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The chlorophenoxy herbicide dicamba and its commercial formulation banvel® induce genotoxicity and cytotoxicity in Chinese hamster ovary (CHO) cells

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Abstract

The sister chromatid exchange (SCE) frequency, the cell-cycle progression analysis, and the single cell gel electrophoresis technique (SCGE, comet assay) were employed as genetic end-points to investigate the geno- and citotoxicity exerted by dicamba and one of its commercial formulation banvel® (dicamba 57.71%) on Chinese hamster ovary (CHO) cells. Log-phase cells were treated with 1.0–500.0 μg/ml of the herbicides and harvested 24 h later for SCE and cell-cycle progression analyses. All concentrations assessed of both test compounds induced higher SCE frequencies over control values. SCEs increased in a non-dose-dependent manner neither for the pure compound ($r = 0.48$; $P > 0.05$) nor for the commercial formulation ($r = 0.58$, $P > 0.05$). For the 200.0 μg/ml and 500.0 μg/ml dicamba doses and the 500.0 μg/ml banvel® dose, a significant delay in the cell-cycle progression was found. A regression test showed that the proliferation rate index decreased as a function of either the concentration of dicamba ($r = -0.98$, $P < 0.05$) or banvel® ($r = -0.88$, $P < 0.01$) titrated into cultures in the 1.0–500.0 μg/ml dose-range. SCGE performed on CHO cells after a 90 min pulse-treatment of dicamba and banvel® within a 50.0–500.0 μg/ml dose-range revealed a clear increase in dicamba-induced DNA damage as an enhancement of the proportion of slightly damaged and damaged cells for all concentrations used ($P < 0.01$); concomitantly, a decrease of undamaged cells was found over control values ($P < 0.01$). In banvel®-treated cells, a similar overall result was registered. Dicamba induced a significant increase both in comet length and width over control values ($P < 0.01$) regardless of its concentration whereas banvel® induced the same effect only within 100.0–500.0 μg/ml dose range ($P < 0.01$). As detected by three highly sensitive bioassays, the present results clearly showed the capability of dicamba and banvel® to induce DNA and cellular damage on CHO cells.

Keywords: Dicamba; Banvel®; Chlorophenoxy herbicides; Sister chromatid exchanges; Cell-cycle kinetics; Comet assay

1. Introduction

Dicamba is a selective systemic herbicide, absorbed by the leaves and roots, acts as an auxin-like growth regulator causing uncontrolled growth. It is used to control annual and perennial broad-leaved weeds and bush species, e.g. cereals, maize, sorghum, sugarcane, asparagus, perennial seed grasses, turf, pastures, rangeland, and non-crop land [1]. Being ranked as class III chemical (slightly hazardous) by WHO (http://www.who.int/ipcs/publications/pesticides_hazard/en/) and included in category D (not classifiable as to human carcinogenicity by EPA [2], dicamba has been proved to have a promot-

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ing activity in two-stage hepatocarcinogenesis [3] and to produce severe damage in hepatic and renal tissues after chronic administration in mice [4]. Furthermore, its ability to induce developmental toxicity in murine preimplantation embryos has been recently reported [5].

The literature on the genotoxicity of dicamba is rife with inconclusive and sometimes with conflictive data ([6,13], and references therein). Different assays have been performed to study the mutagenicity properties of dicamba. Among them, bioassays employing bacteria (*Bacillus subtilis* rec A [8], *Salmonella typhimurium* [9]), plants (*Arabidopsis thaliana* [7]), insects (*Drosophila melanogaster* [10]) and human cells including fibroblasts [11] and lymphocytes [6,12] can be mentioned. However, both positive and negative results on the mutagenicity of dicamba have been reported so far [13].

Previously, we have studied the genotoxicity of herbicide dicamba and the dicamba containing commercial formulation banvel® in *in vitro* human lymphocytes. We were able to demonstrate that dicamba is a DNA damaging agent since enhancement of the frequency of SCEs, alterations in both cell-cycle progression and mitotic indices were found. Thus, the pesticide could be considered as a potentially hazardous chemical to humans [12].

