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Genotoxicity of the herbicide 2,4-dichlorophenoxyacetic and a commercial formulation, 2,4-dichlorophenoxyacetic acid dimethylamine salt. I. Evaluation of DNA damage and cytogenetic endpoints in Chinese Hamster ovary (CHO) cells

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Abstract

Genotoxicity of the 2,4-dichlorophenoxyacetic acid (2,4-D) and a commercially-used derivative, 2,4-D dimethylamine salt (2,4-D DMA), was evaluated in CHO cells using SCE and single cell gel electrophoresis (SCGE) assays. Log-phase cells were treated with 2.0–10.0 μg/ml of herbicides and harvested 24 and 36 h later for SCE analysis. Both agents induced significant dose-dependent increases in SCE, regardless of the harvesting time (2,4-D: $r = 0.98$ and $r = 0.88$, $P < 0.01$, for 24 and 36 h harvesting times; 2,4-D DMA: $r = 0.97$ and $r = 0.88$, $P < 0.01$, for 24 and 36 h harvesting times). Neither test compound altered cell-cycle progression or proliferative replication index ($P > 0.05$), but the higher doses of both compounds reduced the mitotic index of cultures harvested at 24 and 36 h ($P < 0.05$). A 90-min treatment with 2.0–10.0 μg/ml 2,4-D and 2,4-D DMA produced dose-dependent increases in the frequency of DNA-strand breaks detected in the SCGE assay, both in cultures harvested immediately after treatment and in cultures harvested 36 h later. The doses of 2,4-D and 2,4-D DMA were equally genotoxic in all of the assays. The results indicate that 2,4-D induces SCE and DNA damage in mammalian cells, and should be considered as potentially hazardous to humans.

Keywords: 2,4-Dichlorophenoxiacetic acid (2,4-D); Dimethylamine 2,4-D salt (2,4-D DMA); Phenoxyacetic acids; SCE assay; Cell-cycle kinetics; SCGE assay

1. Introduction

Epidemiological and experimental studies have made increasing use of biomarkers that can measure biologically relevant exposure to genotoxic pollutants not only in humans but also in other living species. Among these biomarkers, DNA adducts, DNA strand breaks, and several cytogenetic endpoints, namely chromosomal aberrations, sister chromatid exchange (SCE), micronuclei, and the analysis of cell-cycle progression, have been among the most widely employed (Bolognesi, 2003; IARC, 1971–2002).

The chlorinated aromatic hydrocarbon acid pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) and related chlorophenoxyalkanoic compounds have become substantial environmental pollutants since they are widely used as hormonic herbicides. While at low concentrations 2,4-D acts as an auxin analogue promoting plant growth, at high concentrations it is lethal and used as herbicide against broad-leafed and woody plants (Devine et al., 1993; Sinton et al., 1986; Tripathy et al., 1993). Although the exact mechanisms by which this herbicide is incorporated into cells are not totally understood, 2,4-D has been reported to be a peroxisome

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proliferator (Lundgren et al., 1987). In plant cells 2,4-D induces mitotic and meiotic irregularities both in vivo and in vitro (Bayliss, 1980; Khalatkar and Bhargava, 1982). Furthermore, the most frequent types of aberrations induced are fragments, erosion, bridges, laggards, micronuclei as well as polyploidy and aneuploidy (Fiskesjö et al., 1981; Pavlica et al., 1991).

In mammalian cells in vitro, 2,4-D inhibits cell growth, protein and DNA synthesis, and arrests cells in the G/S phase of the cell cycle (Rivarola et al., 1985). Gollapudi et al. (1999) observed that neither 2,4-D nor several of its derivatives were clastogenic or mutagenic in mammalian cells. Similarly, 2,4-D no increase in chromosomal aberrations in human lymphocyte in vitro and in vivo (Mustonen et al., 1989). Galloway et al. (1987) and Turkula and Jalal (1985), however, reported that 2,4-D increased the frequencies of SCEs in human lymphocytes and in Chinese hamster ovary (CHO) cells treated in the presence of S9.

Using in vivo assays, 2,4-D was positive for chromosomal aberrations in mice (Amer and Aly, 2001; Pilinscaya, 1974) and for micronucleus induction in fish (Abul Farah et al., 2003), while it was negative in the micronucleus assay using mouse bone marrow (Charles et al., 1999). Madrigal-Bujaidar et al. (2001) reported a significant increase in SCE frequencies in the bone marrow and germ cells of mice. In contrast, no SCE induction was observed in three previous in vivo studies (Arias, 1995; Linnainmaa, 1984; Mustonen et al., 1989). Similarly, a commercial formulation of 2,4-D was negative in the single cell gel electrophoresis (SCGE) using bullfrog tadpole erythrocytes (Clements et al., 1997).

