as well as after 1 h-pulse of BLM (1 $\mu\text{g/ml}$, positive control) obtained from three independent experiments. Results revealed statistically significant differences between negative and positive controls ($P < 0.001$). The SCE frequencies observed in CHO-K1 treated cultures were significantly higher than those of negative control cultures when exposed to 100 $\mu\text{g/ml}$ ($P < 0.05$) and 200 $\mu\text{g/ml}$ ($P < 0.01$) of both test compounds. Furthermore, the capacity of pirimicarb and Aficida® to induce SCEs was found

Table 1
Distribution of the different types of chromosomal aberrations (CA) observed in CHO-K1 cells after 12 h treatment with pirimicarb and Aficida®.

Treatment	Concentration ($\mu\text{g/ml}$)	Cells with CA ^a	Breaks ^b		Exchanges ^b		TA ^b
			C	IC	C	IC	
BLM	0	7.00 ± 0.38	3.33 ± 0.32	1.00 ± 0.00	0.33 ± 0.00	2.67 ± 0.71	7.33 ± 0.26
	1	$44.33 \pm 0.68^{***}$	$21.00 \pm 0.22^{***}$	1.00 ± 1.00	$4.00 \pm 0.50^{***}$	$20.00 \pm 0.22^{***}$	$46.00 \pm 0.49^{***}$
Pirimicarb	10	$13.33 \pm 1.11^{**}$	$7.00 \pm 0.38^*$	$3.00 \pm 0.00^*$	1.00 ± 0.00	3.33 ± 0.84	$14.33 \pm 0.31^{**}$
	50	$22.33 \pm 0.86^{***}$	$16.67 \pm 0.28^{***}$	$3.67 \pm 0.80^{**}$	1.00 ± 0.00	2.33 ± 0.76	$23.67 \pm 0.46^{***}$
	100	$32.33 \pm 0.97^{***}$	$20.33 \pm 0.34^{***}$	$10.67 \pm 0.47^{***}$	1.00 ± 0.00	2.67 ± 0.71	$34.67 \pm 0.38^{***}$
	200	$34.33 \pm 0.26^{***}$	$22.33 \pm 0.44^{***}$	$11.67 \pm 0.45^{***}$	0.33 ± 0.00	1.00 ± 0.00	$35.33 \pm 0.22^{***}$
	300	$45.67 \pm 1.44^{***}$	$31.33 \pm 0.84^{***}$	$24.67 \pm 0.84^{***}$	0.33 ± 0.00	3.00 ± 0.58	$59.33 \pm 0.57^{***}$
Aficida®	10	12.00 ± 0.29	$8.00 \pm 0.35^*$	$5.33 \pm 0.25^{***}$	0.33 ± 0.00	1.00 ± 0.00	$14.67 \pm 0.15^{***}$
	50	$19.00 \pm 0.83^{***}$	$12.33 \pm 0.43^{***}$	$7.00 \pm 0.65^{***}$	0.67 ± 0.71	1.00 ± 0.00	$21.00 \pm 0.45^{***}$
	100	$22.00 \pm 0.37^{***}$	$16.00 \pm 0.66^{***}$	$5.67 \pm 0.24^{***}$	0.67 ± 0.71	0.67 ± 0.33	$23.00 \pm 0.49^{***}$
	200	$22.67 \pm 0.44^{***}$	$16.67 \pm 0.51^{***}$	$6.67 \pm 0.89^{***}$	0.33 ± 0.00	2.00 ± 0.71	$25.67 \pm 0.53^{***}$
	300	$27.67 \pm 0.77^{***}$	$19.67 \pm 0.94^{***}$	$8.33 \pm 0.53^{***}$	0.00 ± 0.00	2.67 ± 0.71	$30.67 \pm 0.55^{***}$

^{a,b}Results are expressed as mean values of abnormal cells or number of aberrations \pm S.E. of the mean from three independent experiments. C, chromatid-type aberration; IC, isochromatid-type aberration; TA, total number of aberrations; BLM (BLM 1 $\mu\text{g/ml}$) was used as positive control.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Table 2
Cell-cycle progression, proliferative rate index (PRI) and mitotic index (MI, *f*) values observed in CHO-K1 cells after 24 h treatment with pirimicarb and Aficida®.

