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Dicamba-induced genotoxicity in Chinese hamster ovary (CHO) cells is prevented by vitamin E

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ABSTRACT

In the present study the cytogenetic and genotoxic effect of benzoic herbicide dicamba and its Argentinean commercial formulation banvel® (57.71% dicamba) was evaluated and whether this effect is mediated through oxidative damage or not. The protective role of vitamin E was also studied. Sister chromatid exchange (SCE) frequency, cell-cycle progression, and cell viability analyses in CHO cells were used as *in vitro* end-points. Treatments with the test compounds were performed either during 24 h (Protocol A) or 12 h (Protocol B) before harvesting. Protocol A showed that vitamin E decreased pesticide SCE induction, corrected the cell-cycle delay and partially protected cell-death only in 500 μg/ml dicamba-treated cultures. A similar trend was found in banvel®-treated cultures. Protocol B revealed similar protective role of vitamin E only for dicamba-induced geno- and cytotoxicity. Based on these observations it could be suggested that dicamba injures DNA by delivering reactive oxygen species rather than by another type of mechanism/s. Although banvel® mimics the effect observed by dicamba, its formulation contains other xenobiotic/s agents able to induce cellular and DNA damage by a different mechanism/s. Further investigations are needed to acquire a comprehensive knowledge of the possible mechanism/s through dicamba and banvel® exert their toxic effects.

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1. Introduction

Among of the most widely used herbicides are included the auxinic herbicides. Since the discovery in 1940s, auxinic herbicides have been employed as agrochemicals for their selective control on broadleaf weeds. They comprise several compounds belonging to four different chemical families, namely phenoxyalkanoic acids, pyridinecarboxylic acids, quinolinecarboxylic acids, and benzoic acids [1].

Dicamba is a chlorinated benzoic acid-derivate compound that was first registered in 1967 in the United States as a post-emergent herbicide [2]. It is currently used in agriculture and gardening to control the growth of different unwanted vegetable species, mainly in cereal grain crops but also on sugar cane and soybeans, among others [3].

Dicamba is available in numerous commercial formulations as a single component or co-formulated with a wide variety of other herbicides, such as 2,4-dichlorophenoxy-acetic acid (2,4-D), sulfonylureas or triazines to provide a broader spectrum of weed control. These formulations are registered and sold in many countries all over the world [4].

As dicamba shares characteristics with other acidic herbicides, e.g., high water solubility, low volatility, and heavy agricultural use that facilitate their incorporation into the aqueous environment, a growing number of studies have been performed stressing its environmental effects. Dicamba has been detected as a pollutant in agricultural, urban and agricultural/urban mixed sites [5], in surface drinking-water reservoirs [6], and even in estuarine waters [7]. Furthermore, much is known on its effect on aquatic plants and terrestrial organism risks [3]. Toxicological data of this chlorinated acid herbicide are available and considered adequate to assess its potential hazard not only to humans but also to other living species [3].

In contrast to the environmental assessment of dicamba, there is a limited understanding on its genotoxic potency. So far, dicamba's potential genotoxic hazard has been revealed by diverse *in vivo* [8–10] and *in vitro* assays [8,11–13]. Some recent studies demonstrated that dicamba and banvel® are DNA damaging agents. Induction of sister chromatid exchanges (SCEs), an alteration in both cell-cycle progression and mitotic indices in human lymphocytes *in vitro* have been reported [14]. Additionally, both chemicals were reported to induce an increase in SCE frequencies and a delay in the cell-cycle progression as well as DNA-strand breaks

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revealed by the comet assay on Chinese hamster ovary (CHO) cells [15].

The dicamba DNA damage mechanism is not yet known. Several studies have shown that acidic herbicides like 2,4-D have been proved to cause lipid peroxidation [16,17]. It is known that dicamba induces tissue damage and cell-death in cleavers (*Gallium aparine* L.) by lipid peroxidation [18]. Furthermore, it has been recently suggested that dicamba-induced lesions on DNA could be accounted for reactive oxygen species delivered *in vitro* [14,18].

