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Cytotoxic and genotoxic assessments of 2,4-dichlorophenoxyacetic acid (2,4-D) in *in vitro* mammalian cells

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ABSTRACT

A combined approach employing alkaline single cell gel electrophoresis (SCGE) and cytokinesis-blocked micronucleus (MNs) cytome bioassays was adopted to assess the deleterious properties of the auxinic 2,4-dichlorophenoxyacetic acid (2,4-D) and its microparticulated low volatility product Dedalo Elite (30% a.i.) on Chinese hamster ovary (CHO-K1) cells. Cytotoxicity was estimated by neutral red uptake (NRU), succinic dehydrogenase activity (MTT) and apoptosis assessment. Both compounds were assayed at 0.1–10 μg/ml concentration range. Whereas exposed CHO-K1 cells revealed a statistically significant enhancement of MNs when 10 μg 2,4-D/ml was assayed, MNs were only achieved in cells treated with 2 μg Dedalo Elite/ml. A diminution in the nuclear division index was only achieved after exposure to Dedalo Elite within the 1–10 μg/ml concentration range. Whereas increased genetic damage index was achieved when 6 and 10 μg 2,4-D/ml were assayed, GDI induction was observed in treatments employing 4 μg Dedalo Elite/ml. Both compounds induced cytotoxicity by inhibition of both lysosomal and MTT activities by enhancing the frequencies of early and late apoptotic cells. Our results not only indicate the genotoxic and cytotoxic potential of 2,4-D and its microparticulated marketplace formulation, but also highlight the risk of these agrochemicals present towards the biota and human health.

1. Introduction

Nowadays, our agricultural system and crop production require a large quantity of pesticides to ensure continuous quality in food production. As a direct consequence, human beings are producing and discarding pesticides into the environment causing serious concerns about not only terrestrial and aquatic pollution worldwide but also of aerial compartments (Gavrilescu, 2005; Yadav and Devi, 2017).

The most employed agrochemicals in modern agriculture are herbicides, which represent almost 48% of the total pesticide usage worldwide (Gupta and Gupta, 2014; USEPA, 2017). In the last two decades a new generation of commercial products, including micro- and nano-based pesticide formulations, are available for agricultural application. According to the manufacturers, these new kinds of pesticides have multiple environmentally friendly advantages including the slow release of the active ingredient (a.i.) applied, increased effectiveness, durability and solubility of poorly water-soluble pesticides, good dispersion and wettability of the a.i. and minimization of the toxicity of the a.i., among others (Kah and Hofmann, 2014). Among synthetic auxins, the chlorinated phenol herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is involved in both plant regulation and development by maintaining high concentrations of the auxinic phytohormone. This herbicide is one of the most extensively employed compounds which has been adopted in selective control processes for numerous common weeds in agriculture, forestry, industrial weed control and aquatic ecosystems (USEPA, 2017). Nowadays, over 600 commercial products are available on the market containing 2,4-D as a pure chemical, either alone or in a mixture with other active ingredients (NCBI, 2019; Song, 2014).

Previous genotoxicological studies demonstrated that 2,4-D induces MNs formation in *in vitro* human lymphocytes (Holland et al., 2002; Zeljezic and Garaj-Vrhovac, 2004) as well as sister chromatid exchanges (SCEs) in circulating human lymphocytes (Korte and Jalal, 1982; Soloneski et al., 2007; Turkula and Jalal, 1985; Zeljezic and Garaj-Vrhovac, 2004) and in epithelial-like Chinese hamster ovary (CHO) cells (Galloway et al., 1987; González et al., 2005). However, Linnainmaa (1984) showed that 2,4-D did not modify the basal level of SCEs in the latter cell system. At chromosomal level, several