Concentration (µg/ml)	Cell-cycle progression ^a									
	Pirimicarb			Aficida® (50% pirimicarb)						
	M ₁	M ₂	PRI	MI	<i>f</i>	M ₁	M ₂	PRI	MI	<i>f</i>
0	2.00 ± 1.41	98.00 ± 0.20	1.98 ± 0.14	65.33 ± 0.10	1.00 ± 0.00	2.67 ± 1.87	97.33 ± 0.31	1.97 ± 0.22	68.33 ± 0.06	1.00 ± 0.00
BLM	40.00 ± 0.32 ^{***}	60.00 ± 0.26 ^{**}	1.60 ± 0.16	35.00 ± 0.55 ^{***}	0.54 ± 0.02	32.00 ± 0.35 ^{***}	68.00 ± 0.24 ^{***}	1.68 ± 0.15	38.00 ± 0.16 ^{***}	0.56 ± 0.06
10	2.33 ± 1.65	97.67 ± 0.25	1.98 ± 0.18	44.33 ± 0.62 ^{**}	0.68 ± 0.06	1.67 ± 1.18	98.33 ± 0.15	1.98 ± 0.11	65.67 ± 0.26	0.96 ± 0.02
50	2.67 ± 1.54	97.33 ± 0.26	1.97 ± 0.18	32.00 ± 0.29 ^{***}	0.49 ± 0.05	3.00 ± 1.53	97.00 ± 0.31	1.97 ± 0.19	48.33 ± 0.22 ^{**}	0.71 ± 0.03
100	16.33 ± 2.49 ^{**}	83.67 ± 1.10 ^{**}	1.84 ± 0.74	27.33 ± 0.24 ^{***}	0.42 ± 0.07	4.33 ± 1.54	95.67 ± 0.33	1.96 ± 0.23	37.67 ± 0.34 ^{***}	0.55 ± 0.03
200	64.33 ± 1.99 ^{**}	35.67 ± 0.42 ^{***}	1.36 ± 0.97 ^{**}	20.33 ± 0.10 ^{***}	0.31 ± 0.05	43.33 ± 3.19 ^{***}	56.67 ± 2.79 ^{***}	1.63 ± 1.85 [*]	30.33 ± 0.38 ^{***}	0.45 ± 0.04
300	98.33 ± 0.15 ^{***}	1.67 ± 0.71 ^{***}	1.02 ± 0.15 ^{***}	13.67 ± 0.13 ^{***}	0.21 ± 0.03	100.00 ± 0.00 ^{***}	0.00 ± 0.00 ^{***}	1.00 ± 0.00 ^{***}	19.67 ± 0.47 ^{***}	0.29 ± 0.05

Results are presented as mean values ± S.E. of the mean. BLM (Bleomycin 1 µg/ml) was used as positive control.

^a The proportion of cells in first (M₁) and second (M₂) cell divisions was determined in 100 mitoses for each experimental point in three independent experiments.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

equivalent with both concentrations assessed ($P < 0.05$). No SCE induction was achieved when cells were treated with 10 and 50 µg/ml of both pirimicarb and Aficida® ($P > 0.05$). In those 300 µg/ml pirimicarb- or Aficida®-treated cultures, the frequency of SCEs was unable to be determined since the frequency of M₁ cells reached values as higher as 98–100% of the cell population (Fig. 1).