α-Tocopherol is the most active form of vitamin E being a powerful antioxidant against free radicals and oxidative attacks. It acts as a free radical scavenger and among other effects, it has been proved to be an antigenotoxic agent in several mammalian cells [19–25]. In the present report we analyzed whether the deleterious cytotoxic and genotoxic effects exerted by dicamba and banvel® is mediated by an oxidative damage. Experiments were set up to assess the putative protective role of vitamin E using the SCE frequency, cell-cycle progression, and cell viability analyses as *in vitro* end-points on CHO cells.

2. Materials and methods

2.1. Chemicals

Dicamba (3,6-dichloro-2-methoxybenzoic acid, CAS 1918-00-9), vitamin E (DL-α-tocopherol, CAS 10191-41-0), 5-bromo-2′-deoxyuridine (BrdU, CAS 59-14-3), dimethyl sulfoxide (DMSO, CAS 67-68-5), ethidium bromide (CAS 1239-45-8), colchicine (CAS 64-86-8) and acridine orange (CAS 10127-02-3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol (CAS 64-17-5) was purchased from Merck KGaA (Darmstadt, Germany). Banvel® (57.71% dicamba) was kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina).

2.2. Cell cultures and pesticide treatment for SCE, cell-cycle progression and cell viability assays

CHO cells were grown in Ham's F10 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 10 μg/ml streptomycin (all from Gibco, Grand Island, NY, USA) 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 as previously described elsewhere [15]. 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 Section 3 within the range 0–500 μg/ml. The final solvent concentration was less than <1% for all treatments. Vitamin E was diluted in 96% ethanol and titrated into cultures, when required, immediately after pesticide treatment; at a final concentration of 25 μM. Negative controls (untreated cells and solvents vehicle-treated cells) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Treatments with the test compounds were performed either at 24 h (Protocol A) or 36 h after plating (Protocol B). Regardless of the culture protocol, BrdU (10 μg/ml) was incorporated into cultures 24 h after plating. After BrdU titration, the cells were 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. 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 as previously described elsewhere [26].

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

2.5. Cell-cycle kinetics and proliferative rate index

A minimum of 100 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 BrdU until harvesting [28].

2.6. Sister chromatid exchange analysis

A total of 25 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 75 pooled cells scored per test-compound concentration.

2.7. Cell viability assay

Cell viability was determined using the ethidium bromide/acridine orange assay described elsewhere [29] in cells treated following Protocol A. Briefly, one aliquot of 5 μl of a 1:1 freshly prepared mixture of ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml) was mixed 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. The assay was performed in three independent experiments and cell viability was monitored at 0, 6, 12, 18 and 24 h post-pesticide treatment. At least, 500 cells were counted per experimental point, and results expressed as percentage of viable cells among all cells.

2.8. Statistical analysis

The two-tailed Student's *t*-test was used to compare SCE data between treated and control groups. A χ^2-test was employed for cell-cycle progression and cell viability data. The chosen level of significance was 0.05, unless otherwise indicated.

3. Results and discussion

Fig. 1 shows the results of SCE analysis in CHO cells treated with different concentrations of dicamba or banvel® in the presence or absence of vitamin E following culture Protocols A (Fig. 1A and B) and B (Fig. 1C and D), respectively. Since no differences of SCEs were observed between negative controls (untreated, DMSO- and ethanol-treated cells) regardless of the culture protocol employed, pooled data are presented for control values.

