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Herbicide 2,4-dichlorophenoxyacetic acid (2,4-D)-induced cytogenetic damage in human lymphocytes *in vitro* in presence of erythrocytes

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Abstract

The genotoxic effects of 2,4-D and its commercial derivative 2,4-D DMA were studied by measuring sister chromatid exchange (SCE), cell-cycle progression and mitotic index in human whole blood (WBC) and plasma leukocyte cultures (PLC). Concentrations of 10, 25, 50 and 100 μg herbicide/ml were used during 72 h. In WBC, a significant increase in SCE frequency was observed within the 10–50 μg 2,4-D/ml and 25–100 μg 2,4-D DMA/ml dose range. Contrarily, in PLC, none of the concentrations employed affected the SCEs frequency. A significant delay in cell proliferation was observed in WBC after treatments with 25 and 50 μg 2,4-D/ml and 50 and 100 μg 2,4-D DMA/ml. In PLC, only 100.0 μg 2,4-D/ml altered cell-cycle progression. For both chemicals, a progressive dose-related inhibition of mitotic activity was observed. The results demonstrated that the presence of erythrocytes in the culture system modulated the DNA and cellular damage inflicted by 2,4-D and 2,4-D DMA into human lymphocytes *in vitro* as well as both 2,4-D and 2,4-D DMA were more potent genotoxic agents in the presence of human red cells.

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1. Introduction

Living species are inevitably exposed to pesticides because their use in agriculture has been increasing steadily all over the world. In epidemiological as well as in experimental clastogenesis studies there is increasing interest in biomonitoring markers, which can provide or at least give a clue for the biologically active/passive exposure to genotoxic pollutants. Several epidemiological studies demonstrated that occupational exposure to some pesticides may be related to several cancers types and other diseases (IARC, 1977, 1983, 1986, 1987, 1991). To evaluate genetic damage various *in vivo* and *in vitro* test systems have been assessed in mammalian as well as in prokaryotic cells (Evans and O'Riordan, 1975; Evans, 1986). One of most employed test systems are cultured peripheral lymphocytes, in which the analysis of both sister chromatid exchange (SCE) frequency and cell-cycle proliferation have been widely used as bioassay for clastogenicity (Latt et al., 1980; Wilmer et al., 1981; Palitti et al., 1982; James et al., 1997).

Among the group of chlorinated aromatic hydrocarbon acid pesticides, 2,4-dichlorophenoxyacetic acid (2,4-D) has been in extensive used uninterruptedly since 1944 in agriculture for broad-leaved weeds, control of woody plants, and reforestation programs (IARC, 1977). Despite its decades of usage, there are still data gaps concerning 2,4-D's effects on human health and environmental risk (IARC, 1991). In plants, this chemical mimics the action of auxins, hormones that stimulate growth, but in mammals and other species no mimic hormonal activity was observed (Osterloh et al., 1983). It is known that 2,4-D is taken up by the cells, passes rapidly through the cell membrane, and is not metabolized (Bergesse and Balegno, 1995).

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So far, the exact mechanism/s by which 2,4-D is incorporated into either plant or animal cells are not totally understood, although peroxisome proliferation has been considered as one plausible mediators of 2,4-D genotoxicity (Linnainmaa, 1984; Lundgren et al., 1987; Vainio et al., 1982). It has been suggested that chemicals possessing this type of activity induce peroxisomal β-oxidation increasing the intracellular production of H_2O_2 and other reactive oxygen species (Reddy et al., 1980, 1982). Then, this type of actions may explain an indirect way of action by which the genetic material could be affected by the chemical. It is well documented that when mammalian cells are exposed to reactive oxygen species, lesions appear in the DNA (Meneghini and Hoffmann, 1980; Birboin, 1982; Larramendy et al., 1987). So far, induction of chromosomal aberrations (Emerit et al., 1982a,b; Estervig and Wang, 1984; Phillips et al., 1984; Nicotera et al., 1985), SCE (Emerit et al., 1982a,b; Speit and Vogel, 1982; Estervig and Wang, 1984; Nicotera et al., 1985; Larramendy et al., 1987,1989a,b; James et al., 1997) and delay in cell-cycle progression (Larramendy et al., 1987, 1989a,b) have been determined as a consequence of the damage inflicted in the DNA by reactive oxygen species.

So far, the ability of 2,4-D to induce DNA damage measured by the SCE assay is not fully documented and controversial. Galloway et al. (1987) found an increase SCE in Chinese hamster ovary (CHO) cells after treatment in presence of S9 fraction. However, in a recent study reported by us, a significant increase of SCE was observed in CHO cells treated with 2.0–4.0 μg/ml 2,4-D and 2,4-D DMA dose-range even in the absence the S9 fraction (González et al., 2005). Turkula and Jalal (1985) observed a highly significant increase in SCEs in human lymphocytes *in vitro* treated with 50.0 μg/ml of pure 2,4-D but not when dosages of 100.0 and 250.0 μg/ml were employed. Similarly, Madrigal-Bujaidar et al. (2001) reported a significant increase in SCE frequencies in bone marrow and germ cells of mice after oral administration of 100 and 200 mg/kg of 2,4-D. On the other hand, no SCE induction was observed after a 1 h pulse-treatment of CHO cells with 2,4-D pure and a commercial 2,4-D formulation (2,4-D salt as the active ingredient) with and without S9 activation (2.0–221.0 μg/ml dose-range) (Linnainmaa, 1983). Mustonen et al. (1989) reported that pure 2,4-D was unable to increase the frequency of chromosomal aberrations in human lymphocytes *in vitro* (27.0–75.6 μg/ml dose-range), whereas commercial 2,4-D formulation significantly enhanced it only in the absence of S9 fraction (27.0–27,630.0 μg/ml dose-range). Recently, SCE induction has been reported for chick embryo B-lymphocytes after long-term exposure to either pure 2,4-D and commercial formulation containing 37% of 2,4-D (500.0–4000.0 μg/embryo dose-range) (Arias, 1995).