Results of cell-cycle progression analyses after pirimicarb and Aficida® treatment are summarized in Table 2. BLM treatment produced a significant inhibition of the cell-cycle progression compared with the corresponding negative control values ($P < 0.001$). A significant delay in cell-cycle kinetics was observed in both pirimicarb- and Aficida®-treated cultures within 100–300 µg/ml and 200–300 µg/ml concentration-range, respectively ($P < 0.01$, $P < 0.001$; for pirimicarb- and Aficida®-treated cells, respectively). Similarly, a significant increase in M₁ and a significant decrease M₂ frequencies were observed either when 100–300 µg/ml concentration-range of pirimicarb ($P < 0.01$) and 200–300 µg/ml of Aficida® were titrated into cultures ($P < 0.001$) (Table 2). An associated reduction in the PRI was observed only in those cultures treated with 200 µg/ml ($P < 0.05$) and 300 µg/ml of both pure and formulated compound ($P < 0.001$). Furthermore, a regression test showed that the PRI decreased as a function of either the concentration of pirimicarb ($r = -0.97$, $P < 0.01$) or Aficida® ($r = -0.88$, $P < 0.01$) titrated into cultures (Table 2).

The MI data for pirimicarb- and Aficida®-treated cultures are also presented in Table 2. BLM treatment produced a clear depression of the MI compared with the corresponding control values ($P < 0.01$). For both chemicals, a progressive concentration-related inhibition of MI was observed for all concentrations tested ($P < 0.05$ – $P < 0.001$). A regression test showed that the MI decreased as a function of either the concentration of pirimicarb ($r = -0.85$, $P < 0.05$) or Aficida® ($r = -0.95$, $P < 0.01$) titrated into cultures. When either 300 µg/ml of pirimicarb or Aficida® was used, the MI of cultures decreased over control values ($f = 1.00$) by a mean f of 0.21 ± 0.03 and a mean f of 0.29 ± 0.02 , respectively (Table 2).

4. Discussion

We estimated on CHO-K1 cells the genotoxicity and cytotoxicity induction of *N,N*-dimethylcarbamate pirimicarb and its commercial formulation Aficida® (pirimicarb 50%), by analyzing different end-points namely the analysis of CAs, SCE frequency, cell-cycle progression and MI. The results showed that both products induced a significant concentration-dependent increase in the number of abnormal cells and chromatid-type breaks. In addition, the frequency of SCEs and cell-cycle progression were significantly modified only when concentrations higher than 100 µg/ml of both test compounds were employed. The capacity of pirimicarb and Aficida® to induce SCEs was found equivalent for all concentration assayed. A regression test showed that the PRI decreased as a function of either the concentration of pirimicarb and Aficida® titrated into cultures. For both chemicals, a progressive concentration-related inhibition of MI was observed for all concentrations tested. Then, the results obtained demonstrated that all *in vitro* assays employed were sensitive enough to detect the genotoxicity and cytotoxicity of the insecticide pirimicarb and its formulated product, at least on CHO-K1 cells.

Previous investigations of the genotoxic potential of pirimicarb, using a wide range of assays for mutagenicity and genotoxicity, have revealed non-conclusive and contradictory results [12]. Moreover, in spite of the discrepancies of the results reported so far, pirimicarb has been classified as a moderately hazardous compound (class II) by WHO [10]. Our observations are in agreement with the classification proposed by the WHO for the potential deleterious effect of this *N,N*-dimethylcarbamate.

So far, this is the first report demonstrating the ability of both pure pirimicarb and its formulated product Afcida® to induce SCEs and CAs in mammalian cells *in vitro*. Similar observations have been reported for others *N*-methyl carbamates using these two bioassays. An enhancement of SCEs has been reported after treatment with aldicarb [35,36], carbaryl [37], propoxur [35,38], carbofuran [8,35], and methomyl [35]. Induction of CAs has been also observed after *in vitro* exposure to propoxur [35,39], methomyl [35,40], aldicarb [35,41], carbaryl [42], and carbofuran [35,43], among others. In *in vivo* conditions, the available information on the genotoxic property/ies exerted by pirimicarb is scarce. Pilinskaia [19] reported an enhancement of CAs in occupational workers. However, no increase in SCE frequency related to both occupational and *in vivo* laboratory exposure has been reported so far. However, and in agreement with the positive genotoxic effects we found in our current study, it has been previously observed the ability of the pesticide to induce single-strand breaks in human lymphocytes *in vitro* without metabolic activation [20]. Thus, the capability of mammalian cells to metabolize this carbamate pesticide could not be ruled out. Further experiments are required to elucidate whether this concept is valid for CHO-K1 as well as human lymphocytes or if it is a common mechanism/s for other mammalian cells.