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investigations reported positive results in *in vitro* human lymphocytes (Korte and Jalal, 1982; Mustonen et al., 1986; Pilinskaia, 1974; Zeljezic and Garaj-Vrhovac, 2004) and CHO-treated cells with a metabolic activation system such as the microsomal hepatic S9 mix (Galloway et al., 1987). Finally, negative results were reported by Mustonen and collaborators (1986) when using rat lymphocytes either with or without microsomal hepatic S9 mix and by Gollapudi et al. (1999) when CHO cells were employed as the biotic matrix. A number of references reported the generation of DNA lesions estimated by the single cell gel electrophoresis (SCGE) methodology. Whereas Maire et al. (2007), González et al. (2005) and Sandal and Yilmaz (2011) reported a significant increase of DNA lesions when treated with the auxinic 2,4-D in Syrian golden hamster embryo (SHE) cells, CHO cells and *in vitro* human lymphocytes, respectively; Sandal and Yilmaz (2011) and Sorensen et al. (2005) demonstrated negative effects in human lymphocytes of non-smoker donors and CHO cells, respectively. Finally, the generation of chromosomal aberrations but the generation of MNs in peripheral circulating lymphocytes were observed in occupationally exposed individuals working with 2,4-D (IARC, 2018). Additionally, some reports suggest a positive relationship between exposure to 2,4-D and carcinogenesis in non-Hodgkin's lymphoma through occupational exposure to the herbicide (IARC, 2018). When cytotoxicity effects were evaluated, several findings are worth noting. According to Holland and co-workers (2002), 2,4-D provoked in *in vitro* circulating human lymphocytes an augment in the cell proliferation but in the mitotic index. Whereas González et al. (2005) reported no alterations in the replicative index and cell cycle progression in 2,4-D-exposed CHO-K1 cells, the mitotic index significantly decreased with respect to control values. Cytotoxicity induced by 2,4-D was also reported in several cell types including, *e.g.*, LAMA-84 and U937 (Venkov et al., 2000), CHO-K1 (González et al., 2005), cerebellar granule cells (De Moliner et al., 2002), human lymphocytes (Soloneski et al., 2007) and fibroblasts (Witte et al., 1996). Furthermore, it has been reported that the *in vitro* exposure to 2,4-D provoked apoptosis in hepatocellular carcinoma (HepG2) and cerebellar granule cells as well as human circulating lymphocytes (De Moliner et al., 2002; Kaioumova et al., 2001; Tuschl and Schwab, 2003). On the other hand, Maire et al. (2007) indicated that the herbicide did not produce apoptosis in SHE cells.

Whereas the cytokinesis-block micronucleus cytome (CBMN-cyt) bioassay is widely employed as a robust *in vitro* methodology for estimating DNA lesions at chromosomal level (Corvi et al., 2008; Fenech, 2007; Fenech and Morley, 1985; González et al., 2011; Nikoloff et al., 2012; OECD, 2016), the alkaline SCGE, also recognized as the comet assay, is one of the most frequently applied methodologies for determining both the induction of DNA injure and DNA repair processes in isolated cells (Cavaş and Könen, 2007; Nikoloff et al., 2012; Singh et al., 1988; Speit and Hartmann, 2006).

Actually, an extensive spectrum of cytotoxicity bioassays have been implemented to measure diverse cells' functions and are currently employed in different biological areas (Aslantürk, 2018). Among them, the neutral red uptake (NRU) and the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) bioassays are two of the most commonly employed techniques for the initial screening of basal cytotoxicity exerted by xenobiotics on a variety of cellular systems (Borenfreund et al., 1988; NASEM, 2015; Patravale et al., 2012). In particular, MTT assay evaluates metabolic activity and is the preferred method among others for measuring this end-point (Patravale et al., 2012). NRU bioassay estimates the capability of living cells to assimilate and accumulate the eurhodine neutral red dye in their lysosomes.

Overall, xenobiotic-induced toxicity leading to cell death can involve a necrosis or apoptosis mechanism. Initially, it was thought that necrosis was the predominant, if not the only, pathway implicated in cell death mediated by chemical toxicity. However, it is also well known that many xenobiotics induce cellular toxicity through an apoptotic pathway (Circu and Aw, 2010; Orrenius et al., 2011). The apoptogenic process was initially described by Kerr et al. (1972) as a normal and essential cellular machinery of programmed cell death involving typical biochemical and morphological events. Both the extrinsic mediated cell death receptor and the intrinsic mitochondrial pathways are the two major cellular paths linked with the apoptosis mechanism (Elmore, 2007). Accordingly, the toxicity of contaminants can exert its effects through a passive cell death namely necrosis, or by an activation of the apoptosis pathway (Asweto et al., 2017; Circu and Aw, 2010; Ilboudo et al., 2014; Morcillo et al., 2016; Pallepati and Averill-Bates, 2012).