In agriculture, the 2,4-D is used as the active component of several technical formulations. Accordingly, workers and environment are exposed to the simultaneous action of the active ingredient and a variety of other chemicals contained in its commercial derivatives. Therefore, the present study was carried out to compare the levels of the possible genotoxicity of 2,4-D as a pure active ingredient and 2,4-D DMA, as one of its most widely used technical formulation in Argentina. The DNA-damaging potential of these compounds was monitored on human lymphocytes *in vitro* in presence or absence of red human cells, using the analysis of the SCE frequency and the cell-cycle progression as cytogenetic endpoints.

2. Materials and methods

2.1. Chemicals

2,4-Dichlorophenoxyacetic (2,4-D; CAS No. 94-75) was obtained from Riedel-de Haën (Pestanal®, Hannover, Germany). Dimethylamine 2,4-D salt (2,4-D DMA) was kindly provided by Delente Laboratories SRL (Buenos Aires, Argentina). Acetone was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Blood samples

Blood samples were obtained from six healthy men under 30 years of age (non-smokers, non-alcohol drinkers, and under no medication or food supplements intake) selected according to previously described recommendations reported elsewhere (Bianchi et al., 1979). Twenty ml of blood were drawn by venipuncture from each donor immediately prior culturing at the Buenos Aires Province Blood Bank (Argentina) under approval granted by the ethical committee for studies involving human subjects. The donors were thoroughly questioned regarding their lifestyle, working environment and recent exposures to potential mutagens to exclude possible confounding factors affecting the endpoints employed in the analysis, e.g. drugs intake, recent viral infections, vaccinations, or X-ray exposure.

2.3. Whole blood cultures (WBC) and plasma leukocyte cultures (PLC). Pesticide treatment for SCE assay

Human WBC were set up by inoculating 1.0 ml of whole blood in 9.0 ml of culture medium [90% Ham's F10 medium (Gibco, Grand Island, NY, USA), 10% fetal calf serum (Gibco), 100 U penicillin/ml (Gibco), 10 μg streptomycin/ml (Gibco) and 10 μg BrdUrd/ml (Sigma Chemical Co.)]. Human PLC was carried out according to Moorhead et al. (1960) and Larramendy and Reigosa (1986). Briefly, after gravity sedimentation of whole blood (approximately 20 ml sample) for 1–2 h at room temperature, aliquots of 1 ml of plasma-leukocyte suspension were added to 9 ml of complete culture medium. The final concentration of leukocytes was approximately 1.2×10^6 cells/ml. Immediately after seeding, 2,4-D and 2,4-D DMA were dissolved in acetone prior to use and then were diluted in culture medium so that the addition of 100 μl to cultures allowed to reach the required concentration specified in results section. 2,4-D and 2,4-D DMA were used at the final concentration of 10, 25, 50 and 100 μg/ml. WBC and PLC from donors 1–3 and from donors 4–6 were treated with pure 2,4-D and 2,4-D DMA, respectively. The final solvent concentration was <1% for all the treatments. Negative controls [untreated cell and solvent-vehicle-treated cells (50 μl acetone/10 ml)] were processed concurrently with herbicide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Cultures were duplicated for each experimental point, during at least three independent experiments. Immediately after herbicide treatment, 0.3 ml of phytohaemagglutinin M (Gibco) was added to each culture (0 h). After treatment, cells were incubated at 37 °C in a 5% CO_2 for 72 h. During the last 3 h of culture, the cells were treated with colchicine (0.1 μg/ml, Sigma Chemical Co.), collected by centrifugation, treated with hypotonic solution (0.075 M KCl, 37 °C, 15 min) and fixed in methanol-acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique. The same batches of culture medium, sera and reagents were used throughout the study.

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

Chromosome spreads were stained using the FPG technique for sister chromatid differentiation described in detail by Larramendy and Knuutila (1990). Slides were coded and scored blind by one cytogeneticist.

2.5. Cell-cycle kinetics and mitotic index

For each donor, a minimum of 200 metaphases per sample were scored to determine the percentage of cells which had undergone one (M_1), two (M_2), and three or more mitoses (M_{3+}). The proliferative rate index (PRI) was calculated for each experimental point according to the formula $PRI = [(\%M_1) + 2(\%M_2) + 3(\%M_{3+})]/100$ which indicated the average number of times cells had divided in the medium since the incorporation of BrdUrd until harvesting (Lamberti et al., 1983). The mitotic index (MI) was determined by scoring 2000 cells from each donor and 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) ($f = MI_t/MI_c$) (Miller and Adler, 1989).

2.6. Sister chromatid exchange analysis

For the SCE assay a total of 50 well spread diploid metaphases were scored per treatment for each experimental condition in M_2 cells. The data were expressed as the mean number of SCEs per cell ± SE.

2.7. Statistical analysis

The Kruskal–Wallis one-way analysis of variance was used to compare differences among six donors and the treatments. The two-tailed Student's *t*-test and linear regression analyses were used to compare SCE frequencies between treated and control groups. A χ^2 test was used for cell-cycle progression and mitotic index data. The level of significance chosen was 0.05 unless indicated otherwise.