Our results clearly demonstrated that both pirimicarb and Afcida® induced perturbations of the cell-cycle progression when 100–300 µg/ml concentration-range of pirimicarb and 200–300 µg/ml of Afcida® were titrated into cultures, respectively. This cell-cycle arrest may be an adaptative process in which surveillance mechanism delays the cell-cycle when DNA lesions occur. It is well known the ability of cells to delay their cell-cycle in order to repair to take place [44,45].

Studies of toxicity of have been conducted of a number of metabolites of pirimicarb: three carbamate metabolites, three hydroxypyrimidine metabolites and three guanidine metabolites. Two out of the three carbamate metabolites, namely desmethyl pirimicarb and desmethylformamido pirimicarb, were found to be of the same toxicity order as that of pirimicarb itself whereas lower toxicity has been reported for the remaining seven derivatives [12]. Accordingly, it has been suggested that the genotoxicity of the pirimicarb could be mostly related to the effect of the desmethyl pirimicarb and/or the desmethylformamido pirimicarb metabolites [12]. So far, the mechanism/s by which *N,N*-dimethylcarbamate and their carbamate metabolites exert genotoxicity is not fully established. However, our findings verify previous results depicting the genotoxicity of pirimicarb through the induction of chromosomal damage as well as single-strand DNA breaks revealed by the comet assay in human lymphocyte cells both *in vivo* and *in vitro* [19,20]. Our observations confirmed the findings of other authors revealing that pirimicarb is able to exert DNA damage in cultured cells without the presence of a microsomal metabolic S-9 fraction during culturing [20]. Thus, the non-consistent results reported so far by the different research groups about the deleterious effects of pirimicarb could be attributed to the ability to convert this type of pesticide into its carbamate derivatives by the different cellular systems employed [12,17,19,20]. Then, it could be assumed that the deleterious effect induced by insecticide on mammalian cells is committed to the pesticide itself or to any metabolite/s or any other subproducts generated during the treatment period, at least in human lymphocytes and CHO-K1 cells *in vitro*.

Several investigations have proved that commercial formulations have the ability to induce DNA damage by themselves [8,22,23,27,46–48]. As a commercial formulation, Afcida® contains a 50% of excipients that theoretically not possessing deleterious genotoxic and/or cytotoxic effect/s. However, our results demonstrated that the excipient contained in Afcida® affects the genotoxic potential of the insecticide when using the SCE end-point. We observed that although only 50% of pirimicarb was

present at each of the concentration tested of Afcida®, the capacity of SCE induction was found equivalent for all concentration assayed. Three plausible explanations for this peculiar observation could be suggested. Though almost improbable, the possibility that the concentration of pirimicarb in the technical formulation could be higher than 50% could not be discarded. Second, that the theoretically inert excipients may be, in fact, genotoxicants by themselves and able to induce SCEs. Finally, the possibility that during the manufacture of the Afcida®, the parental pirimicarb could be converted into any carbamate derivative cannot be ruled out. In spite of any putative explanation, it seems evident that the component/s of the excipient from the Argentinean pirimicarb-containing technical formulation Afcida® were able to enhance the pirimicarb-induced SCEs but not CAs, at least in CHO-K1 cells. In agreement with this possibility, a positive linear relationship was found between the pirimicarb-concentration-range and the number of induced abnormal cells but not for Afcida® treatment. Unfortunately, the identity of the excipients present in the commercial formulation we evaluated was not made available to us by the manufacturer. It is well known that SCEs are considered to be a reflection of recombinational repair of double-strand breaks [49]. On the other hand, unrepaired or mis-repaired lesions introduced in the DNA such as single or double-strand breaks, base damages, base alkylations, DNA cross-links, among others, lead to chromosomal aberrations [50]. Although the frequencies of CAs and SCEs bioassays estimate different deleterious events induced into DNA, as previously stated, it can be suggested that the former resulted to be a higher sensitive end-point to detect DNA damage on CHO-K1 cells exposed to pirimicarb than the frequency of SCEs does.