The cyto- and genotoxic capability of 2,4-D as an a.i. and as a component of its commercial microformulation Dedalo Elite (30% a.i.) was assayed on CHO-K1 cells. We employed the CBMN-cyt, SCGE, MTT and NRU bioassays to estimate the cellular CHO-K1 response exerted by this herbicide. In addition, the cellular response in terms of exploring a plausible mechanism for the apoptosis exerted by the herbicide was also analyzed.

2. Materials and methods

2.1. Chemicals

2,4-dichlorophenoxyacetic acid (CAS 94-75-7; purity, 99%), cytochalasin B (CAS 14930-96-2), ethanol (EtOH; CAS 64-17-5), dimethyl sulfoxide (DMSO; CAS 67-68-5), neutral red dye (CAS 553-24-2), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; CAS 57360-69-7) and propidium iodide (PI; CAS 25535-16-4) were obtained from Sigma Chemical Co. (St. Louis, MO). Dedalo Elite (30% a.i.) was supplied by Red Surcos S.A. (Santa Fe, Argentina). Annexin V-FITC was obtained from Invitrogen Molecular Probes® (Carlsbad, CA). Whereas acetone (ACTN) was obtained from Merck KGaA (Darmstadt, Germany), bleomycin (BLM; Blocamycin®) was supplied by Gador S.A. (Buenos Aires, Argentina). All other chemicals and reagents of analytical grade were acquired from Sigma Chemical Co., unless otherwise indicated.

2.2. Cell cultures and 2,4-D treatment for the CBMN-cyt assay

CHO-K1 cells were acquired from American Type Culture Collection (Gaithersburg, MD) and maintained in Ham's F-10 medium plus 10% foetal bovine serum, 100 units penicillin/ml, 10 μg streptomycin/ml (both from Gibco, Grand Island, NY) and 8 μg tylosin/ml (TiloZur®, Weizur, Buenos Aires, Argentina) (37 °C, 5% CO_2 atmosphere). Cells were harvested by trypsinization after 2 days from plating in the culture flask, resuspended in Ham's F-10 medium and then plated onto sterilized 22 × 22 mm coverslips in 35 mm Petri dishes at 1.2×10^4 cells/3 ml of culture medium. Treatments with the a.i. and the microformulated product were realized 24 h after plating as suggested previously (González et al., 2006, 2007; Molinari et al., 2009; Soloneski and Larramendy, 2010). A stock solution of herbicide was performed in ACTN, and dilutions of 2,4-D were performed in culture media and then incorporated to cultures. ACTN was present in less than 1% (v/v) of cell assays. Dilutions of Dedalo Elite were directly prepared in culture medium. The pure compound and the microformulated product were prepared by incorporation of 100 μl into 2.9 ml of cultures allowing the herbicide to reach the required concentration within the range of 0.1–10 μg/ml. To determine the concentrations used in the CBMN-cyt assay, preliminary bioassays were performed according the recommendations proposed by the OECD (2016) selecting the range of concentrations based on cytotoxic response observed in NRU experiments after 24 h of treatment. Simultaneously with the treatments, 3 μg cytochalasin B/ml was incorporated into the cultures. The cultures were maintained for another 24 h (37 °C, 5% CO_2). Untreated, solvent vehicle-treated and 1.0 μg BLM/ml-treated cells were used as negative and positive controls, respectively, and run simultaneously with the herbicide-treated cultures. Culture medium pH was not affected by the treatments (range 7.2–7.4). The experiments were performed three

times. Identical batches of culture medium, serum and reagents were employed during the experiments.

2.3. CBMN-cyt assay

CBMN-cyt assay was conducted according to Fenech (2007) with minor modifications as reported elsewhere (González et al., 2011; Nikoloff et al., 2012). At least 1000 binucleated (BN) cells were blind-scored at 1000× magnification and the number of BN cells with zero, one, two or three MNs was analyzed in BN cells, whereas necrotic or apoptotic cells were not incorporated in the score according to Fenech (2007). At least, 500 viable cells per experimental point were scored and the nuclear division index (NDI) was analyzed following recommendations of Eastmond and Tucker (1989). The NDI value represents the proportion of nuclear divisions present in the system culture as follow: [M1 + (2× M2) + (3 × M3) + (4× M4)]/N, where M1 to M4 represents the number of cells with 1 to 4 nuclei, respectively, and *N* is the total number of cells.