3. Results

The frequencies of SCEs and cell-cycle progression obtained in WBC and PLC after treatment with different doses of both test compounds and tested in three separate experiments are summarized in Table 1. Since no differences of SCEs were observed between the untreated and vehicle-treated (negative control) cultures for both WBC and PLC, pooled data are presented for control cultures. In WBC, concentrations of 10.0, 25.0 and 50.0 μg/ml of 2,4-D induced a significant increase in SCEs frequency over control values ($P \leq 0.01$, $P \leq 0.001$) (Table 1). When 100.0 μg/ml of 2,4-D was assayed a cytotoxicity effect was observed due to a complete cellular death in all cultures. SCEs increased in a dose-dependent manner. Regression tests showed that SCEs increased as a function of the concentration of 2,4-D titrated into cultures ($r = 0.91–0.96$, $P \leq 0.001$). Treatment with commercial formulation 2,4-D DMA yielded similar results than those found in 2,4-D-treated WBC, except for the lowest dose employed (10.0 μg/ml) which was unable to alter the frequency of SCEs with respect to the control values ($P > 0.05$) (Table 1). A significant increase in the SCEs frequency was observed within concentrations of 25.0–100.0 μg/ml dose-range ($P \leq 0.01$, $P \leq 0.001$) (Table 1). Similarly, regression tests showed that SCEs increased as a function of the concentration of 2,4-D DMA titrated into cultures ($r = 0.83–0.87$,

Table 1
Sister chromatid exchange (SCE) frequencies and cell-cycle progression in control and 2,4-D- and 2,4-D DMA-treated human lymphocytes grown in whole blood (WBC) and plasma leukocyte cultures (PLC)

Culture type	Treatment μg/ml	Mean SCE[a]		Cell cycle progression[a]					
		2,4-D	2,4-D DMA	2,4-D			2,4-D DMA		
				M_1	M_2	M_{3+}	M_1	M_2	M_{3+}
WBC	0.0	7.72 ± 0.39	7.96 ± 0.48	13.33 ± 0.88	28.33 ± 0.33	58.33 ± 0.66	15.00 ± 0.58	22.30 ± 0.33	63.00 ± 0.58
	10.0	9.36 ± 0.53*	8.64 ± 0.51	14.00 ± 0.58	28.33 ± 0.88	57.67 ± 0.88	10.00 ± 0.58	23.00 ± 0.58	67.00 ± 0.88
	25.0	10.52 ± 0.64**	9.81 ± 0.53*	21.00 ± 0.58***	38.33 ± 0.88***	40.67 ± 0.33**	13.00 ± 0.58	24.33 ± 0.88	62.67 ± 0.58
	50.0	11.35 ± 0.54**	10.24 ± 0.45**	24.67 ± 0.33***	37.33 ± 0.33***	38.00 ± 0.58**	21.00 ± 0.58	30.00 ± 0.58***	49.00 ± 0.68*
	100.0	ND	10.37 ± 0.46**	ND	ND	ND	27.33 ± 0.88**	32.00 ± 0.88***	40.67 ± 0.33**
PLC	0.0	10.73 ± 0.53	11.31 ± 0.70	16.00 ± 0.58	46.00 ± 0.58	38.00 ± 0.88	14.00 ± 0.58	42.00 ± 0.33	44.00 ± 0.33
	10.0	10.59 ± 0.54	10.12 ± 0.58	16.33 ± 0.67	44.67 ± 0.33	39.00 ± 0.58	11.00 ± 0.58	40.00 ± 0.66	49.00 ± 0.58
	25.0	10.64 ± 0.65	10.90 ± 0.64	14.00 ± 0.58	43.67 ± 0.33	42.33 ± 0.33	13.00 ± 0.67	40.00 ± 0.67	47.00 ± 0.58
	50.0	11.07 ± 0.65	12.35 ± 0.61	17.33 ± 0.33	45.00 ± 0.58	37.67 ± 0.67	12.67 ± 0.67	42.33 ± 0.66	45.00 ± 0.58
	100.0	11.83 ± 0.59	11.73 ± 0.51	45.67 ± 0.88**	46.67 ± 0.88	7.67 ± 0.33**	16.00 ± 0.58	38.33 ± 0.67	45.67 ± 0.67

Lymphocytes were treated with 2,4-D and 2,4-D DMA immediately after stimulation with PHA, and harvested 72 h later. ND, not determined. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.05$, significant differences with respect to control values.

[a] Results are expressed as mean values of pooled data from three independent experiments ± SE from three donors for each pesticide employed.

$P \leq 0.01$). Over the broad dose range of concentrations, minimal genotoxicity was observed in PLC either after treatment with 2,4-D or 2,4-D DMA. For both test compounds, a slight increase in the SCEs frequencies was found although not reaching statistical significance when compared to control values ($P > 0.05$) (Table 1).

Results from the analysis of cell-cycle progression shown that 2,4-D exerted a toxic effect on lymphocytes cultures, since a significant delay in cell-cycle progression was observed in both WBC and PLC cultures. A significant increase in the frequency of M_1 and M_2 and a significant decrease in the frequency of M_3 were observed in those WBC treated with 25.0 and 50.0 μg/ml of 2,4-D ($P \leq 0.05$, $P \leq 0.001$). The highest 2,4-D dose employed in WBC (100.0 μg/ml) rendered a complete cell death. On the other hand, in PLC system, the only noticeable significant changes observed were an increase in the M_1 and a decrease in M_3 frequencies when 100.0 μg/ml of 2,4-D was assayed ($P \leq 0.001$). When 2,4-D DMA was employed, a significant increase in the frequency of M_1 and M_2 and a significant decrease in the frequency of M_3 were observed when 50.0 and 100.0 μg/ml of the compound was used ($P \leq 0.05$; $P < 0.001$). In PLC, no deleterious effect in cell-cycle progression was observed with any 2,4-D DMA treatments respect to control values ($P > 0.05$) (Table 1).