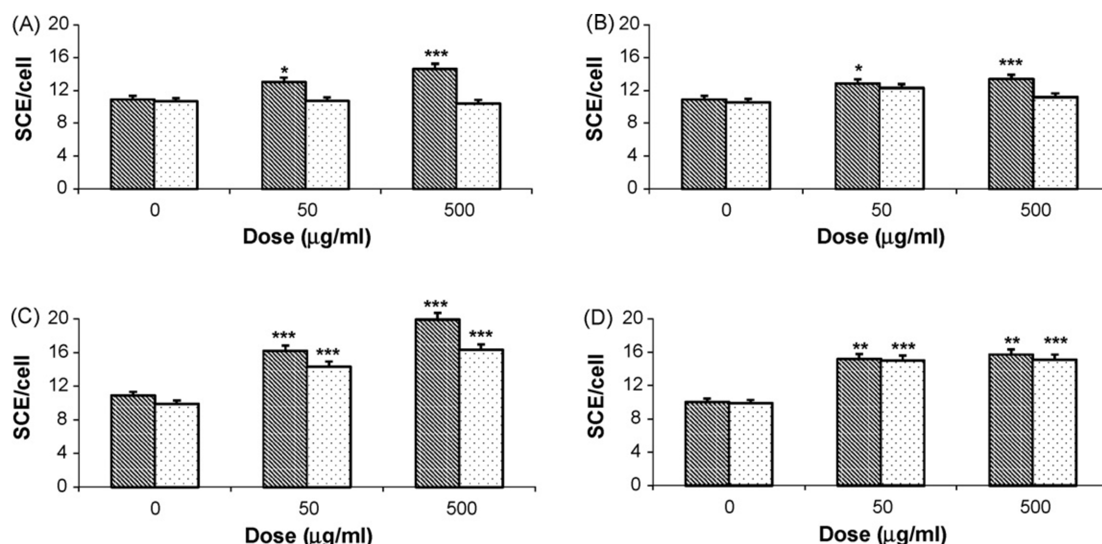

Fig. 1. Effect of dicamba (A and C) and banvel® (B and D) on SCE frequency from CHO cells in absence (stripped bars) or presence (dotted bars) of vitamin E. Cultures were harvested after 24 h (A and B) or 12 h (C and D) treatment. The mean SCE values ± S.E. (*y*-axis) are plotted against each dose of the test-compound (0, 50 and 500 μg/ml; *x*-axis). *$P<0.05$; **$P<0.01$; ***$P<0.001$.

The SCE frequencies induced by dicamba in cultures performed under Protocol A were significantly higher than those of control values ($P<0.05$ and $P<0.001$ for 50 and 500 μg/ml, respectively; Fig. 1A). Similar results were found in banvel®-treated cultures (Fig. 1B).

Fig. 1A and B also shows that the presence of vitamin E during the culture period did not alter the basal level of SCEs of negative control cultures ($P>0.05$). However, its presence decreased the frequency of SCE from dicamba-treated cultures independently of the pesticide concentration ($P<0.01$ and $P<0.001$ for 50 and 500 μg/ml of dicamba, respectively; Fig. 1A). On the other hand, when banvel®-treated cultures were co-incubated with vitamin E only a similar protective trend was achieved although not reaching statistical value ($P>0.05$; Fig. 1B).