Considering the world-wide use of this herbicide, the possible human exposure due to inhalation, skin contact or ingestion of contaminated food, and the inconsistent results in previous in vivo and in vitro genotoxicity assays, we thought it prudent to re-examine the genotoxic potential of 2,4-D. In the present study, we have evaluated the genotoxicity of 2,4-D and a dimethylamine (DMA) derivative currently used in Argentina (2,4-D DMA) in CHO cells. Besides measuring SCE frequency, mitotic index, and cell-cycle progression analyses as cytogenetic endpoints, we have measured DNA damage induction using the highly sensitive, relatively recently developed SCGE assay.

2. Material and methods

2.1. Chemicals

2,4-Dichlorophenoxyacetic (2,4-D; CAS No. 94-75) was obtained from Riedel-de Haën (Pestanal®, Hannover, Germany). Commercial formulation "Delente Selectivo Amina 50" (Dimethylamine 2,4-D salt) was kindly provided by Delente Laboratories SRL (Buenos Aires, Argentina). Dimethylamine 2,4-D salt (2,4-D DMA) contains 2,4-D as an active ingredient in the concentration of 60,2 g/100 ml. Acetone was purchased from Sigma (St. Louis, MO).

2.2. Cell culture and pesticide treatment for cytogenetic studies

CHO cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and in a medium consisting of Ham's nutrient mixture F10 supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 10 μg/ml of streptomycin (all cell culture reagents from GIBCO-Invitrogen, Carlsbad, CA). The cells were seeded in T75 flasks at a density of 1×10^6 cells/flask. Treatments with test compounds were performed 24 h later, when the cultures were in the log phase of growth. Both 2,4-D and 2,4-D DMA were dissolved in acetone prior to use so that the addition of 100 μl to the cultures produced the desired concentration (2.0–10.0 μg/ml). The employed doses were chosen from a preliminary study in which a dose–response was analyzed (data not published) and only those doses unable to induce in vitro citotoxicity (cell death) but able to show a deletereous effect were selected. The final solvent concentration was less than 1% for all the treatments. Immediately afterwards, bromodeoxyuridine (BrdUrd) was added to cultures at a final concentration of 10 μg/ml, and the cells were incubated in complete culture medium under safety light until harvesting 24 or 36 h later. Negative controls (untreated cells and vehicle-treated cells) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Each treatment was performed in duplicate in at least three independent experiments. The same batches of culture medium, serum, and reagents were used throughout the study.

2.3. Chromosome preparations and staining

During the last 3 h of culture, the cells were treated with 0.2 μg/ml colchicine (Sigma). The cells were detached by trypsinization, collected by centrifugation, treated with hypotonic saline (0.075 M KCl, 37 °C, 15 min), and fixed in 3:1 methanol–acetic acid. Chromosome spreads were prepared on coded slides using the air-drying technique. The spreads were stained using the FPG technique for sister chromatid differentiation and examined by a single cytogeneticist.

2.4. Cell-cycle kinetics and mitotic index

A minimum of 200 metaphase cells 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 calcu-

lated for each experimental point according to the formula, $PRI = [(\%M_1) + 2(\%M_2) + 3(\%M_{3+})]/100$, which estimates the average number of cell divisions from the addition of BrdUrd to the culture medium until harvesting (Lamberti et al., 1983). The mitotic index (MI) was determined by scoring 2000 cells from each experimental point and was expressed as the number of mitoses/1000 nuclei. Changes in the MI were expressed as *f*, the mean MI from the treated culture (MI_t) divided by the mean MI of the control (MI_c) ($f = MI_t/MI_c$) (Miller and Adler, 1989).

2.5. Sister chromatid exchange analysis

Treatments were conducted on at least three separate occasions, with a total of 50 well-spread diploid metaphases from each treatment scored for SCE (a total of 150 cells scored per data point). SCE were scored in M_2 cells, 24 and 36 h after the addition of 2,4-D and 2,4-D DMA. The data are expressed as the mean number of SCEs per cell ± SE.

2.6. Cell cultures and pesticide treatment for single cell gel electrophoresis

Exponentially growing CHO cells were detached by trypsinization, collected by centrifugation, resuspended in complete culture medium, and then counted. Aliquots containing 3.5×10^5 cells/ml were incubated for 90 min at 37 °C in a 5% CO_2 atmosphere in culture medium containing the test compounds. Both 2,4-D and 2,4-D DMA were dissolved in acetone and added to the cultures as described above. Negative controls (untreated cells and vehicle-treated cells) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. After the treatment, the cells were washed twice with pesticide-free complete culture medium, collected by centrifugation, and resuspended in 1.0 ml of pesticide-free complete culture medium. The SCGE and cell viability assays were performed immediately after the treatment or after a post-treatment incubation period of 36 h at 37 °C in a 5% CO_2 atmosphere. Each treatment was performed in duplicate in at least three independent experiments. The same batches of culture medium, serum, and reagents were used throughout the study.