Table 2 presents the PRI, MI and *f* mean values obtained after treatment with different doses of both 2,4-D and 2,4-D DMA in WBC and PLC. Significant reductions in PRI values were observed in both WBC and PLC after treatment with 50.0 and 100.0 μg/ml of 2,4-D ($P \leq 0.05$, $P \leq 0.01$). In 2,4-D DMA-treated cultures, only a significant reduction of PRI was achieved in those WBC treated with 100.0 μg/ml ($P \leq 0.01$) (Table 2).

Significant reductions in MI were found in human lymphocytes after treatment with 50.0 and 100.0 μg/ml of 2,4-D in WBC and PLC, respectively ($P \leq 0.01$). For 2,4-D DMA, the mitotic activity in WBC showed a significant decrease over control values only when lymphocytes were treated with 100.0 μg/ml ($P \leq 0.01$). On the other hand, no significant alterations in mitotic activity were observed when lymphocytes of PLC were treated with commercial formulation ($P > 0.05$).

4. Discussion

Among pesticides, the phenoxy acid derivative 2,4-D and its commercial formulations widely used as herbicides are noted for their strong genotoxic effects in various organisms (Seiler, 1991; Garabrant and Philbert, 2002). In the present study, the genotoxicities of 2,4-dichlorophenoxyacetic acid (2,4-D) and its derivative dimethylamine 2,4-D salt (2,4-D DMA) were evaluated *in vitro* using human lymphocytes cultured in WBC and PLC systems and applying three different cytogenetic endpoints, namely, the analysis of the frequency of sister chromatid exchanges, a follow-up of cell-cycle progression, and assessment of mitotic activity. As an attempt to elucidate/evaluate the plausible cause/s of their geno- and citoxicity, we investigated their genotoxic effect using the same cell type, human lymphocytes, cultured under two different cell culture conditions, WBC and PLC systems; in other words, in the presence and absence of erythrocytes during the culture period, respectively.

Our results demonstrated that either 2,4-D or 2,4-D DMA enhanced the frequency of SCEs in WBC but not when lymphocytes were cultured in absence of erythrocytes (PLC). Furthermore, they also showed that both test compounds modulated the cell-cycle kinetics of human lymphocytes from both WBC and PLC. Finally, while 2,4-D was able to reduce the mitotic activity of lymphocytes from WBC and PLC, 2,4-D DMA exerted this deleterious effect in WBC but not in PLC.

Although the use of a wide range of assays for mutagenicity and genotoxicity, previous studies have revealed conflicting or even opposite results of the genotoxic potential of 2,4-D (Gandhi et al., 2000). These inconsistencies, as suggested by Kaya et al. (1999), could be most probably due to: (1) the use of both pure compounds and commercial formulations with unknown impurities; and/or (2) the differential sensitivities of the specific systems/endpoints used. The latter is illustrated

Table 2
Proliferative rate index (PRI), mitotic indices (MI) and mitotic index factors (*f*) values in control, 2,4-D- and 2,4-D DMA-treated human lymphocytes grown in whole blood (WBC) and plasma leukocyte cultures (PLC)

Culture type	Treatment μg/ml	Mean PRI[a]		Mean MI		Mean *f*	
		2,4-D	2,4-D DMA	2,4-D	2,4-D DMA	2,4-D	2,4-D DMA
WBC	0.0	2.44 ± 0.39	2.48 ± 0.48	24.00 ± 3.08	28.67 ± 0.33	1.00 ± 0.00	1.00 ± 0.00
	10.0	2.37 ± 0.53	2.57 ± 0.51	18.67 ± 5.13	23.67 ± 0.88	0.72 ± 0.11	0.80 ± 0.04
	25.0	2.20 ± 0.64	2.49 ± 0.53	18.33 ± 4.49	23.00 ± 0.88	0.74 ± 0.08	0.77 ± 0.07
	50.0	2.13 ± 0.54**	2.28 ± 0.45	12.00 ± 3.85*	24.00 ± 0.33	0.45 ± 0.09	0.82 ± 0.04
	100.0	ND	2.25 ± 0.46**	ND	11.67 ± 0.33*	ND	0.40 ± 0.13
PLC	0.0	2.22 ± 0.53	2.30 ± 0.70	25.00 ± 5.00	29.67 ± 0.58	1.00 ± 0.00	1.00 ± 0.00
	10.0	2.22 ± 0.54	2.38 ± 0.58	19.00 ± 4.62	25.00 ± 0.33	0.81 ± 0.07	0.84 ± 0.04
	25.0	2.30 ± 0.65	2.16 ± 0.64	20.33 ± 6.80	25.67 ± 0.33	0.65 ± 0.04	0.79 ± 0.10
	50.0	2.20 ± 0.65	2.35 ± 0.61	18.67 ± 5.13	23.33 ± 0.58	0.65 ± 0.02	0.75 ± 0.03
	100.0	1.61 ± 0.59**	2.30 ± 0.51	5.00 ± 1.15*	22.33 ± 0.88	0.19 ± 0.05	0.71 ± 0.04

Lymphocytes were treated with 2,4-D and 2,4-D DMA immediately after stimulation with PHA, and harvested 72 h later. ND, not determined. *, $P < 0.001$; **, $P < 0.05$, significant differences with respect to control values.

[a] Results are expressed as mean values of pooled data from three independent experiments ± SE from three donors for each pesticide employed.

by the studies, e.g., performed in *Drosophila melanogaster* by Graf and Würgler (1996) who reported that while the white-ivory eye spot test did not detect any genotoxic effect for the phenoxy herbicide, the wing somatic mutation and recombination tests gave positive results.