In the present study we employed the sister chromatid exchange (SCE) frequency, the cell-cycle progression analysis, and the single cell gel electrophoresis technique (SCGE, comet assay) as genetic end-points to further characterize the genotoxicity and cytotoxicity exerted by dicamba and one of its commercial formulation banvel® (dicamba 57.71%) on Chinese hamster ovary (CHO) cells.

2. Materials and methods

2.1. Chemicals

Dicamba (3,6-Dichloro-2-methoxybenzoic acid, CAS n° 1918-00-9) was obtained from Riedel-de-Haën (Pestanal®; Hannover, Germany). Banvel® (57.71% dicamba) was kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell cultures and pesticide treatment for cytogenetic assay

CHO cells were grown in Ham's F10 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), 100 units/ml penicillin (Gibco) and 10 μg/ml streptomycin (Gibco) at 37 °C in a 5% CO_2 atmosphere. Experiments were set up with cultures in the log phase of growth. The cells were seeded in T75 flasks at a density of 10^6 cells per flask. Treatments with the test compounds were performed 24 h after plating. Prior to use, dicamba was first dissolved in DMSO and then diluted in culture medium while banvel® was directly diluted in culture medium. Both dicamba and banvel® were diluted so that addition of 100 μl into cultures allowed to reach the required concentration specified in Results section within the range 0.0–500.0 μg/ml. The final solvent concentration was <1% for all treatments in the different experiments. Negative controls (untreated cells and solvent vehicle-treated cells) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Afterwards, 10 μg/ml bromodeoxyuridine (BrdUrd) (Sigma Chemical Co.) was incorporated into cultures and then the cells incubated at 37 °C in a 5% CO_2 atmosphere under a safety light for an additional 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.

2.3. Chromosome preparations

During the last 3 h of culture, the cells were treated with 0.2 μg/ml colchicine (Sigma Chemical Co.). Cells were detached with a rubber-policeman, collected by centrifugation, hypotonically shocked (0.075 M KCl, 37 °C, 17 min) and fixed in methanol/acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique.

2.4. 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 [14]. Slides were coded and scored blind by one researcher.

2.5. Cell-cycle kinetics

A minimum of 200 metaphase cells per sample were scored to determine the percentage of cells that had undergone one (M_1), two (M_2) and three or subsequent 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 the cells had divided in the medium since the addition of BrdUrd until harvesting [15].

2.6. Sister chromatid exchange analysis

A total of 50 well-spread diploid M_2 cells metaphases were scored per experimental point from each treatment. The data were expressed as the mean number of SCEs per cell ± S.E. from 150 cells.

2.7. Cell cultures and pesticide treatment for single cell gel electrophoresis (SCGE)

Prior to test chemical treatment, exponentially CHO cell were detached by a rubber-policeman, collected by centrifugation, resuspended in complete culture medium, and then counted. Afterwards, 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 dicamba and banvel® were used at a final concentration between 50.0 and 500.0 μg/ml. The final solvent concentration was <1% for all the treatments in all experiments. Negative controls (untreated cells and solvent vehicle-treated cells) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Thereafter, the cells were washed twice with pesticide-free complete culture medium. The SCGE and cell viability assays were performed immediately after the 90 min treatment. Cultures were duplicated for each experimental point, during at least three independent experiments. The same batches of cultures medium, sera and reagents were used throughout the study.

2.8. Cell viability assay

Cell viability was determined using the ethidium bromide/acridine orange assay described elsewhere [16]. Briefly, one aliquot of 5 μl of a 1:1 freshly prepared mixture of ethidium bromide (100 μg/ml, Sigma Chemical Co.) and acridine orange (100 μg/ml, Sigma Chemical Co.) was mixtured with 50 μl of the cell suspension. Afterwards, cells were analyzed using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. Viable cells appeared green-fluorescent whereas orange-stained nuclei indicated dead cells. At least, 500 cells were counted per experimental point, and results expressed as percentage of viable cells among all cells. Cell viability was expressed as proportion of living cells.