2.7. Cell viability assay

Cell viability was determined using the ethidium bromide/acridine orange assay described by McGahon et al. (1995). Briefly, one 5 μl aliquot of a freshly prepared mixture of ethidium bromide (100 μg/ml, Sigma) and acridine orange (100 μg/ml, Sigma) were mixed with 50 μl of the cell suspension. The cells then were analysed using an Olympus BX50 fluorescence photomicroscope equipped with the appropriate filters. Viable cells appeared green-fluorescent whereas dead cells had orange-stained nuclei. At least 500 cells were counted per experimental point, and the results expressed as the percentage of viable cells in the culture.

2.8. Single cell gel electrophoresis assay

The remaining cell culture (950 μl) was used for microgel electrophoresis. The SCGE assay was performed following the alkaline procedure described by Singh et al. (1988) and Klaude et al. (1996) with minor modifications. Slides were cleaned with 100% ethanol and air-dried. Two solutions containing 0.5% normal melting agarose (NMA), and 0.5% low melting agarose (LMA) solution in Ca^{2+}–Mg^{2+}-free PBS were performed. Briefly, 75 μl of 0.5% NMA was transferred onto a pre-cleaned slide, spread evenly, and placed at 37 °C to solidify the agarose. Afterwards, 95 μl of 0.5% LMA together with 7×10^3 cells (20 μl cell suspension +75 μl 0.5% LMA) was applied, covered with a coverslip, and placed at 4 °C for 15 min. After this layer had solidified, a third layer of 75 μl of 0.5% LMA was added, and slides placed at 4 °C for 15 min. Immediately after, slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris pH 10.0, 1% Triton X-100, 10% DMSO) and then lysed in the dark at 4 °C for an overnight period. After the overnight period, slides were placed in a horizontal electrophoresis device filled with freshly prepared electrophoresis buffer (1 mM Na_2 EDTA, 300 mM NaOH) for 20 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer at 4 °C for 20 min at 25 V and 250 mA. Afterwards, slides were neutralised with a solution comprising 0.4 M Tris HCl (pH 7.5) and stained with 4′,6-diamidino-2-phenylindole (DAPI) (Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Slides were coded and scored blind by a single individual. Analysis of the slides was performed with an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filters and an X63 fluorescence objective. Cellular images were acquired with the Leica IM50 Image Manager (Imagic Bilderarbeitung AG), which employed an integrated high-sensitivity monochrome charge-coupled device (CCD) camera and automated capture image software. The cellular nucleus diameter and the comet length, determined as the diameter of the nucleus plus migrated DNA, were measured by CarioFISH 1.2 software. Widths of the nucleus and comet length (expressed in μm) were determined from 50 randomly captured cells per experimental point of each experiment. Two parallel slides were analysed for each experimental point. The cells were visually

graded into four DNA damage categories according to Lebailly et al. (1997). The categories were as follows: undamaged (no comet tail, diameter $\leqslant 30\,\mu m$), slightly damaged (diameter 31–45 μm), damaged (tail comet length > 45 μm) and highly damaged (dying or dead cells).

2.9. Statistical analysis

The two-tailed Student's *t*-test was used to compare pooled data of three independent experiments as mean values of SCE frequencies and SCGE measurements between treated and control groups. A χ^2 test was used for cell-cycle progression and MI data. The non-parametric Kruskal–Wallis test was used to compare the effect of pesticide exposure in the SCGE assay, while individual comparisons between pairs of data were performed using the Mann–Whitney test. SCE, mitotic index and SCGE data were evaluated by regression analysis. The level of significance was $P < 0.05$, unless indicated otherwise.

3. Results

Since no significant differences were observed for cell-cycle progression, SCE, PRI and SCGE values in untreated and acetone-treated cells, these data were pooled and used as the negative control values.