Finally, the study clearly demonstrated that pirimicarb by itself and even in a greater extend its Argentinean formulation Afcida® exert both genotoxicity and cytotoxicity in mammalian cells in culture, at least in CHO-K1 cells. Further studies are required in order to analyze whether these effects are committed to CHO-K1 cells or can be extensive to other cell types either *in vivo* or *in vitro*.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by grants from National Council of Scientific and Technological Research (CONICET, PIP 6386), National University of La Plata (Grant Number 11/N493 and 11/N564) and National Agency of Scientific and Technological Promotion (BID 1728/OC-AR-PICT 2004 number 26116) from Argentina.

References

- [1] D.J. Kirkland, L. Henderson, D. Marzin, L. Müller, J.M. Parry, G. Speit, D.J. Tweats, G.M. Williams, Testing strategies in mutagenicity and genetic toxicology: an appraisal of the guidelines of the European scientific committee for cosmetics and non-food products for the evaluation of hair dyes, *Mutat. Res.* 588 (2005) 88–105.
- [2] J.M. Garcia-Sagredo, Fifty years of cytogenetics: a parallel view of the evolution of cytogenetics and genotoxicology, *Biochim. Biophys. Acta* 1779 (2008) 363–375.
- [3] OECD, In Vitro mammalian chromosome aberration test, in: OECD Guidelines for the Testing of Chemicals, Organization for Economic Co-operation and Development (OECD), OECD, Paris, 1997.
- [4] C.B. Brezden, R.A. McClelland, A.M. Rauth, Apoptosis and 1-methyl-2-nitroimidazole toxicity in cho cells, *Br. J. Cancer* 76 (1997) 180–188.
- [5] S. Soloneski, M. González, E. Piaggio, M. Apezteguía, M.A. Reigosa, M.L. Larramendy, Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. I. Genotoxic evaluation on cultured human lymphocytes exposed *in vitro*, *Mutagenesis* 16 (2001) 487–493.
- [6] S. Soloneski, M.A. Reigosa, M.L. Larramendy, Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. II. Clastogenesis on immunophenotyped human lymphocytes assessed by the micronucleus test, *Environ. Mol. Mutagen.* 40 (2002) 57–62.