2.9. Single cell gel electrophoresis (SCGE) assay

The SCGE assay was performed following the alkaline procedure described by Singh and collaborators [17] with minor modifications. Briefly, 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. Seventy-five microliters NMA were transferred onto 100% ethanol precleaned slide, spread evenly, and placed at 37 °C for 20–30 min. Afterwards, 95 μl of LMA together with 7.0×10^3 cells (20 μl cell suspension + 75 μl 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 LMA was added and slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcocinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris pH 10.0, 1% Triton X-100, 10% DMSO) and then lysed at darkness for an overnight period (4 °C). When this period concluded, slides were placed in a horizontal electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 20 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 20 min at 25 V and 250 mA. Finally, the slides were neutralized with a solution comprising 0.4 M Tris–HCl, pH 7.5, stained with 4′6-diamino-2-phenylindole (DAPI) (Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA, USA). Slides were coded and scored blind by one cytogenetist. Analysis of the slides was performed under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The cellular nucleus diameters of the nucleus plus migrated DNA were individually measured using a calibration scale with an X63 fluorescence objective from 50 randomly selected cells per experimental point for each experiment. Cells were visually graded into four categories as suggested elsewhere [18,19] depending on DNA damage level as undamaged, slightly damaged, damaged, and highly damaged, respectively.

2.10. Statistical analysis

The two-tailed Student's *t*-test was used to compare SCE frequencies and measures from comets between treated and control groups. A χ^2-test was employed for cell-cycle progression. The non-parametric Kruskal–Wallis test was used to compare the effect of a pesticide over control cells in comet assay experiments while individual comparisons between pairs of data were performed using the Mann–Whitney test. The level of significance chosen was 0.05 unless otherwise indicated.

3. Results

Since no difference of cell-cycle progression, SCEs and PRI values were observed between negative controls (untreated and DMSO-treated cells), pooled data are presented for control values.

Fig. 1 shows the results of SCE analysis in CHO cells treated for 24 h with different doses of dicamba or the dicamba-containing commercial herbicide banvel®. The SCE frequencies observed either in dicamba- or banvel®-treated cultures were significantly higher than those of control cultures ($P < 0.05$, $P < 0.01$, respectively). SCE frequencies induced by the pure compound and the commercial formulation increased in a non-dose-dependent manner ($r = 0.48$, $P > 0.05$, and $r = 0.58$, $P > 0.05$ for dicamba and banvel®, respectively). The maximal SCEs increase over control values was detected when a 200.0 μg/ml dicamba dose was tested (12.55 ± 0.55 SCEs/cell versus 9.33 ± 0.51 SCEs/cell) (Fig. 1). In the banvel®-treated cultures the highest SCEs value was observed when a 500.0 μg/ml dose was used (13.40 ± 0.55 SCEs/cell versus 9.47 ± 0.50 SCEs/cell) (Fig. 1).

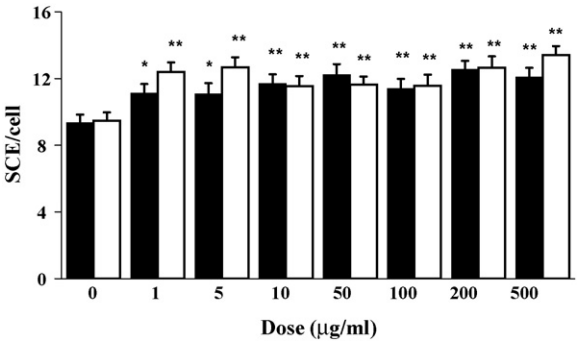

Fig. 1. Effect of the chlorophenoxy dicamba (black bars) and its commercial formulation banvel® (empty bars) on SCE frequency from CHO cells. Cultures were harvested at 24 h from pesticide treatment and the frequency of SCEs were determined in 50 M_2 mitoses for each experimental point. For each herbicide, pool data from three independent experiments are reported as mean SCE values ± S.E. (*y*-axis) and plotted against the herbicide concentration (0.0–500.0 μg/ml dose-range; *x*-axis). $^*P < 0.05$; $^{**}P < 0.01$.