Fig. 1 shows the frequencies of SCE in CHO cells treated for 24 or 36 h with different concentrations of 2,4-D and 2,4-D DMA. The SCE frequencies observed in cultures treated with 2.0–10.0 μg/ml of either 2,4-D or 2,4-D DMA were all significantly higher than control

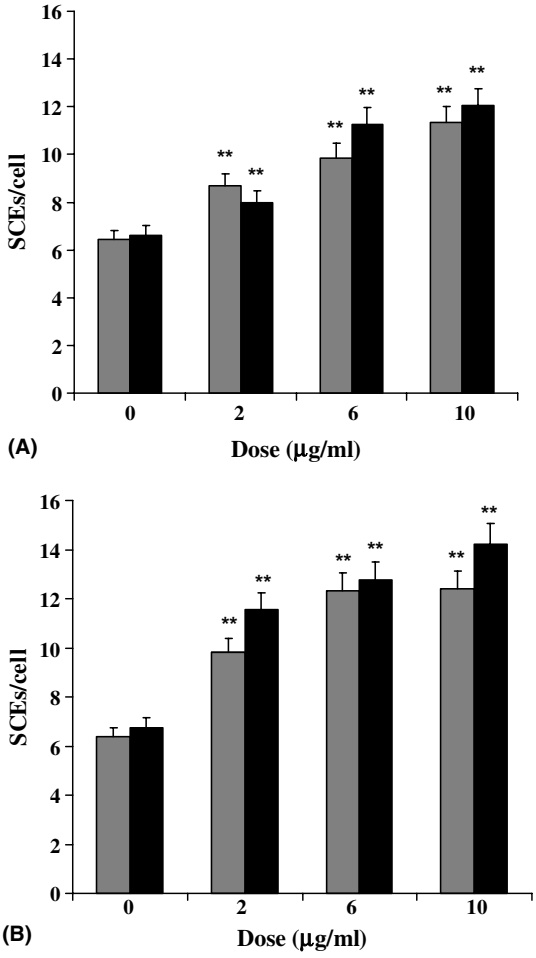

Fig. 1. Effect of 2,4-dichlorphenocyacetic acid (2,4-D) (stripped bars) and dimethylamine 2,4-D salt (2,4-D DMA) (black bars) on SCE frequency in CHO cells. Cultures were harvested at 24 h (A) and 36 h (B) after beginning the pesticide treatment. ** $P < 0.01$.

Table 1
Cell-cycle progression, proliferative rate index (PRI), mitotic index and mitotic index factor (*f*) values for control, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-D dimethylamine salt (2,4-D DMA)-treated Chinese hamster ovary (CHO) cells[a]

Length of treatment (h)	Dose (μg/ml)	2,4-D						2,4-D DMA					
		Cell-cycle progression[b]			PRI	Mitotic Index	*f*	Cell-cycle progression[b]			PRI	Mitotic Index	*f*
		M_1	M_2	M_{3+}				M_1	M_2	M_{3+}			
24	0	1.3	98.3	0.3	1.99 ± 0.01	49.7	1.00 ± 0.00	1.7	97.7	0.7	1.99 ± 0.02	66.7	1.00 ± 0.00
	2	3.3	96.7	0.0	1.97 ± 0.02	38.7	0.78 ± 0.14	0.0	100.0	0.0	2.00 ± 0.00	69.3	0.91 ± 0.03
	6	5.7	94.3	0.0	1.98 ± 0.00	37.3*	0.75 ± 0.08	0.3	99.0	0.7	2.00 ± 0.01	42.0**	0.65 ± 0.05
	10	2.7	97.3	0.0	1.97 ± 0.02	32.3*	0.65 ± 0.13	3.3	99.7	0.0	1.97 ± 0.04	41.0**	0.51 ± 0.13
36	0	0.0	20.7	79.3	2.79 ± 0.03	79.3	1.00 ± 0.00	0.0	40.0	60.0	2.60 ± 0.21	76.7	1.00 ± 0.00
	2	3.7	19.0	77.3	2.74 ± 0.09	67.0	0.84 ± 0.17	0.0	28.0	72.0	2.72 ± 0.09	66.7	0.87 ± 0.20
	6	0.0	21.3	78.7	2.79 ± 0.03	45.3**	0.57 ± 0.23	0.0	29.7	70.3	2.70 ± 0.10	56.0*	0.73 ± 0.19
	10	0.0	15.0	85.0	2.83 ± 0.02	42.7**	0.54 ± 0.21	0.0	30.0	70.0	2.70 ± 0.15	55.3*	0.72 ± 0.17

[a] CHO cells were treated 24 h after seeding with different test compounds and harvested 24 h or 36 h later. Results are presented as mean values of pooled data from three independent experiments ± SE of the mean.

[b] The proportion of cells in first (M_1), second (M_2), and third or subsequent cell divisions (M_{3+}) were determined in 600 mitoses for each experimental point.

* $P < 0.05$.