- [7] S. Soloneski, M.A. Reigosa, M.L. Larramendy, Vitamin E prevents ethylene bis(dithiocarbamate) pesticide zineb-induced sister chromatid exchange in Chinese hamster ovary cells, *Mutagenesis* 18 (2003) 505–510.
- [8] S. Soloneski, M.A. Reigosa, G. Molinari, N.V. González, M.L. Larramendy, Genotoxic and cytotoxic effects of carbofuran and furadan® on Chinese hamster ovary (CHO_{K1}) cells, *Mutat. Res.* 656 (2008) 68–73.
- [9] A. Aouadene, C. Di Giorgio, L. Sarrazin, X. Moreau, L. De Jong, F. Garcia, A. Thiery, A. Botta, M. De Méo, Evaluation of the genotoxicity of river sediments from industrialized and unaffected areas using a battery of short-term bioassays, *Environ. Mol. Mutagen.* 49 (2008) 283–299.
- [10] WHO, The WHO Recommended Classification of Pesticides by Hazard and Guidelines to the Classification 1988–1989, World Health Organization, Geneva, 1988, pp. 1–200.
- [11] WHO, International Programme of Chemical Safety, World Health Organization, Geneva, 2000–2002.
- [12] WHO-FAO, Pesticides Residues in Food-2004, FAO Plant Production and Protection Paper, World Health Organization and Food and Agriculture Organization of the United Nations, Rome, 2004, pp. 154–161.
- [13] EPA, The Grouping of a Series of Dithiocarbamate Pesticides Based on a Common Mechanism of Toxicity, US Government Printing Office, Washington, DC, 2001.
- [14] EPA, Compendium of Registered Pesticides, US Government Printing Office, Washington, DC, 1974.
- [15] IARC, Some Carbamates, Thiocarbamates and Carbazides, International Agency for Research on Cancer, Lyon, 1976.
- [16] EPA, Pesticide Fact Sheet: Pirimicarb, US Government Printing Office, Washington, 1974, pp. 1–18.
- [17] I. Aguirrezabalaga, I. Santamaría, M.A. Comendador, The w/w+ SMART is a useful tool for the evaluation of pesticides, *Mutagenesis* 9 (1994) 341–346.
- [18] D. Anderson, C.R. Richardson, C.A. Howard, C. Bradbrook, M.J. Salt, Pirimicarb: a cytogenetic study in the rat, *WHO* (1980).
- [19] M.A. Pilinskaia, Cytogenetic effect of the pesticide pirimor in a lymphocyte culture of human peripheral blood *in vivo* and *in vitro*, *Tsitol. Genet.* 16 (1982) 38–42.
- [20] Ü. Undeğer, N. Başaran, Effects of pesticides on human peripheral lymphocytes *in vitro*: induction of DNA damage, *Arch. Toxicol.* 79 (2005) 169–176.
- [21] N. Lin, V.F. Garry, *In vitro* studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota, *J. Toxicol. Environ. Health* 60 (2000) 423–439.
- [22] N.V. González, S. Soloneski, M. Larramendy, Dicamba-induced genotoxicity of Chinese hamster ovary (CHO) cells is prevented by vitamin E, *J. Hazard. Mater.* 163 (2009) 337–343.
- [23] N.V. González, S. Soloneski, M.L. Larramendy, The chlorophenoxy herbicide dicamba and its commercial formulation banvel induce genotoxicity in Chinese hamster ovary cells, *Mutat. Res.* 634 (2007) 60–68.
- [24] K. Syberg, A. Elleby, H. Pedersen, N. Cedergreen, V.E. Forbes, Mixture toxicity of three toxicants with similar and dissimilar modes of action to *Daphnia magna*, *Ecotoxicol. Environ. Safe.* 69 (2008) 428–436.
- [25] T.H. Andersen, R. Tjørnhøj, L. Wollenberger, T. Slothuus, A. Baun, Acute and chronic effects of pulse exposure of *Daphnia magna* to dimethoate and pirimicarb, *Environ. Toxicol. Chem.* 25 (2006) 1187–1195.
- [26] R. Alvarez, M.P. Honrubia, M.P. Herráez, Skeletal malformations induced by the insecticides ZZ-Aphox and Folidol during larval development of *Rana perezi*, *Arch. Environ. Contam. Toxicol.* 28 (1995) 349–356.
- [27] G. Molinari, S. Soloneski, M.A. Reigosa, M.L. Larramendy, *In vitro* genotoxic and cytotoxic effects of ivermectin and its formulation ivomec® on Chinese hamster ovary (CHO_{K1}) cells, *J. Hazard. Mater.* 165 (2009) 1074–1082.
- [28] A.D. Bolzán, M.S. Bianchi, Detection of incomplete chromosome elements and interstitial fragments induced by bleomycin in hamster cells using a telomeric PNA probe, *Mutat. Res.* 554 (2004) 1–8.