We observed that 2,4-D and 2,4-D DMA caused SCEs in human lymphocytes indicating that they have clastogenic activity. It has been suggested that at the chromosomal level, the induction of SCE is a reliable indicator for the screening of clastogens, since the bioassay is more sensitive than the analysis of clastogen-induced chromosomal aberrations (Palitti et al., 1982).

In the present study, WBC and PLC systems were respectively chosen since they are routinely used for SCE assays although differences in baseline SCE frequencies and cell-cycle progression, of the same cell between these two types of cultures, have been reported to occur in humans lymphocytes most probably committed to the presence or absence of erythrocytes therein (Ray and Altenburg, 1978; Larramendy and Reigosa, 1986; Larramendy et al., 1990; Van Hummelen and Kirsch-Volders, 1992). We have previously reported that human lymphocytes when cultured in the absence of erythrocytes within the culture media (PLC) show at least a two-fold increase in the basal frequency of SCEs and lengthened of cell-cycle over the values observed when they are cultured in the presence of red blood cells (WBC) (Larramendy and Reigosa, 1986; Larramendy et al., 1990, 1996). Besides, the titration of red blood cells into PLCs induces a decrease of basal frequency of SCEs in a dose-dependent manner as well as a faster proliferation rate, achieving values similar to those observed in WBC when the concentration of erythrocytes incorporated into PLCs is equivalent to the present in WBC (Larramendy and Reigosa, 1986; Larramendy et al., 1990, 1996). Furthermore, previous reports showed differential response in clastogen-induced SCE induction between these types of cultures after treatment with the same xenobiotic agent (Ray and Altenburg, 1978; Norppa et al., 1983; Larramendy and Reigosa, 1986; Norppa and Jarventaus, 1992; Landi et al., 1995).

The results of this study showed that both 2,4-D and 2,4-D DMA induced a significant increase of SCE in human WBC. Although usually the enzymatic activity in erythrocytes act as a detoxification system by presence of glutathione *S*-transferase complex (Gutteridge, 1989), our results suggested that 2,4-D-induced SCEs was a result of metabolic activation by erythrocytes present in those cultures, effect not found when the lymphocytes were cultured in absence of red cells as the PLC. In agree with these results, Norppa and co-workers (Norppa et al., 1983) demonstrated that styrene induces SCEs in human whole-blood lymphocytes cultures without exogenous metabolizing system. Accordingly, taking into account the numerical preponderance of erythrocytes over the amount of leukocytes present in culture, it could be suggested that red blood cells may also play a major role in the metabolic activation of the compound under study in WBC-treated culture system.

Insufficient information is currently available on the molecular background about toxic properties of phenoxy herbicides not only for humans but also for others species (IARC, 1991). Examination of the chemical structure of the phenoxy group indicates a potential for the generation of reactive intermediates capable of eliciting toxic effects. Some authors have reported that 2,4-D causes lipid bilayer damage by lipid peroxidation as well as increases in membrane fluidity by damaging membrane proteins in erythrocytes (Duchnowicz and Koter, 2003). Accordingly, the labile iron pool becomes compromised and the cellular concentration of free Fe^{II} is then increased. The alteration in the ratio of iron pool results in increased lipid peroxidation and the accumulations of lipid hydroperoxide-derived reactive aldehydes (Gutteridge, 1989) which can induce DNA lesions and apoptosis (West et al., 2004; Lee et al., 2005). Several reports have shown that injure introduced into DNA by these metabolic intermediate compounds is closely related to a result of membrane lipid peroxidation (Larramendy et al., 1987; Fraga and Tappel, 1988; Zastawny et al., 1995). However, this evidence is in contrast to the fact that membrane lipid peroxidation is often a late stage in oxidative damage and that the intermediates of lipid peroxidation such as $ROO\cdot$ and $RO\cdot$ are not always able to reach DNA (Halliwell, 1978; Gutteridge, 1989).

It is well known that reactive oxygen species formed by normal cellular metabolism or by exogenous sources play a significant role in several biological processes including mutagenesis, carcinogenesis, reproductive cell death and aging (Halliwell, 1978; Gutteridge, 1989). Among these species, $O_2\cdot$ and H_2O_2 do not cause DNA damage under physiological conditions. The toxicity of $O_2\cdot$ and H_2O_2 is thought to result from their transition metal ion-catalyzed conversion by the Haber-Weiss reaction into hydroxyl radical ($\cdot OH$), that is now considered to be the major mechanism by which the highly reactive hydroxyl radicals react with the cellular DNA (Larramendy et al., 1987; Zastawny et al., 1995). Years ago, we demonstrated by the first time that the induction of SCEs by $O_2\cdot$ and H_2O_2 mammalian cells *in vitro* is mediated by the presence of Fe^{II}/Fe^{III} in the culture system (Larramendy et al., 1987). In agreement with our present observations, it is well documented that when mammalian cells are exposed to reactive oxygen species, lesions appear in the DNA (Meneghini and Hoffmann, 1980; Birboin, 1982; Larramendy et al., 1987; Bolzán et al., 1992). As a consequence of the damage inflicted on DNA by reactive oxygen species, several studies have reported the induction of chromosomal aberrations (Emerit and Cerutti, 1981; Estervig and Wang, 1984; Phillips et al., 1984; Nicotera et al., 1985; Larramendy et al., 1989a,b), sister chromatid exchange (Emerit et al., 1982a,b; Speit and Vogel, 1982; Estervig and Wang, 1984; Nicotera et al., 1985; Larramendy et al., 1987, 1989a,b) and delays in cell-cycle progression (Larramendy et al., 1987, 1989a,b).