** $P < 0.01$.

Table 2
Frequencies of undamaged cells (cells without comets) and damaged cells (cells with comets) in control and 2,4-dichlorophenoxyacetic acid (2,4-D)- and 2,4-D dimethylamine salt (2,4-D DMA)-treated Chinese hamster ovary (CHO) cells [a]

Treatment (+incubation)	Dose (μg/ml)	Number cells examined	Percentage of cells[b]		Viability
			Undamaged	Damaged	
2,4-D					
90 min	0	300	98.0 ± 2.0	2.0 ± 2.0	94.0 ± 1.0
	2.0	150	20.7 ± 10.7**	79.3 ± 10.7**	89.6 ± 1.3
	6.0	150	12.0 ± 6.9**	88.0 ± 6.9**	92.3 ± 1.6
	10.0	150	0.0 ± 0.0**	100.0 ± 0.0**	90.0 ± 1.1
+36 h	0	300	97.3 ± 1.3	2.7 ± 1.3	88.3 ± 4.1
	2.0	150	0.0 ± 0.0**	100.0 ± 0.0**	89.0 ± 4.5
	6.0	150	0.0 ± 0.0**	100.0 ± 0.0**	85.3 ± 4.7
	10.0	150	0.0 ± 0.0**	100.0 ± 0.0**	87.3 ± 1.2
2,4-D DMA					
90 min	0	300	91.3 ± 2.4	8.7 ± 2.4	91.0 ± 3.2
	2.0	150	12.0 ± 9.2**	88.0 ± 9.2**	86.0 ± 2.3
	6.0	150	2.7 ± 1.8**	97.3 ± 1.8**	98.7 ± 1.3
	10.0	150	1.3 ± 1.3**	98.7 ± 1.3**	97.7 ± 0.3
+36 h	0	300	95.3 ± 0.6	4.7 ± 0.7	89.3 ± 4.8
	2.0	150	1.3 ± 1.3**	98.7 ± 1.3**	88.6 ± 4.7
	6.0	150	0.0 ± 0.0**	100.0 ± 0.0**	86.3 ± 0.6
	10.0	150	0.0 ± 0.0**	100.0 ± 0.0**	87.0 ± 2.6

[a] Cells were treated with test compounds for 90 min, harvested either immediately or 36 h later, and assayed both for viability and for DNA damage using the single cell gel electrophoresis assay.
[b] Results are expressed as mean values ± standard error of three experiments.
** $P < 0.01$.

cultures regardless of the harvesting time ($P < 0.01$). SCEs increased in a dose-dependent manner. Regression analysis indicated that SCE frequencies increased as a function of the concentration of 2,4-D ($r = 0.98$ and $r = 0.88$, $P < 0.01$, for the 24 and 36 h harvest times, respectively), and 2,4-D DMA ($r = 0.96$ and $r = 0.88$, $P < 0.01$, for the 24 and 36 h harvest times, respectively). Furthermore, the SCE frequencies for each concentration of 2,4-D and 2,4-D DMA were higher in the 36-h cultures than in cultures harvested at 24 h after treatment ($P < 0.01$).

No effect on cell-cycle progression was observed in 2,4-D and 2,4-D DMA-treated cultures at either harvest time (Table 1). For both test compounds, no significant alteration in the frequency of M_1, M_2, M_{3+}, relative to control values, was observed in cultures treated with 2.0–10.0 μg/ml of either 2,4-D or 2,4-D DMA ($P > 0.05$). Thus, no alteration in the rate of cell proliferation (PRI) was found for either agent ($P > 0.05$ for 24- and 36-h harvest times) (Table 1).

Reductions in MI were found at both harvest times for CHO cells treated with 6.0 and 10.0 μg/ml 2,4-D ($P < 0.05$ and $P < 0.01$, for the 24 and 36 h harvest time, respectively) and 6.0 and 10.0 μg/ml 2,4-D DMA ($P < 0.01$ and $P < 0.05$, for the 24 and 36 h harvest time, respectively) (Table 1). Overall, regression analysis indicated that the mitotic activity decreased at both harvest times as a function of the concentration of 2,4-D ($r = -0.88$ and $r = -0.94$, $P < 0.01$, for 24 and 36 h harvest times, respectively) and 2,4-D DMA ($r = -0.99$ and $r = -0.88$, $P < 0.01$, for 24 and 36 h harvest times, respectively) (Table 1). When 10.0 μg/ml of the herbicides were used, 2,4-D reduced the mitotic activity of the cultures from a control value of $f = 1.00$ to a mean f of 0.65 ± 0.13 and 0.54 ± 0.21 (for 24-h and 36-h treatments, respectively), and 2,4-D DMA reduced the f to means of 0.51 ± 0.13 and 0.72 ± 0.17 (for 24-h and 36-h treatments, respectively) (Table 1).

Table 2 summarises the results of SCGE assays performed on CHO cells treated with 2.0–10.0 μg/ml 2,4-D and 2,4-D DMA for 90 min. The total cell count and viability of the treated cells were similar to the controls, regardless of whether the assays were performed immediately after the treatment or after 36 h of incubation ($P > 0.05$). Overall, the viability was above 85% for 2,4-D and 2,4-D DMA-treated cells at both harvest times.