- [29] M.L. Larramendy, D. López-Larraz, L. Vidal-Rioja, N.O. Bianchi, Effect of the metal chelating agent o-phenanthroline on the DNA and chromosome damage induced by bleomycin in Chinese hamster ovary cells, *Cancer Res.* 49 (1989) 6583–6586.
- [30] A.D. Bolzán, N.O. Bianchi, M.L. Larramendy, M.S. Bianchi, Chromosomal sensitivity of human lymphocytes to bleomycin. Influence of antioxidant enzyme activities in whole blood and different blood fractions, *Cancer Genet. Cytogenet.* 64 (1992) 133–138.
- [31] J. Sánchez, M.S. Bianchi, A.D. Bolzán, Effect of bleomycin on interstitial telomeric sequences of immortalized Chinese hamster ovary cells, *Mutat. Res.* 669 (2009) 139–146.
- [32] L. Lamberti, P. Bigatti-Ponzetto, G. Ardito, Cell kinetics and sister chromatid exchange frequency in human lymphocytes, *Mutat. Res.* 120 (1983) 193–199.
- [33] B.M. Miller, I.D. Adler, Suspect spindle poisons: analysis of c-mitotic effects in mouse bone marrow cells, *Mutagenesis* 4 (1989) 208–215.
- [34] R. Mateuca, N. Lombaert, P.V. Aka, I. Decordier, M. Kirsch-Volders, Chromosomal changes: induction, detection methods and applicability in human biomonitoring, *Biochemie* 88 (2006) 1515–1531.
- [35] C.M. Lin, L.Y. Wei, T.C. Wang, The delayed genotoxic effect of N-nitroso N-propoxur insecticide in mammalian cells, *Food Chem. Toxicol.* 45 (2007) 928–934.
- [36] S. Kevekordes, T. Gebel, K. Pav, R. Edenharder, H. Dunkelberg, Genotoxicity of selected pesticides in the mouse bone-marrow micronucleus test and in the sister-chromatid exchange test with human lymphocytes *in vitro*, *Toxicol. Lett.* 89 (1996) 35–42.
- [37] A. Onfelt, I. Klusterska, Sister Chromatid exchanges and thioguanine resistance in V79 Chinese hamster cells after treatment with the aneuploidy-inducing agent carbaryl+S9 mix, *Mutat. Res.* 125 (1984) 269–274.
- [38] P.S. Lin, S. Quamo, K.C. Ho, K. Baur, The diethyldithiocarbamate concentration effects and interactions with other cytotoxic agents on Chinese hamster cells (V79), *Rad. Res.* 102 (1985) 271–282.
- [39] H.H. Kuo, S.S. Shyu, T.C. Wang, Genotoxicity of low dose N-nitroso propoxur to human gastric cells, *Food Chem. Toxicol.* 46 (2008) 1619–1626.
- [40] S. Bonatti, C. Bolognesi, P. Degan, A. Abbondandolo, Genotoxic effects of the carbamate insecticide methomyl. I. *In vitro* studies with pure compound and the technical formulation “lannate 25”, *Environ. Mol. Mutagen.* 23 (1994) 306–311.
- [41] M. González-Cid, E. Matos, Chromosomal aberrations in cultured human lymphocytes treated with aldicarb, a carbamate pesticide, *Mutat. Res.* 191 (1987) 99–103.
- [42] S.D. Sivanesan, K. Krishnamurthi, S.D. Wachasunder, T. Chakrabarti, Genotoxicity of pesticide waste contaminated soil and its leachate, *Biomed. Environ. Sci.* 17 (2004) 257–265.
- [43] R. Naravani, K. Jamil, Cytogenetic biomarkers of carbofuran toxicity utilizing human lymphocyte cultures *in vitro*, *Drug Chem. Toxicol.* 28 (2005) 359–372.
- [44] D.P. Lane, p53, guardian of the genome, *Nature* 358 (1992) 15–16.
- [45] D.K. Orren, L.N. Petersen, V.A. Bohr, A UV-responsive G2 checkpoint in rodent cells, *Mol. Cell Biol.* 15 (1995) 3722–3730.
- [46] R.M. Brand, C. Mueller, Transdermal penetration of atrazine, alachlor, and trifluralin: effect of formulation, *Toxicol. Sci.* 68 (2002) 18–23.
- [47] D. Zeljezic, V. Garaj-Vrhovac, P. Perkovic, Evaluation of DNA damage induced by atrazine and atrazine-based herbicide in human lymphocytes *in vitro* using a comet and DNA diffusion assay, *Toxicol. In Vitro* 20 (2006) 923–935.
- [48] B. Kaya, A. Yanikoglu, R. Marcos, Genotoxicity studies on the phenoxyacetates 2,4-D and 4-CPA in the *Drosophila* wing spot test, *Teratogen. Carcin. Mutagen.* 19 (1999) 305–312.
- [49] A.T. Natarajan, G. Obe, M. Hayashi, Chromosomal aberrations, *Mutat. Res.* 657 (2008) 1–2.
- [50] A.T. Natarajan, J.J. Boei, Formation of chromosome aberrations: insights from FISH, *Mutat. Res.* 544 (2003) 299–304.