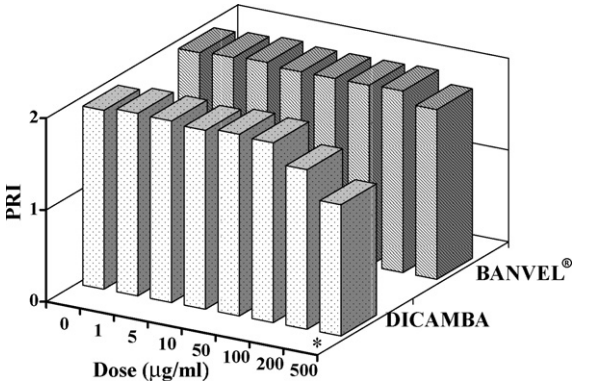

Fig. 3. Effect of the chlorophenoxy dicamba and its commercial formulation banvel® on proliferative rate index (PRI) from CHO cells. Cultures were harvested at 24 h from pesticide treatment and the PRIs were determined in 300 mitoses for each experimental point. For each herbicide, pool data from three independent experiments are reported as mean frequencies ± S.E. (*y*-axis) and plotted against the herbicide concentration (0.0–500.0 μg/ml dose-range; *x*-axis). $^*P < 0.05$.

Cytotoxicity, measured as cell-cycle kinetics, was observed in those dicamba- (Fig. 2A) and banvel®-treated (Fig. 2B) cultures since both a significant delay in cell-cycle progression (Fig. 2) and a significant reduction of the PRI were induced (Fig. 3). For dicamba, a significant increase in the frequency of M_1 ($P < 0.001$) and a significant decrease in the M_2 frequency ($P < 0.001$) were registered in cultures titrated with both 200.0 μg/ml and 500 μg/ml (Fig. 2A). A similar effect was only found in cultures treated with 500.0 μg/ml banvel® ($P < 0.001$) (Fig. 2B). On the other hand, no significant alteration in the frequencies of M_1, M_2, and M_{3+} was observed for both test compounds within 1.0–100.0 μg/ml and 1.0–200.0 μg/ml of dicamba and banvel®, respectively ($P > 0.05$) (Fig. 2). A decrease in the PRI was observed in cultures treated with 200.0–500.0 μg/ml and 500.0 μg/ml of dicamba and banvel®, respectively. However, statistical significance was only achieved after treatment with 500.0 μg/ml of dicamba ($P < 0.05$) (Fig. 3). A regression test showed that the PRI decreased as a function of either the concentration of the dicamba ($r = -0.98$, $P < 0.05$) or banvel® ($r = -0.88$, $P < 0.01$) titrated into cultures in the 1.0–500.0 μg/ml dose-range (Fig. 3).

Table 1 summarizes the results of the SCGE performed on CHO cells after a 90 min pulse-treatment of dicamba and banvel® in a 50.0–500.0 μg/ml dose-range. Since no differences of frequency of undamaged/damaged cells were observed between negative controls (untreated and DMSO-treated cells) pooled data are presented for control cultures. The SCGE revealed