The 2,4-D and 2,4-D DMA treatments resulted in increased DNA damage in CHO cells as revealed by an increased proportion of damaged cells and a concomitant decrease in the frequency of undamaged cells in cultures assayed by SCGE both immediately after the treatment and 36 h later ($P < 0.01$). No slightly damaged or highly-damaged cells were found in the treated cultures. Regression analysis indicated that, when assayed immediately after treatment, the proportion of damaged cells increased as a function of the concentration of both 2,4-D and 2,4-D DMA ($r = 0.80$, $P < 0.05$ and $r = 0.74$,

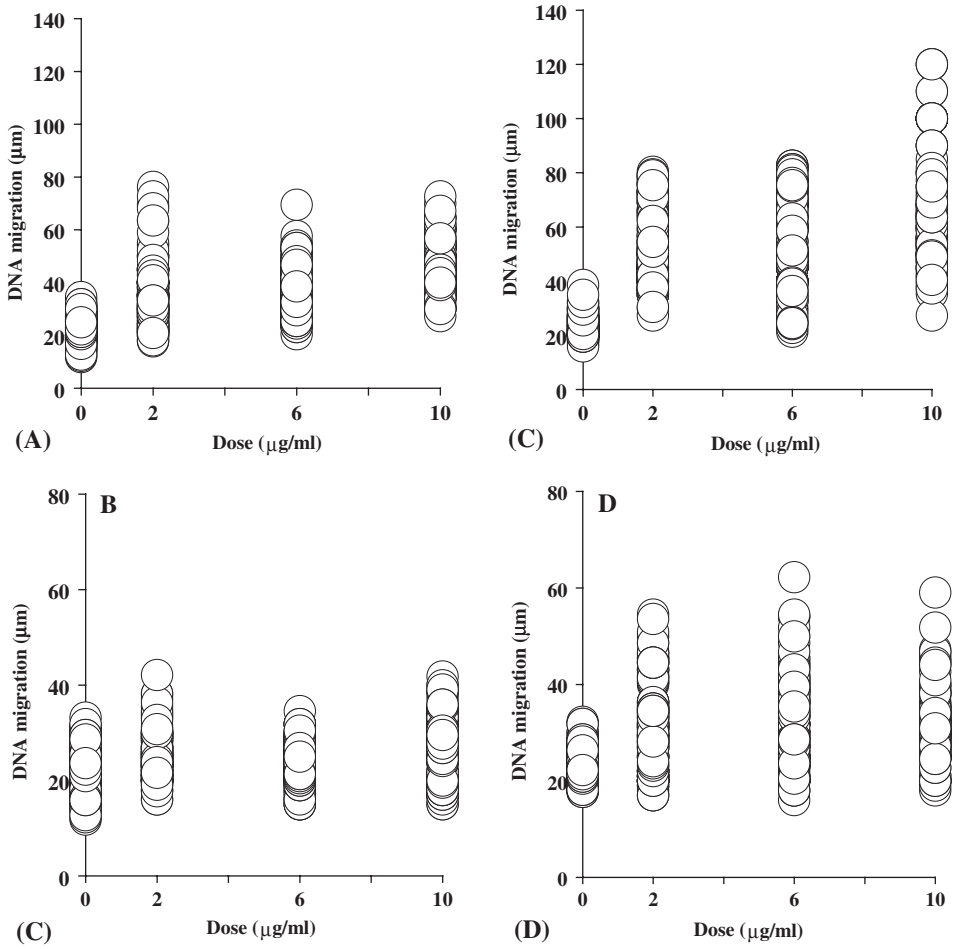

Fig. 2. Effect of 2,4-D treatment of Chinese CHO cells on the length of comet tails (A and C) and on the diameter at the estimated trailing edge of comet tails (B and D). Cultures were harvested immediately after a 90 min treatment (A and B) or 36 h later (C and D) and assayed by SCGE.

$P < 0.05$, respectively). In contrast, when the assay was performed with cultures harvested 36 h after treatment, no dose–response relationships for damaged-cell induction were observed since all cells were damaged ($r = 0.67$, $P > 0.05$ and $r = 0.68$, $P > 0.05$, respectively) (Table 2). Overall and regardless of the harvest time, image analysis showed a positive relationship between 2,4-D concentration and the frequency of DNA-strand breaks, as measured by comet length (Fig. 2A and C, $r = 0.90$ and $r = 0.91$, $P < 0.01$, for the immediate and 36-h harvests, respectively) and comet width (Fig. 2B and D, $r = 0.72$ and $r = 0.81$, $P < 0.05$, for immediate and 36-h harvests, respectively). Similarly, when cells were treated with the commercial formulation 2,4-D DMA, image analysis showed a positive relationship between concentration and DNA-strand breaks, as measured by both comet length (Fig. 3A and C, $r = 0.94$ and $r = 0.95$, $P \leqslant 0.01$, for immediate and 36-h harvests, respectively) and comet width (Fig. 3B and D, $r = 0.77$ and $r = 0.76$, $P < 0.05$, for immediate and 36-h harvests, respectively).