It is well documented that one of the most major cellular response observed both *in vitro* and *in vivo* after exposure to chemicals, ionizing radiation, and/or other genotoxic agents is the inhibition or the delay of cell-cycle progression. This event is commonly observed after pesticide exposure (Sobti et al., 1982; Rupa et al., 1989; Soloneski et al., 2001,2002). Investigations on the possible biologic mechanisms for 2,4-D have been primarily focused on the genotoxicity of the

herbicide, whereas cell-cycle progression analysis was rarely assessed. Our observations revealed that human lymphocytes *in vitro* when exposed to 100.0 μg/ml of both 2,4-D and 2,4-D DMA differentially altered their cell-cycle proliferation and mitotic index upon the presence/absence of red cells in the culture. Besides, our results also demonstrated that 2,4-D has a higher cell-cycle delay capability than 2,4-D DMA since this concentration induced cellular death in WBC while only a delay in cell-cycle progression was observed in PLC. On the other hand, our current results showed that both cell-cycle proliferation and mitotic index of human lymphocytes were altered by both 2,4-D and 2,4-D DMA regardless of the presence of red cells when lower doses were titrated into cultures. In agreement with our observations, Holland et al. (2002) observed a significant inhibition of replicative index when human lymphocytes were treated with 2-4-D in *in vitro* both in the presence or absence of red blood. It is worth mentioning here that the herbicide doses used in the present report were higher than the dose-range of 2,4-D concentrations employed in our previous investigations in CHO cells (González et al., 2005) and in the study reported by Pilinscaya (1974). Previously, we have recently reported the ineffectiveness of both 2,4-D and 2,4-D DMA within the dose-rage of 2.0–10.0 μg/ml in inducing alterations of the cell-cycle progression of CHO cells (González et al., 2005). These observations could highlight that most probably the mechanism/s by which the herbicide inflicted DNA and cellular damage are different and/or that different cellular pathways are involved depending the cellular target, i.e., cell proliferation and DNA damage. Two plausible possibilities could explain these controversial findings. The doses of 2,4-D and 2,4-D DMA used in the present study were higher to the dose-range assayed in our previous studies in CHO cells (González et al., 2005). Moreover, the possibility that human lymphocytes are more sensitive than CHO cells to the deleterious effect of the 2,4-D in inducing alterations in the cell-cycle progression cannot be ruled out.

In summary, we clearly have showed that the presence of erythrocytes in the culture system modulated the DNA and cellular damage inflicted by 2,4-D and 2,4-D DMA into human lymphocytes *in vitro*. As far as cytotoxicity and genotoxicity are concerned, since both 2,4-D and 2,4-D DMA were more potent genotoxic agents in the presence of human red cells, further studies are required to elucidate the plausible/s toxic mechanism/s implicated in these processes.

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References

Arias E. Cytogenetic and cytokinetic effects of 2,4-D on B lymphocytes during the development of chick embryos. Toxicol In Vitro 1995;1:65–9.

Bergesse JR, Balegno HF. 2,4-Dichlorophenocyacetic acid influx is mediated by an active transport system in Chinese hamster ovary cells. Toxicol Lett 1995;81:167–73.

Bianchi NO, Bianchi MS, Larramendy ML. Kinetics of human lymphocyte division and chromosomal radiosensitivity. Mutat Res 1979;63:317–24.

Birboin HC. DNA strand breakage in human leukocytes exposed to a tumor promoter, phorbol myristate acetate. Science 1982;215:1247–9.

Bolzán AD, Bianchi NO, Larramendy ML, Bianchi MS. Chromosomal sensitivity of human lymphocytes to bleomycin. Influence of antioxidant enzyme activities in total blood and different blood fractions. Cancer Genet Cytogen 1992;64:133–8.

Duchnowicz P, Koter M. Damage to the erythrocyte membrane caused by chlorophenoxyacetic herbicides. Cell Mol Biol Lett 2003;8:25–30.

Emerit I, Cerutti P. The tumor promoter phorbol-12-myristate-13-acetate induces chromosomal damage and polyploidization in human lymphocytes. Nature 1981;293:144–6.

Emerit I, Cerutti P, Levy A, Jalbert P. Chromosome breakage factor in the plasma of two Bloom's syndrome patients. Hum Genet 1982a;61:65–7.

Emerit I, Keck M, Levy A, Feigold J, Michelson AM. Activated oxygen species at the origin of chromosome breakage and sister-chromatid exchanges. Mutat Res 1982b;140:27–31.

Estervig D, Wang RJ. Sister chromatid exchanges and chromosome aberrations in human cells induced by H_2O_2 and other photoporducts generated by fluorescent light-exposed medium. Photochem Photobiol 1984;40:333–6.

Evans HJ. The role of human cytogenetics in studies of mutagenesis and carcinogenesis. Prog Clin Biol Res 1986;209A:42–69.

Evans HJ, O'Riordan ML. Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mutat Res 1975;31:135–48.

Fraga C, Tappel AL. Damage to DNA concurrent with lipid peroxidation in rat liver slices. Biochem J 1988;252:893–6.

Galloway SM, Armstrong MJ, Reuben C, Colman S, Brown B, Cannon C, et al. Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. Environ Mol Mutagen 1987;10:1–175.

Gandhi R, Wandji S, Snedeker S. Critical evaluation of cancer risk form 2,4-D. Rev Environ Contam Toxicol 2000;167:1–33.

Garabrant DH, Philbert MA. Review of 2,4-dichlorophenoxyacetic acid (2,4-D) epidemiology and toxicology. Crit Rev Toxicol 2002;32:233–57.