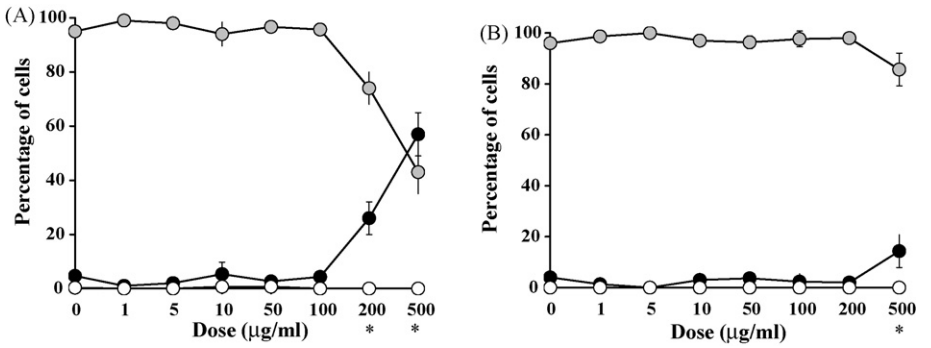

Fig. 2. Effect of the chlorophenoxy dicamba and its commercial formulation banvel® on cell-cycle progression from CHO cells. Cultures were harvested at 24 h from pesticide treatment and the proportion of cells in first (white circles, M_1), second (gray circles, M_2), and third or subsequent cell divisions (black circles, M_{3+}) were determined in 200 mitoses for each experimental point. For each herbicide, pool data from three independent experiments are reported as mean frequencies ± S.E. (*y*-axis) and plotted against the herbicide concentration (0.0–500.0 μg/ml dose-range; *x*-axis). $^*P < 0.01$.

Table 1
Frequencies of undamaged, slightly damaged (non-migrant) and damaged (migrant) in control, dicamba- and banvel®-treated CHO cells[a]

Doses (μg/ml)	Number of cells examined	Percentage of cells[b]			Viability[c]
		Undamaged	Slightly damaged	Damaged	
Dicamba					
0	300	80.33 ± 0.33	19.66 ± 0.33	0.00 ± 0.00	96.50 ± 0.50
50	150	10.00 ± 1.33*	77.00 ± 2.33*	13.00 ± 3.67*	96.00 ± 2.10
100	150	9.00 ± 3.00*	73.00 ± 2.33*	18.00 ± 3.33*	91.00 ± 1.20
200	150	8.00 ± 0.67*	81.00 ± 1.00*	11.00 ± 0.33*	96.75 ± 0.75
500	150	7.50 ± 0.33*	85.00 ± 0.33*	7.50 ± 0.33*	91.75 ± 2.50
Banvel®					
0	300	81.33 ± 0.67	17.67 ± 0.67	1.00 ± 0.67	96.50 ± 0.50
50	150	11.00 ± 2.33*	77.00 ± 0.33*	12.00 ± 2.67*	92.50 ± 1.40
100	150	9.00 ± 1.67*	72.00 ± 2.00*	19.00 ± 0.33*	93.50 ± 1.50
200	150	13.00 ± 4.33*	79.00 ± 3.67*	8.00 ± 0.67*	90.50 ± 2.50
500	150	1.00 ± 0.33*	75.00 ± 1.67*	24.00 ± 1.33*	91.00 ± 1.20

[a] Cells were treated with test compounds and harvested 24 h thereafter, and processed following procedure for SCGE. Electrophoresis was performed at 4 °C for 20 min at 25 V and 250 mA. Cells were stained with DAPI.
[b] Undamaged cells, width and length <30 μm; slightly damaged cells, width and length 31–45 μm; damaged cells, width and length >45 μm; results are expressed as mean values ± S.E. of the mean.
[c] Results are expressed as mean values ± S.E. of the mean.
* $P < 0.01$.