4. Discussion

In the present study, the genotoxicities of 2,4-D and its derivative 2,4-D DMA were evaluated in cultures of CHO cells using four different genetic endpoints, namely the analysis of SCE frequency, cell-cycle progression and mitotic index following treatments of 24 h and 36 h, and the alkaline SCGE assay after a treatment of 90 min. Our results show that all but one of the bioassays detected a genotoxic effect by the herbicides. Both compounds induced SCE and DNA-strand breaks as well as inhibited mitotic activity, but neither chemical modified the cell-cycle kinetics of CHO cells, at least with the 2.0–10.0 μg/ml dose-range employed for the assays. These data support the view of many authors who indicate that both SCE and SCGE are sensitive bioassays for detecting genotoxic activity (WHO, 1985).

Previous studies of the genotoxic potential of 2,4-D, using a wide range of assays for mutagenicity and genotoxicity, have produced conflicting results (Gandhi et al., 2000). These inconsistencies, as suggested by Kaya

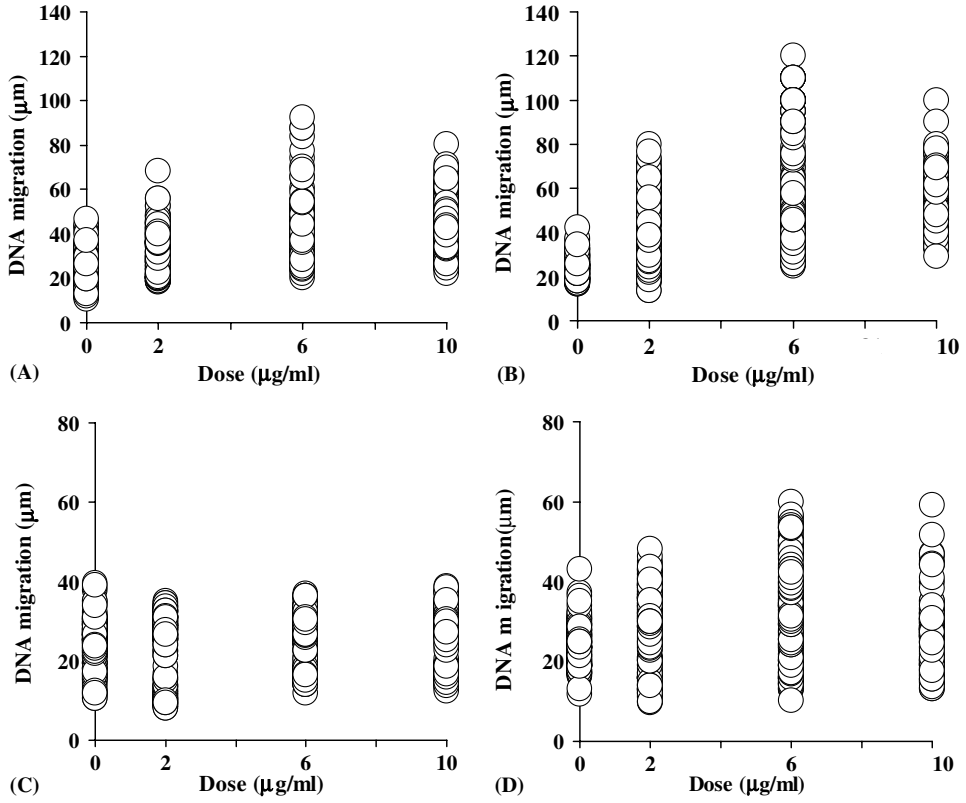

Fig. 3. Effect of 2,4-D DMA treatment of Chinese CHO cells on the length of comet tails (A and C) and on the diameter at the estimated trailing edge of comet tails (B and D). Cultures were harvested immediately after a 90 min treatment (A and B) or 36 h later (C and D) and assayed by SCGE.

et al. (1999), most probably are due to (1) the use of both pure compounds and commercial formulations with unknown impurities; or/and (2) the differential sensitivities of the specific systems/endpoints used. The latter point is illustrated by the opposite responses of 2,4-D in two somatic assays performed in *Drosophila melanogaster* by Graf and Wurler (1996). These authors observed that while the white-ivory eye spot test did not detect any genotoxic effect, the wing somatic mutation and recombination test gave positive results.