2.4. Cell cultures and 2,4-D treatment for SCGE

Exponentially growing CHO-K1 cells were seeded in aliquots including 3.0×10^4 cells/ml and maintained for 90 min in 2 ml of culture media containing the test compounds (37 °C, 5% CO_2 atmosphere). 2,4-D and its commercial microformulation Dedalo Elite were employed at a final concentration between 1 and 10 μg/ml. To determine the concentrations used in the SCGE assay, preliminary viability assay after a short pulse treatment lasting 90 min was performed showing that this exposure time was sufficient to yield observable genotoxic effects. The amount of ACTN in cell assays was less than 1% (v/v). Simultaneously with the herbicide-treated cultures, negative controls (untreated cells and solvent vehicle ACTN-treated cells) and positive control (1.0 μg BLM/ml) were performed. None of the treatments produced pH variations in the culture medium (pH 7.2–7.4). The SCGE and cell viability bioassays were run immediately after a 90 min pulse period. Each experiment was repeated three times and the cultures were performed in duplicate for each experimental point.

2.5. SCGE assay

The alkaline adaptation of the SCGE bioassay was carried out following Singh et al. (1988) with minor modifications reported elsewhere (Molinari et al., 2009; Nikoloff et al., 2012). The extent of DNA lesions was analyzed by visual scoring of 100 randomly selected and non-overlapping nucleoids. The nucleoids were sorted into five categories (0-I: undamaged; II: minimum damage; III: medium damage; IV: maximum damage) (Cavaş and Könen, 2007). Results are expressed as the mean number of damaged nucleoids (sum of classes II, III and IV) and the mean comet scored for each treatment group. Additionally, a genetic damage index (GDI) was calculated for each test compound using the formula GDI = [(I) + 2(II) + 3(III) + 4(IV)]/$N_{(I\text{-}IV)}$, where I-IV represent the nucleoid type and N_I–N_{IV} represent the total number of nucleoids scored (Pitarque et al., 1999).

2.6. Cell viability assay

Viability of cells was measured employing the ethidium bromide/acridine orange assay (McGahon et al., 1995). All experiments were performed in triplicate. Viability was measured at 90 min after herbicide treatment. At least, 500 cells were registered per experimental point, and results were expressed as the percentage of viable cells among all cells.

2.7. NRU and MTT assays

Cytotoxicity of 2,4-D and Dedalo Elite was monitored as previously described by Borenfreund and Puerner (1985) and Kosmider et al. (2004) for the NRU and MTT bioassays, respectively. Briefly, CHO-K1 cells were seeded on a 96-well microplate at a density of 1×10^5 cells per well. After 24 h, the culture medium was removed and then the cells were incubated with 2,4-D or Dedalo Elite (0.1–100 μg/ml) in Ham's F-10 medium for an additional period of 24 h. EtOH (5.0%)-treated and ACTN (0.1%)-treated cells were employed as positive and solvent vehicle controls, respectively. Experiments were performed in triplicate. Absorbance at 550 nm was estimated with a microplate spectrophotometer (Sunrise Absorbance Reader, Tecan Austria GmbH, Salzburg, Austria). Results were expressed as the mean percentage of lysosomal and mitochondrial activities.

2.8. Cell cultures and 2,4-D treatment for apoptosis analysis

CHO-K1 cells were seeded in 35 mm Petri dishes (2.5×10^5 cells/ml) and exposed for 24 h after plating (37 °C, 5% CO_2). Either 2,4-D or Dedalo Elite were incorporated into cultures to reach the final concentrations of 4 and 10 μg/ml. Negative controls (untreated cells and ACTN solvent vehicle-treated cells) were run simultaneously with 2,4-D-exposed cultures. After treatment, cells were processed for flow cytometry using Annexin V-FITC following the manufacturer's instructions to determine phosphatidyl serine externalization and cellular membrane integrity. Viability was determined by flow cytometry (BD FACSAria™ Fusion; Biosciences, San Jose, CA). Cells were designated as viable (annexin V negative/PI negative), early-stage apoptotic (annexin V positive/PI negative), late-stage apoptotic (annexin V positive/PI positive) and necrotic (annexin V negative/PI positive).