González M, Soloneski S, Reigosa MA, Larramendy ML. Effect of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and its derivative 2,4-D dichlorophenoxyacetic acid dimethylamine salt (2,4-D DMA). I. Genotoxic evaluation on Chinese hamster ovary (CHO) cells. Toxicol In Vitro 2005;19:289–97.

Graf U, Würgler F. The somatic white-ivory eye spot test does not detect the same spectrum of genotoxic events as the wing somatic mutation and recombination test in *Drosophila melanogaster*. Environ Mol Mutagen 1996;27:219–26.

Gutteridge JM. Iron and oxygen: a biologically damaging mixture. Acta Paediatr Scand 1989;361(Suppl.):78–85.

Halliwell B. Superoxide-dependent formation by hydroxil radical in the presence of iron salts. FEBS Lett 1978;96:238–42.

Holland NT, Duramad P, Rothman N, Figgs LW, Blair A, Hubbard A, et al. Micronucleus frequency and proliferation in human lymphocytes after exposure to herbicide 2,4-dichlorophenoxyacetic acid in vitro and in vivo. Mutat Res 2002;521:165–78.

IARC. Some fumigants, the herbicides 2,4-D and 2,4,5-T, chlorinated dibenzodioxins and miscellaneous industrial chemicals. IARC Monogr Eval Carcinog Risk Chem Man 1977;15:111–48.

IARC. Miscellaneous pesticides. IARC Monogr Eval Carcinog Risk Chem Man 1983;30:1–430.

IARC. Some halogenated hydrocarbons and pesticide exposures. IARC Monogr Eval Carcinog Risk Chem Man 1986;4:1–434.

IARC. Overall evaluation of carcinogenicity: an updating of IARC monographs vols. 1–42. IARC Monogr Eval Carcinog Risk Chem Man 1987;(Suppl. 7):1–440.

IARC. Occupational exposures in insecticide application, and some pesticides. Monogr Eval Carcinog Risk Chem Man 1991;53:1–612.

James LA, Mitchell EL, Menansce L, Varley JM. Comparative genomic hybridization of ductal carcinoma in situ of the breast: identification of regions of DNA amplification and deletion in common with invasive breast carcinoma. Oncogene 1997;14:1059–65.

Kaya B, Yanikoglu A, Marcos R. Genotoxicity studies on the phenoxyacetates 2,4-D and 4-CPA in the *Drosophila* wing spot test. Teratogen Carcinogen Mutagen 1999;19:305–12.

Lamberti L, Bigatti-Ponzetto P, Ardito G. Cell kinetics and sister chromatid exchange frequency in human lymphocytes. Mutat Res 1983;120:193–9.

Landi S, Ponzanelli I, Barale R. Effect of red cells and plasma blood in determining individual lymphocytes sensitivity to diepoxybutane assessed by in vitro induced sister chromatid exchanges. Mutat Res 1995;348:117–23.

Larramendy ML, Knuutila S. Immunophenotype and sister chromatid differentiation: a combined methodology for analyzing cell proliferation in unfractionated lymphocyte cultures. Exp Cell Res 1990;188:209–13.

Larramendy ML, Reigosa MA. Variation in sister chromatid exchange frequencies between human and pig whole blood, plasma leukocyte, and mononuclear leukocyte cultures. Environ Mutagen 1986;8:543–54.

Larramendy ML, Mello-Filho A, Leme-Martins E, Meneghini R. Iron mediated induction of sister chromatid exchanges by hydrogen peroxide and superoxide anion. Mutat Res 1987;178:57–63.

Larramendy ML, López-Larraza D, Vidal-Rioja L, Bianchi NO. The effect of the metal chelating agent *o*-phenanthroline on the DNA and chromosome damage induced by bleomycin in Chinese hamster ovary cells. Cancer Res 1989a;49:6583–6.

Larramendy ML, Bianchi MS, Padrón J. Correlation between the anti-oxidant enzyme activities of blood fractions and the yield of bleomycin-induced chromosome damage. Mutat Res 1989b;214:129–36.

Larramendy ML, Reigosa MA, Bianchi MS. Erythrocytes modulate the baseline frequency of sister-chromatid exchanges and the kinetics of lymphocyte division in culture. Mutat Res 1990;232:63–70.

Larramendy ML, Reigosa MA, Garcia CF, Soloneski S, Knuutila S. Variation in sister chromatid exchange frequencies and cell cycle kinetics between human whole blood and plasma leukocyte cultures. Braz J Genet 1996;18:63–8.

Latt SA, Schreck RR, Loveday KS, Dougherty CP, Shuler CF. Sister chromatid exchanges. Adv Hum Genet 1980;10:267–331.

Lee SH, Oe T, Arora JS, Blair IA. Analysis of Fe^{II}-mediated decomposition of a linoleic acid-derived lipid hydroperoxide by liquid chromatography/mass spectrometry. J Mass Spectrom 2005;40:661–8.

Linnainmaa K. Sister chromatid exchanges among workers occupationally exposed to phenoxy acid herbicides 2,4-D and MCPA. Teratogen Carcinogen Mutagen 1983;3:269–79.

Linnainmaa K. Induction of sister chromatid exchanges by the peroxisome proliferator 2,4-D, MCPA, and clofibrate *in vivo* and *in vitro*. Carcinogenesis 1984;5:703–7.

Lundgren B, Meijer J, DePierre JW. Induction of cytosolic and microsomal epoxide hydrolases and proliferation of peroxisomes and mitochondria in mouse liver after dietary exposure to p-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid. Chem Biol Interact 1987;36:815–21.