To the best of our knowledge, this is the first report demonstrating an increased frequency of SCE in CHO cells after continuous treatment with both pure 2,4-D and a commercial 2,4-D formulation, in this case 2,4-D DMA. In contrast, Linnainmaa (1984) reported no increase in SCE frequency after a 1 h pulse-treatment of CHO cells with pure 2,4-D and a commercial 2,4-D formulation (2,4-D amine salt as the active ingredient) with and without S9 activation. The discrepancy between our present results and those of Linnainmaa (1984) may be related to the different exposure times rather than differences in the purity of the compounds. However, two previous reports indicate that SCEs were elevated significantly when human lymphocytes were treated in vitro with 2,4-D (Turkula and Jalal, 1985). Also, in studies in vivo, Madrigal-Bujaidar et al. (2001) reported a significant increase in SCE frequency in bone marrow and germ cells of mice treated with 2,4-D, while Arias (2003) reported increased SCE frequencies in chick embryos.

Our results indicated that 2,4-D and 2,4-D DMA had no effect on cell-cycle progression but that the higher test concentrations (6 and 10 μg/ml) reduced mitotic activity in both the 24 h and 36 h treatments. What might account for the reduced numbers of metaphases cells in the absence of any indication of an altered cell cycling? Tuschi and Schwab (2003) whose recently observed 2,4-D induced mitochondrial damage and apoptosis in HepG2 cells. It could be that this effect might be occurring here as well to reduce the mitotic indices. Moreover, the lack of highly damaged cells in the SCGE results corresponding to dying or dead cells according in the classification of Lebailly et al. (1997) could argue against this plausible explanation. In agreement with our observations, no significant changes in the cell-cycle progression were reported in rodent studies after in vivo treatment with 2,4-D (Linnainmaa, 1984; Mustonen et al., 1989). However, treatment with 2,4-D slowed the cell-cycle and reduced the MI in chick embryos and in cultured human hepatoma cells (Arias, 2003; Tuschi and Schwab, 2003). There is little information on the ability of specific pesticides to induce DNA-strand breaks in the SCGE assay, especially pesticides of the chlorinated aromatic hydrocarbon acid group.

To the best of our knowledge this is the first study in which this methodology was employed for analyzing the DNA damage produced by pure 2,4-D. Previously, DNA-strand break induction was evaluated by SCGE in erythrocytes from bullfrog (*Rana catesbeiana*) tadpoles after in vivo exposure for 24 h to Amsol (2,4-D amine) with negative results (Clements et al., 1997). Our results demonstrate that 2,4-D and 2,4-D DMA induce similar levels of DNA damage after a treatment of 90 min, increasing not only the proportion of damaged cells but also the comet length. These results are clear evidence that both herbicides are able to damage mammalian cell DNA, and, therefore, are genotoxic.

The genotoxicity of 2,4-D and 2,4-D DMA in the SCGE assay did not appear to be due to any effects on cell viability, since the treatments producing dose-dependent increases in DNA-strand breaks had little effect on the proportion of non-viable cells relative to untreated cultures. Furthermore, SCGE indicated that there were no highly damaged cells in the treated samples, which correspond, according to Lebailly et al. (1997), to dying or dead cells. These observations suggest that the treatments for 90 min with both 2,4-D and 2,4-D DMA trigger a process that is able to damage DNA and induce DNA-strand breaks prior to any loss of membrane integrity as revealed by the viability test. Consistent with this suggestion, it has been demonstrated that 2,4-D is taken up by CHO cells, passes rapidly through the cell membrane, and is not metabolised. However, the herbicide penetrates to the cell interior and possibly combines temporarily with cell constituents, thereby interfering with their normal functions suggest an indirect mode of action for 2,4-D. The herbicide has been included in a list of possible hypolipidermic carcinogens known as peroxisome proliferators (Reddy et al., 1980). In relation to chemicals possessing this type of activity, it has been proposed that the induction of peroxisomal beta-oxidation causes oxidative stress and increases the intracellular levels of DNA-damaging hydrogen peroxide and other reactive oxygen species (Yeldandi et al., 2000).

In summary, our present results in CHO cells indicate that 2,4-D and 2,4-D DMA have similar effects on SCE induction, cell proliferation kinetics, MI alteration and DNA damage detected in the SCGE assay. In agreement with these observations, it is has been reported that contamination in commercial preparations of 2,4-D is usually minimal, and comparisons of the toxicity produced by the pure herbicide and its commercial preparations usually reveal similar levels of activity (Cochrane et al., 1981). Our findings indicating that 2,4-D and 2,4-D DMA produce DNA damage and SCE in CHO cells provide additional evidence for the genotoxicity of these herbicides. Therefore, further investigation into the genotoxicity of 2,4-D will be required to understand its carcinogenic potential.

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