a clear increase in dicamba-induced DNA damage as an enhancement of the proportion of slightly damaged and damaged cells for all concentrations used ($P < 0.01$); concomitantly, a decrease of undamaged cells was found over control values ($P < 0.01$). In banvel®-treated cells, a similar overall result was registered since a significant increase in the frequency of slightly damaged and damaged cells for all concentrations used ($P < 0.01$). The results demonstrated that cultures showed the lowest proportion of undamaged cells and the highest frequency of damaged cells when treated with 500.0 μg/ml banvel®. Furthermore SCGE revealed that banvel® at a dose of 500.0 μg/ml induced a seven-fold decrease in the frequency of undamaged cells and a three-fold enhancement of damaged cells with regard to the same dicamba concentration (Table 1). Effects of dicamba and banvel® concentrations on DNA damage, measured by comet length and width, are presented in Fig. 4. Dicamba induced a significant increase both in comet length (Fig. 4A) and width (Fig. 4B) over control values ($P < 0.01$) regardless of its concentration. Banvel® induced significant enhancement over control values ($P < 0.01$) either in comet length (Fig. 4C) or comet width (Fig. 4D) in the 100.0–500.0 μg/ml dose range. A regression analysis revealed a positive relationship between dicamba concentration and the frequency of DNA-strand breaks as measured as comet length ($r = 0.81$, $P < 0.01$). On the other hand, no statistical association was observed between dicamba concentrations and comet width ($r = 0.58$, $P > 0.05$), and between banvel® concentrations and comet length ($r = 0.29$, $P > 0.05$) or width ($r = 0.29$, $P > 0.05$).

4. Discussion

The aim of this study was to evaluate the genotoxic and cytotoxic effects of dicamba and its technical formulation banvel® in CHO cells. The investigation was conducted using the sister chromatid exchange (SCE) frequency, the cell-cycle progression analysis, and the SCGE assay as genetic end-points. Both chemicals induced higher SCE frequencies and a delay in the cell-cycle progression compared to control values, as well as DNA-strand breaks revealed by the comet assay. Thus, either dicamba- or banvel®-treated cultures' results found, at least under the experimental conditions used in the present work, showed the ability of these compounds to induce genotoxicity and cytotoxicity *in vitro*.

Diverse genetic end-points and test-systems have been utilized for research on dicamba, and results emerging from those investigations, although being diverse, have been also inconclusive. Specifically concerning animal systems, dicamba has been demonstrated to cause an increase in SCE frequency in human peripheral blood lymphocytes *in vitro* [6,12] but failed to induce genetic damage such as structural chromosomal aberrations in rat bone marrow cells [13] or sex-linked recessive lethal mutations in *D. melanogaster* [20]. CHO cells have been recently applied in pesticides soil clays-mediated