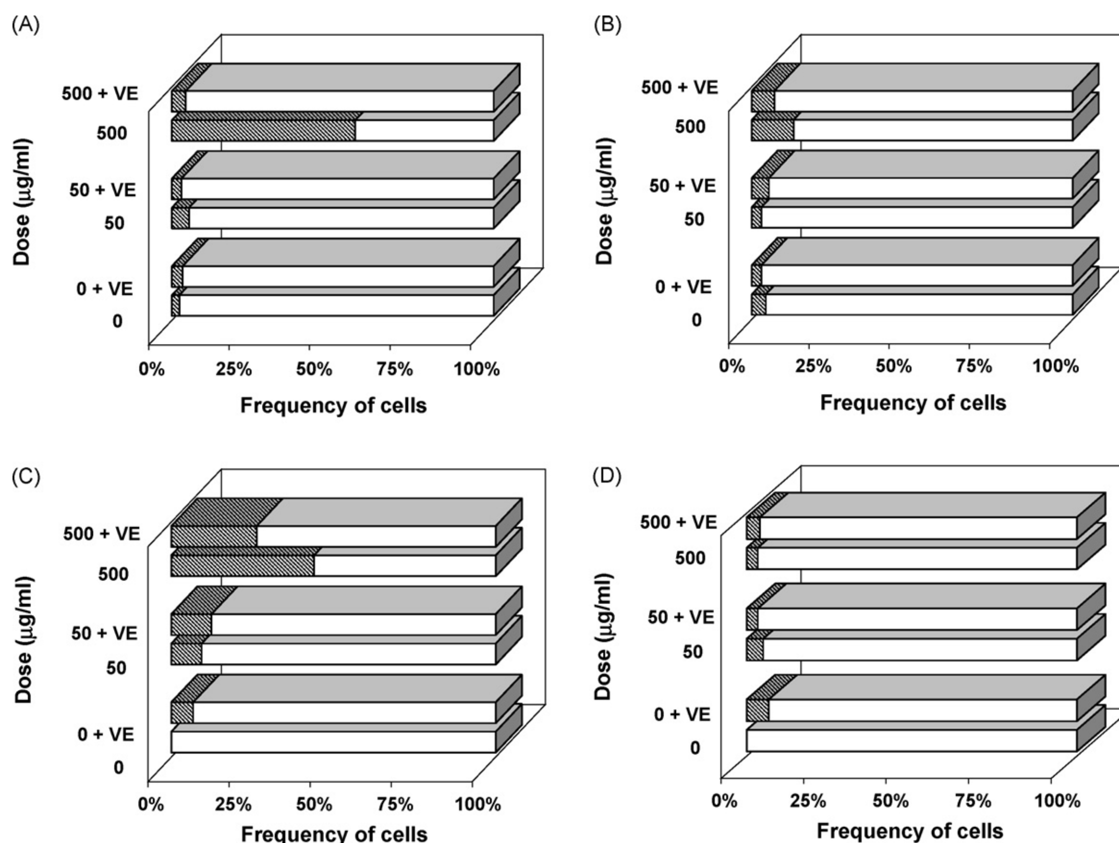

Fig. 2. Effect of dicamba (A and C) and banvel® (B and D) on cell-cycle progression in CHO cells after 24 h (A and B) or 12 h (C and D) treatment. M_1: stripped bar areas; M_2: white bar areas. The mean frequencies ± S.E. (*x*-axis) are plotted against each dose of the test-compound (0, 50 and 500 μg/ml; *y*-axis).

Table 1
Proliferative rate index (PRI) in control, dicamba- and banvel®-treated CHO cells with and without vitamine E (50 μg/ml)[a]

Dose (μg/ml)	PRI[b]							
	Protocol A				Protocol B			
	Dicamba		banvel® (57.71%)		Dicamba		banvel® (57.71%)	
	Without VE	With VE	Without VE	With VE	Without VE	With VE	Without VE	With VE
0	1.98	1.97	1.97	1.95	2	1.93	2	1.93
50	1.95	1.97	1.96	1.94	1.91	1.88	1.95	1.97
500	1.49***	1.96	1.92	1.88	1.56**	1.74	1.97	1.94

[a] CHO cells were treated with dicamba or banvel® and harvested 24 h (Protocol A) or 12 h (Protocol B) later.
[b] The proportion of cells in first (M_1) and second (M_2) cell divisions were determined in 300 mitoses for each experimental point.
** $P \le 0.01$.
*** $P \le 0.001$.