2.9. Statistical analysis

The data were analyzed using Statgraphics 5.1 Plus software. SCGE and MNs values were compared by applying one-way ANOVA. Variables were tested for normality with the Kolmogorov–Smirnov test and homogeneity of variances between groups was verified by the Levene test. Pairwise comparisons between the different groups were made using the *post hoc* least significant difference test. Differences in GDI, NDI, apoptosis and viability in treated and control cells were evaluated by χ^2 test. CBMN-cyt data were analyzed by χ^2 test. The two-tailed Student's *t*-test was used to compare MTT and NRU data between treated and negative control groups. To check for a concentration-dependent response to the treatments, Spearman's rank order linear correlation analysis was also performed. The chosen level of significance was 0.05 unless indicated otherwise.

3. Results

Table 1 depicts the results obtained from the analysis of 2,4-D- and Dedalo Elite-induced MNs in CHO-K1 cells. Increased level of MNs was evidenced in those BLM-treated cells when compared to negative control values ($p < 0.001$). ACTN did not alter MNs frequency compared with negative control values ($p > 0.05$). A significant induction of MNs was observed after 10 μg 2,4-D/ml ($p < 0.01$) and 2 μg Dedalo Elite/ml ($p < 0.001$) treatments. A correlation test revealed that the proportion of MNs augmented as a positive function of the concentration of 2,4-D ($r = 0.98$, $p < 0.001$) or Dedalo Elite ($r = 1$, $p < 0.001$) titrated into cultures (Table 1). In addition, the frequency of MNs enhanced by 10 μg 2,4-D/ml treatment was due to an augment in the proportion of BN cells presenting one ($p < 0.05$) and three MNs ($p < 0.01$). Finally, in 2 μg Dedalo Elite/ml-treated cultures the proportion of cells with one and two MNs was significantly higher than the negative control values ($p < 0.01$). A noticeable modification in cell morphology not allowing MN quantification was observed in 4–10 μg Dedalo Elite/ml-treated cultures.

NDI values induced by 2,4-D and Dedalo Elite are showed in Table 1. No variations in the NDI were observed in 2,4-D-exposed

Table 1
Micronuclei (MNs) induction and nuclear division index (NDI) values for control, 2,4-D-, and 2,4-D-based technical formulation Dedalo Elite-treated binucleated cytokinesis-blocked Chinese hamster ovary (CHO-K1) cells[a].

Compound	Concentration	MN frequencies[b]			Micronucleated cell numbers[c]									NDI[d]		
	(μg/ml)				1 MN			2 MN			3 MN					
Negative control		19.73	±	0.72	19.09	±	0.54	0.64	±	0.18	0.00	±	0.00	2.13	±	0.05
ACTN[e]		22.14	±	0.55	20.38	±	0.63	1.76	±	0.52	0.00	±	0.00	2.13	±	0.01
2,4-D	0.1	19.61	±	0.32	18.95	±	0.44	0.66	±	0.38	0.00	±	0.00	2.08	±	0.01
	1	20.08	±	0.24	19.42	±	0.15	0.66	±	0.19	0.00	±	0.00	2.14	±	0.03
	2	22.31	±	1.34	21.37	±	1.42	0.94	±	0.30	0.00	±	0.00	2.02	±	0.01
	4	23.75	±	1.41	22.47	±	1.38	1.28	±	0.48	0.00	±	0.00	2.02	±	0.01
	6	25.11	±	1.68	23.21	±	1.57	1.90	±	0.53	0.00	±	0.00	2.08	±	0.02
	10	31.87	±	3.22**	29.60	±	2.58*	1.96	±	0.67	0.31	±	0.18**	2.02	±	0.05
Dedalo Elite	0.1	19.74	±	0.93	18.09	±	0.81	1.65	±	0.19	0.00	±	0.00	2.04	±	0.08
	1	24.40	±	0.28	23.74	±	0.30	0.66	±	0.19	0.00	±	0.00	1.83	±	0.03*
	2	35.76	±	5.84***	33.01	±	5.75**	2.75	±	1.02**	0.00	±	0.00	1.69	±	0.05**
	4	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	1.05	±	0.00***
	6	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	1.03	±	0.01***
	10	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	1.02	±	0.01***
BLM[f]		76.63	±	2.35***	64.43	±	3.31***	9.72	±	1.20***	2.48	±	0.29***	1.97	±	0.01***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significant differences with respect to control values.
ND, not determined.
[a] CHO-K1 cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later.
[b] Results are presented as mean MN/1000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.
[c] Results are presented as number of cells carrying 1, 2 or 3 MNs among 3000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.
[d] Results are presented as mean value of pooled data from three independent experiments ± S.E. of the mean.
[e] Acetone (ACTN, 0.1%) was used as 2,4-D-solvent control.
[f] Bleomycin (BLM, 1.0 μg/mL) was used as positive control.