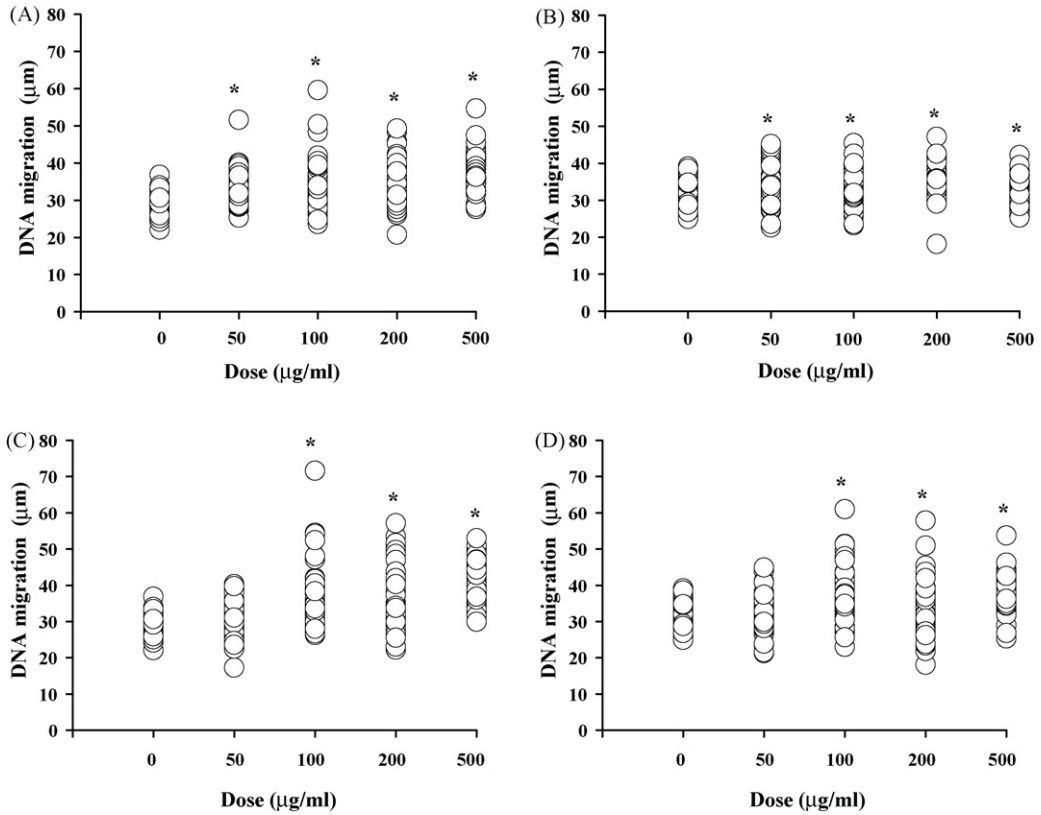

Fig. 4. Effect of the chlorophenoxy dicamba (A and B) and its commercial formulation banvel® (C and D) on the length of the comet tails (A–C) and on the diameter (B–D) at the estimated trailing edge of comet tails in CHO cells. Cultures were harvested at 90 min from herbicide treatment and processed following procedure for single cell gel electrophoresis. Electrophoresis was performed at 4 °C for 20 min at 25 V and 250 mA, and cells stained with DAPI. DNA migration (μm) were determined in 150 cells for each experimental point. For each herbicide, pool data from three independent experiments are reported as DNA migration (*y*-axis) and plotted against the herbicide concentration (0.0–500.0 μg/ml dose-range; *x*-axis). $*P < 0.01$.

toxicity assessments, including dicamba among others agricultural agents [21,22]. In the current investigation, we have combined the extensively used CHO *in vitro* system and three highly sensitive bioassays for geno- and cytotoxicity [23,24].

Our results demonstrated that all dicamba and banvel® doses titrated into CHO cultures induced a similar level of SCE frequency in a non-dose-dependent relationship. This is in contrast with our previous findings on human lymphocytes *in vitro* which exhibited similar augments only for the 200.0 μg/ml dicamba and the 500.0 μg/ml banvel® doses. Furthermore, the highest concentration tested in CHO cells (500.0 μg/ml) did not cause cell-death as it resulted in human lymphocytes. Furthermore, it is worth mentioning that the latter when treated with doses ranging from 10.0 to 100.0 μg/ml and 10.0 to 200.0 μg/ml of dicamba and banvel®, respectively, failed to show any sign of cytotoxicity [12]. Thus, the survival of CHO cells together with their higher resistance to dicamba and banvel® deleterious effects when compared to human lymphocytes, point out that this cell line constitutes a well suited system to investigate these chlorobenzoic compounds. However, the possibility that human lymphocytes are able to repair the damage introduced into their DNA by low doses of the pesticide during G_0 cannot be ruled out.

To our knowledge this is the first time the cell-cycle kinetics is used as an alternative approach to quantify dicamba and banvel® cytotoxicity. The results presented here showed that pure pesticide treatment of 200.0 μg/ml and 500.0 μg/ml delayed significantly the cell-cycle progression and produced an overall valuable PRI reduction, results obtained for the maximal banvel® concentration assayed only. These three test compound doses rendered a matched combination of M_1 increase and M_2 reduction that differed from our previous results on human lymphocytes *in vitro* which exhibited a varied combination of M_1, M_2, and M_{3+} reductions and/or increases [12]. Plausible explanation for this discrepancy could be most probably related to differences both in the harvesting

times we employed and in the length of the cell-cycle between CHO cells and human lymphocytes *in vitro*.