Fig. 2 summarizes the results on cell-cycle kinetics analyses from CHO cells treated with different concentrations of dicamba or banvel® in the presence or absence of vitamin E following culture Protocols A (Fig. 2A and B) and B (Fig. 2C and D) while PRI are presented in Table 1. Cytotoxicity, measured as cell-cycle kinetics, rendered diverse outcomes. Only cells exposed to 500 μg/ml of dicamba during 24 h (Protocol A) showed a delay in the cell-cycle progression over control values due to both a significant M_1 frequency increase ($P < 0.001$) and a significant M_2 frequency decrease ($P < 0.001$; Fig. 2A), and a concomitant reduction in the PRI ($P < 0.001$; Table 1). Similarly, only 500 μg/ml banvel® yielded a significant M_1 frequency increase ($P < 0.001$; Fig. 2B) without significant modification of the PRI ($P > 0.05$; Table 1). Fig. 2A and B also shows that the presence of vitamin E did not alter the cell-cycle progression of negative control cultures ($P > 0.05$). However, vitamin E co-incubation with pesticide-treated cells returned the cell-cycle kinetics of those 500 μg/ml dicamba-treated cultures to control values by inducing a significant reduction of M_1 frequency and a significant increase of M_2 frequency ($P < 0.01$; Fig. 2A). On the other hand, when banvel®-treated cultures were co-incubated with vitamin E only a similar protective trend was achieved although not reaching statistical value ($P > 0.05$; Fig. 2B). The analysis of the PRI revealed that the presence of vitamin E during the 24 h culture period normalized the alteration of the proliferation rate induced by 500 μg/ml dicamba to levels similar to control values ($P < 0.01$; Table 1).

Cell viability was assessed in cultures set up under Protocol A conditions with and without the presence of vitamin E during the whole length of the culture period. The results are presented in Fig. 3. Only 500 μg/ml of dicamba reduced significantly the cell viability after 6–12 h of treatment ($P < 0.01$) maintaining a plateau value until 24 h post-treatment (Fig. 3B). The results revealed that although vitamin E was able to protect 500 μg/ml dicamba-induced cell-death ($P < 0.01$), the frequency of survival cells did not return to control values ($P < 0.01$; Fig. 3B). On the other hand, no significant alterations in cell viability were found in those cultures in the presence or absence of vitamin E when 50 μg/ml of dicamba (Fig. 3A), and 50 or 500 μg/ml of banvel® (Fig. 3C and D) were used ($P > 0.05$).

In previous investigations the induction of SCEs by several concentrations of both test compounds have been analyzed on CHO cells [15] and human whole blood lymphocyte cultures [14]. The results revealed a higher sensitivity of the former to the deleterious effects of dicamba and banvel® when compared to the later. Prior studies showed differential response to clastogen-induced SCE when human lymphocytes were cultured in the presence or absence of erythrocytes after treatment with the same xenobiotic agent [30–36]. Those investigators have suggested that erythrocytes, when present in culture media exert a protective effect most probably due to the antioxidant enzymes contained in these cells [30,35–37]. These findings could indicate an indirect mode of action of these xenobiotics, most probably via free radical reactions and production of reactive oxygen species [14,15] which are well known to introduce lesions into DNA leading to geno- and cytotoxic effects like induction of SCEs, chromosomal aberrations, delay in cell-cycle progression, among others [36,38–41]. Several reports agree in demonstrating that vitamin E can prevent reactive oxygen species-induced geno- and cytotoxicity damages [19,42–44]. In the present study we employed vitamin E to test whether oxidative damage was involved in the effects induced by dicamba and banvel®. The results of Protocol A showed that this free radical scavenger significantly protected not only target cells from the SCE induction but also corrected the cell-cycle delay caused by the highest concentration of dicamba employed. In regard to banvel®-treated CHO cells, a similar protective effect was found though not reaching statistical significance. Additionally, the present observations revealed that vitamin E partially protected cell-death in those 500 μg/ml dicamba-treated cultures.