cultures compared to negative control values ($p > 0.05$). Conversely, a high level of cytotoxicity due to a significant decrease of the NDI was observed in 1–10 μg Dedalo Elite/ml-treated cells ($0.05 > p < 0.001$). The NDI diminished in a concentration-dependent manner ($r = -0.98$, $p < 0.001$).

Table 2 presents the SCGE bioassay results obtained in CHO-K1 cells after treatment of both compounds within a 1–10 μg/ml concentration range. Pooled data are presented for negative control cultures since no differences in the viability and undamaged/damaged cell frequencies between negative controls (untreated and ACTN-treated cells) were observed. BLM treatment produced a significant increase in the proportion of damaged cells and an increment in the GDI values compared to the negative control values ($p < 0.001$), although this enhancement did not significantly alter cell viability. Moreover, 6 and 10 μg 2,4-D/ml treatments induced an enhancement of GDI values due to an increase of type III ($p < 0.05$ and 0.001) and IV nucleoids ($p < 0.001$). Viability was not altered in any of the 2,4-D treatments ($p > 0.05$). The GDI values achieved in the 4 and 6 μg Dedalo Elite/ml treatments varied significantly from those of the control ($p < 0.001$). The increase in the GDI ratio of both concentrations was due to an enhancement of type III

Table 2
Analysis of DNA damage measured by SCGE assay in Chinese hamster ovary (CHO-K1) cells exposed during 90 min to 2,4-D and 2,4-D-based technical formulation Dedalo Elite.

Compound	Concentration	Proportion of damaged nuclei (%)[a]					DNA damage (%)[b] (II + III + IV)			GDI[c]			Viablility (%)[b]		
	(μg/ml)	Type 0	Type I	Type II	Type III	Type IV									
Negative control		0.00	88.20	9.94	1.55	0.31	11.8	±	2.64	1.14	±	0.03	99.51	±	0.40
ACTN[d]		0.00	86.37	9.63	2.33	1.19	13.62	±	4.50	1.19	±	0.12	99.13	±	0.10
2,4-D	1	0.00	85.44	11.11	2.30	1.15	14.56	±	2.06	1.19	±	0.05	98.10	±	1.90
	2	0.00	82.20	14.38	2.06	1.37	17.81	±	5.72	1.23	±	0.09	98.60	±	0.51
	4	0.00	83.61	8.70	2.67	5.02***	16.39	±	3.82	1.29	±	0.12	99.55	±	0.45
	6	0.00	76.09	13.77	4.71*	5.43***	23.91	±	2.43***	1.39	±	0.12*	97.83	±	2.17
	10	0.00	73.65	11.49	6.08***	8.78***	26.35	±	3.94***	1.50	±	0.11**	98.57	±	0.44
Dedalo Elite	1	0.00	85.44	10.34	2.30	1.92**	14.56	±	1.34	1.21	±	0.03	97.10	±	0.70
	2	0.00	78.47	14.60	3.65	3.28***	21.53	±	5.25**	1.32	±	0.10	94.25	±	1.47
	4	0.00	52.14***	14.53	8.12***	25.21***	47.86	±	3.57***	2.06	±	0.05***	76.88	±	3.22*
	6	ND	ND	ND	ND	ND	ND	±	ND	ND	±	ND	ND	±	ND
	10	ND	ND	ND	ND	ND	ND	±	ND	ND	±	ND	ND	±	ND
BLM[e]		0.00	31.72***	26.49***	7.09***	34.70***	68.28	±	1.54***	2.45	±	0.09***	96.17	±	0.01