Madrigal-Bujaidar E, Hernandez-Ceruelos A, Chamorro G. Induction of sister chromatid exchanges by 2,4-dichlorophenoxyacetic acid in somatic and germ cells of mice exposed in vivo. Food Chem Toxicol 2001;39:941–6.

Meneghini R, Hoffmann ME. The damaging action of hydrogen peroxide on DNA of human fibroblasts is mediated by a non-dialyzable compound. Biochem Biophys Acta 1980;608:167–73.

Miller BM, Adler ID. Suspect spindle poisons: analysis of c-mitotic effects in mouse bone marrow cells. Mutagenesis 1989;4:208–15.

Moorhead PS, Nowell PC, Mellman WJ, Barttips DM. Chromosome preparations of leukocyte cultures from human peripheral blood. Exp Cell Res 1960;20:613–6.

Mustonen R, Elovaara E, Zitting A, Linnainmaa K, Vainio H. Effects of commercial chlorophenolate, 2,3,7,8-TCDD, and pure phenoxyacetic acids on hepatic peroxisome proliferation, xenobiotic metabolism and sister chromatid exchange in the rat. Arch Toxicol 1989;63:203–8.

Nicotera TM, Block AW, Gibas Z, Sandberg AA. Induction of superoxide dismutase, chromosomal aberrations and sister chromatid exchanges by paraquat in Chinese hamster fibroblasts. Mutat Res 1985;151.

Norppa H, Jarventaus H. Induction of sister-chromatid exchanges by 2-aminofluorene in cultured human lymphocytes with and without erythrocytes. Mutat Res 1992;282:135–8.

Norppa H, Vainio H, Sorsa M. Metabolic activation of styrene by erythrocytes detected as increased sister chromatid exchanges in cultured human lymphocytes. Cancer Res 1983;43:3579–82.

Osterloh J, Lotti M, Pond SM. Toxicologic studies in a fatal overdose of 2,4-D, MCPP, and chlorpyrifos. J Anal Toxicol 1983;7:125–9.

Palitti FC, Tanzarella C, Cozzi R, Ricondy E, Vitagliana A, Fiori M. Comparison of frequencies of SCEs induced by chemical mutagens in bone-marrow spleen and spermatogoneal cell of mice. Mutat Res 1982;103:191–5.

Phillips BJ, James TEB, Andersen D. Genetic damage in CHO cells exposed to enzymatically generated active oxygen species. Mutat Res 1984;126:265–71.

Pilinscaya M. Cytogenetic effect of the herbicide 2,4-D on human and animal chromosomes. Tsitol Genet 1974;8:202–6.

Ray JH, Altenburg LC. Sister-chromatid exchange induction by sodium selenite: dependence on the presence of red blood cells or red cell lysate. Mutat Res 1978;54:343–54.

Reddy JK, Azarnoff DL, Hignite CE. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. Nature 1980;283:397–8.

Reddy JK, Lalwani ND, Reddy MK, Qureshi SA. Excessive accumulation of autofluorescent lipofucsion in the liver during hepatocarcinogens by methyl clofenape and other hypopolipidemic peroxisome proliferators. Cancer Res 1982;42:259–66.

Rupa DS, Reddy PP, Reddi OS. Analysis of sister-chromatid exchanges, cell kinetics and mitotic index in lymphocytes of smoking pesticide sprayers. Mutat Res 1989;223:253–8.

Seiler JP. Pentachlorophenol. Mutat Res 1991;257:27–47.

Sobti RC, Krishan A, Pfaffenberger CD. Cytokinetic and cytogenetic effects of some agricultural chemicals on human lymphoid cells in vitro: organophosphates. Mutat Res 1982;102:89–102.

Soloneski S, González M, Piaggio E, Apezteguía M, Reigosa MA, Larramendy ML. Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. I. Genotoxic evaluation on cultured human lymphocytes exposed *in vitro*. Mutagenesis 2001;16:487–93.

Soloneski S, González M, Piaggio E, Reigosa MA, Larramendy ML. Effect of dithiocarbamate pesticide zineb and its commercial formulation azzurro. III. Genotoxic evaluation on Chinese hamster ovary (CHO) cells. Mutat Res 2002;514:201–12.

Speit G, Vogel W. The effect of sulphydryl compounds on sister chromatid exchanges. II. The question of cell specificity and the role of H_2O_2. Mutat Res 1982;93:175–83.

Turkula TE, Jalal SM. Increased rates of sister chromatid exchanges induced by the herbicide 2,4-D. J Hered 1985;76:213–4.

Vainio H, Nickels J, Linnainmaa K. Phenoxy acid herbicides cause peroxisome proliferation in Chinese hamsters. Scand J Work Environ Health 1982;8:70–3.

Van Hummelen P, Kirsch-Volders M. Analysis of eight known or suspected aneugens by the in vitro human lymphocyte micronucleus test. Mutagenesis 1992;7:447–55.

West JD, Ji C, Duncan ST, Amarnath V, Schneider C, Rizzo CJ, et al. Induction of apoptosis in colorectal carcinoma cells treated with 4-hydroxy-2-nonenal and structurally related aldehydic products of lipid peroxidation. Chem Res Toxicol 2004;17:453–62.

Wilmer JL, Kligerman AD, Erexson GL. Sister chromatid exchange induction and cell cycle inhibition by aniline and its metabolites in human fibroblasts. Environ Mutagen 1981;3:627–38.

Zastawny TH, Altman SA, Randers-Eichhorn L, Madurawe R, Lumpkin JA, Dizdaroglu M, et al. DNA base modifications and membrane damage in cultured mammalian cells treated with iron ions. Free Radic Biol Med 1995;18:1013–22.