Results from Protocol A were obtained after a continuous pesticide treatment lasting 24 h which approximately cover the time required for CHO cells to perform two cell-cycles. This assumption is not only revealed by the proportion of M_2 cells at this harvesting time found in this study but also from previous observations reported elsewhere [15,22,41,45]. In spite of the clear dicamba and banvel® geno- and cytotoxic properties, the present observations demonstrated some effects related to the *in vitro* culture condition/s (Protocol A) that could lead to the underestimation of the damage assessed by the end-points used in this study. Among them (a) the induction of a selective cell loss by pesticide-induced cell-death of the most damaged cells during the first cell-cycle, being only a reduced proportion of cells capable of reaching the M_2 status, (b) the inability of most severe damaged cells to proliferate and perform a second cell-cycle, and/or (c) an exponential dilution of damage due to cell division if the deleterious effect induced by the pesticide only takes place during the first but not during the second cell-cycle, can be included. Furthermore, the possibility that CHO cells could be able to repair the damage induced by dicamba during the second cell-cycle cannot be ruled out. Thus, in order to achieve a further elucidation of dicamba and banvel® damaging mechanism, the level of dicamba- and banvel®-induced damage in cells exposed to the pesticide one cell-cycle prior harvesting (Protocol B) was analyzed.

The SCE frequencies induced by dicamba and banvel® in those cells cultured under Protocol B conditions are presented in Fig. 1C and D, respectively. Results on cell-cycle kinetics in cultures developed following Protocol B are shown in Fig. 2C and D while PRI data are presented in Table 1. The results from Protocol B, in brief revealed an increase in the SCE frequencies and a delay in cell-cycle progression induced by both test compounds while vitamin E protective role was only achieved in those dicamba-treated CHO cultures.