Disparity between past and present results can be related to the culture systems employed and the ability of the systems to predict toxic effects. The assessment of dicamba and banvel® on human lymphocytes *in vitro* was performed in whole blood cultures to exploit the metabolic capability of whole blood versus other erythrocytes-free systems, e.g., purified lymphocyte cultures [25–28]. Previous studies have suggested a protective effect for the erythrocytes present in whole blood cultures against the induction of genotoxic damage [27–31]. They all agree in demonstrating that the protective effect exerted by red blood cells, most probably could be committed to the antioxidant enzymes contained in these cells [29,32]. Similar results concerning identical culture systems have been reported by us when testing the genotoxicity of herbicides of the same chemical group 2,4-dichlorophenoxy-acetic acid (2,4-D) and its commercial formulation [33] suggesting a detoxification role for the erythrocytes from the whole blood cultures but absent in CHO cultures. In accord to the statement by Plewa et al. [34] reckoning that the *S. typhimurium* assay did not predict toxic effects with respect to CHO cells, we have found that toxicity data in human lymphocytes did not quantitatively predict the toxic effects of dicamba and banvel® in mammalian cell systems, at least between CHO cells and human lymphocytes *in vitro*. Moreover, the possibility that CHO cells are more sensitive than human lymphocytes to the deleterious effect of dicamba and banvel® in inducing alterations in the cell-cycle cannot be ruled out, as we stated previously.

There is little information on the ability of dicamba and banvel® to induce DNA-strand breaks quali- and quantitative analyzed by the SCGE assay. Previously, Sorensen et al. [21,22] reported the genotoxic effect of several pesticides, including dicamba, before and after smectite clay–pesticide interactions. These authors found that dicamba was not genotoxic when directly analyzed in spite of the longer time of exposure (4 h versus 90 min) and the highest concentration assessed (four-fold higher than the maximal dose employed in our experiments). In contrast to those results, our current results allow us to communicate for the first time the *in vitro* evaluation of DNA damage produced by dicamba and its technical formulation banvel®. A brief pulse-treatment of 50.0–500.0 μg/ml and 100.0–500.0 μg/ml dicamba and banvel®, respectively, resulted in manifest levels of DNA-strand break induction. Thus, the positive results found by us could be related to a genotoxic effect exerted by the herbicide minimizing the possibility of a cytotoxic effect which most probably could be responsible for the negative results previously reported [21,22].

The dicamba damage mechanism is not yet known. Several studies have shown that chlorophenoxy herbicides compounds like 2,4-dichlorophenoxy-acetic acid (2,4-D) have been proved to cause lipid peroxidation [35,36]. Dicamba also belongs to the chlorophenoxy herbicide group and as far it is known, it induces tissue damage and cell death in cleavers (*Gallium aparine* L.) by lipid peroxidation [37]. However, none of the endpoints we employed in the present study allow us to correlate the DNA and cellular damage with lipid peroxidation. Further studies are required to prove or discard this plausible mechanism/s of action. What is clear is that the pure herbicide and its commercial formulation banvel® have genotoxic and cytotoxic properties.

As the totality of banvel® concentrations employed induced SCEs in CHO these results led us to the consideration of the excipients contained in the commercial formulation. Currently, there are a number of registered products containing dicamba for use in non-food producing situations (mainly lawn, turf and recreational areas) or in food producing crops (barley, rye, oats, wheat, grass pastures, grain sorghum, maize, rice and sugarcane) (http://www.apvma.gov.au/residues/ARDS-dicamba.html). Banvel®, a technical formulation produced in Argentina is included in this group of agrochemicals. Unfortunately, the identity of the components of the commercial formulation product was not made available to us. On the other hand, though almost improbable, the possibility that the amount of dicamba incorporated in the technical Argentinean formulation could be higher than 57.71% cannot be discarded.

Dicamba and several dicamba-containing formulations have been pointed out as air, water and soil pollutants [38,39] but these phenoxy herbicides are not to be considered hazardous to the environment only. Occupational and accidental exposures to dicamba have been reported for several countries, including Argentina (http://bases.bireme.br). Thus, further investigation is needed to acquire a comprehensive knowledge of the possible mechanism/s through dicamba and banvel® exert their toxic effects. Regardless of the mechanism, the present study, employing three reliable genetic endpoints, showed that both test compounds are DNA- and cellular-damage inducing agents.

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