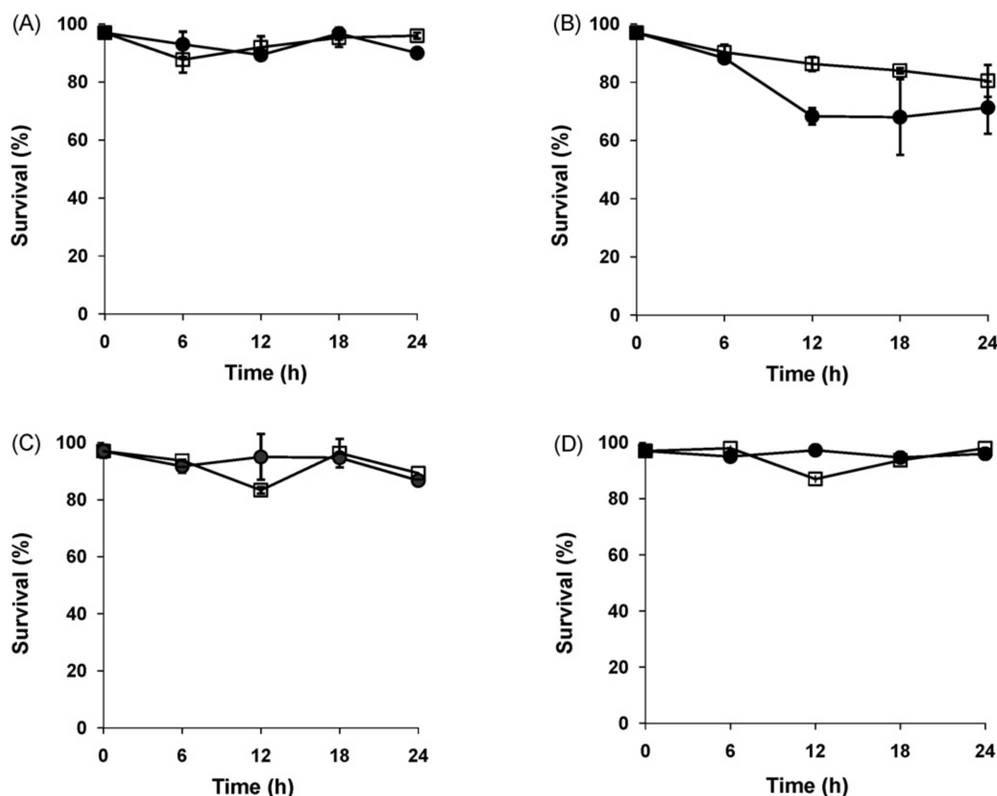

Fig. 3. Effect on *in vitro* treatment with dicamba-treated (50 μg/ml, A; 500 μg/ml, B) and banvel®-treated (50 μg/ml, C; 500 μg/ml, D) cultures in the absence (black circles) or presence (white squares) of vitamin E determined by the ethidium bromide/acridine orange assay and expressed as the proportion of living cells monitored at 0, 6, 12, 18 and 24 h post-pesticide treatment. The mean values ± S.E. (*y*-axis) are plotted against incubation time (*x*-axis).

When comparing the SCE frequencies induced by dicamba and banvel® in those CHO cells cultured under Protocols A and B some remarkable findings are observed (Fig. 4A and B). Overall, treatments with both test-compounds induced a significantly higher frequency of SCEs when target cells were cultured under Protocol B than in Protocol A ($P < 0.001$). Additionally, co-incubation with vitamin E rendered similar results when comparing outcomes from each culture protocol, since a significant higher frequency of SCEs were registered in those cultures developed under Protocol B than in Protocol A ($P < 0.001$; Fig. 4). The increase in SCE response found in the former could be most likely due to the decrease of culturing time which presumably reflects the ineffectiveness of CHO cells in repairing dicamba- or banvel®-inflicted DNA lesions leading to SCE induction and/or the requirement of a second round of DNA synthesis to allow the removal of at least some of them.

The possibility that the most severely damaged cells were unable to proliferate after injury, and perform a second cell-cycle could be ruled out since it was observed that only 500 μg/ml of dicamba but any concentration of banvel® altered cell viability in CHO cultures. Furthermore, another end-point assessed in both culture protocols was the cell-cycle progression analysis. Results clearly demonstrated that both dicamba and banvel® induced perturbations of the cell-cycle progression increasing M_1 frequencies under either Protocol A or B, but comparatively the delay was more extended in cells cultured by the conditions of the former ($P < 0.05$). This cell-cycle arrest may be an adaptative process in which surveillance mechanism delays the cell-cycle when DNA lesions occur. Other studies have demonstrated the ability of cells to delay their multiplication cycle in order for repair to take place [46,47]. In view of the present results concerning dicamba, a damaging mechanism mediated by oxidation may be hypothesized for vitamin E prevented cell-death, cell-cycle delay and SCE induction. As the level of genotoxic damage was less severe in target cells grown under Protocol A, this observation could further suggest that the DNA repairing systems required two cell-cycles to correct, at least partially, the DNA pesticide-inflicted lesions.

The exponential dilution of pesticide induced-SCEs due to cell division assuming that the deleterious effect exerted by the pesticide only takes place during the first but not during the second cell-cycle is a possibility that could not be totally ruled out with the methodologies adopted in the present study. Further molecular studies are required to elucidate whether dicamba- or banvel®-introduced lesions into DNA is an event committed only to first cell-cycle but not during the second division cycle after cellular pesticide treatment.

These findings verify previous results depicting the genotoxicity and cytotoxicity of dicamba and banvel® in CHO and human lymphocyte cells through the induction of SCEs and delaying the cell-cycle progression [14,15]. The present results also confirmed the observations of other authors [8,48] revealing that dicamba is able to exert DNA and cellular damage in cultured cells without the presence of a microsomal metabolic S-9 fraction during culturing [14,15]. Then, it can be assumed that the deleterious effect induced by dicamba is committed to the pesticide itself and not to any metabolite/s or any other subproducts generated during its cellular metabolic activation, at least in human lymphocytes and CHO cells *in vitro*.

It is worth mentioning that the Argentinean dicamba-containing technical formulation banvel®-induced geno- and cytotoxic effects on CHO cells were not totally corrected by the presence of vitamin E during pesticide treatment. Thus, this evidence for an incomplete recovery led us to consider that banvel® exerts its deleterious effect by a different mechanism rather than only through reactive oxygen species generation. Moreover, as a commercial herbicide formula-

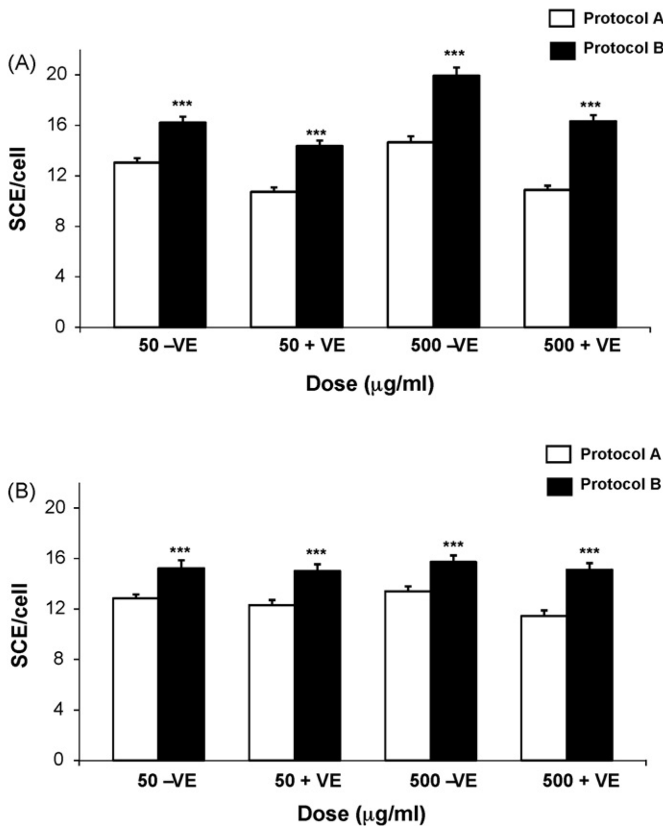

Fig. 4. Comparison of SCE frequency induced by dicamba and banvel® on CHO cells. Cultures were harvested after 24 or 12 h treatment. The mean SCE values ± S.E. (*y*-axis) are plotted against each dose of the test-compound (0, 50 and 500 μg/ml; *x*-axis). $^{***}P < 0.001$.

tion, banvel® contains a 42.29% of excipients. Unfortunately, the identity of the components present in the excipient formulation was not made available by the manufacturer. Several investigations have proved that commercial formulations have ability to induce DNA damage [49–51]. Thus, the possibility that the excipients contained in banvel® might affect the genotoxic potential of the herbicide cannot be discarded. On the other hand, it could be also probable, that the amount of dicamba present in the technical Argentinean formulation could be lower than 57.71% as indicated by the manufactures. Whether this latter possibility is true, the amount of lesions mediated by reactive oxygen species would be less than theoretically expected. Moreover, it seems evident that such excipient/s is/are able to enhance the dicamba-induced cytotoxicity.

4. Conclusions

The results presented here employing several *in vitro* bioassays to evaluate the geno- and cytotoxic effects of a chlorinated benzoic acid-derivate pesticide, as the dicamba, as well as one of the Argentinean including-dicamba commercial formulation, as the banvel®, resulted very useful short-term tools which could be used for quick screening methods directly committed not only to human but to other living species toxicology. Further investigations are needed to acquire a comprehensive and exhaustive knowledge of the possible mechanism/s through dicamba and banvel® exert their toxic effects. Finally, it should be highlighted that geno- and cytotoxicity risk to the exposure to commercial formulation was higher than that of the exposure to the pure compound. In this context, it was demonstrated that although banvel® mimics the deleterious effect registered by dicamba, it possesses into its formulation other xenobiotic/s agents able to induce cellular and DNA damage by a different mechanism/s, and thus increasing the risk for a synergism between the chemical/s included into the “apparent” inert excipient.

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