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significant diferences with respect to control values.
ND, not determined.
[a] I–IV indicate grades of DNA damage as mean values of pooled data from three independent experiments.
[b] Results are presented as mean values of pooled data from three independent experiments ± S.E. of the mean.
[c] Genetic damage index (GDI).
[d] Acetone (ACTN, 0.1%) was used as solvent control.
[e] Bleomycin (BLM, 1.0 μg/ml) was used as positive control.

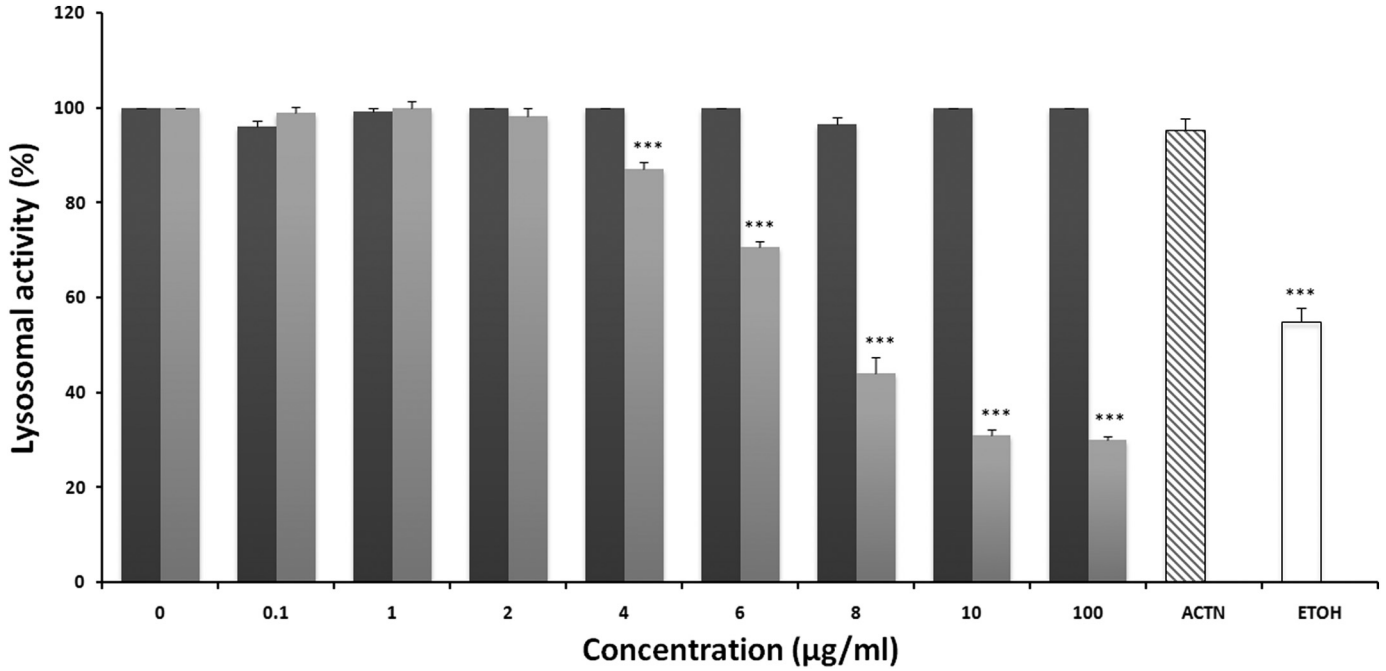

Fig. 1. Lysosomal activity assessed by NRU assay in 2,4-D- (dark grey bars) and Dedalo Elite- (light grey bars) treated CHO-K1 cells. 5% ethanol-treated (white bar) and 0.1% acetone-treated (striped bar) cells were used as positive and solvent controls, respectively. *** $p < 0.001$; significant differences with respect to control values.

and IV nucleoids ($p < 0.001$) with a simultaneous diminution of type I nucleoids ($p < 0.001$). Additionally, a decline in cell viability was found in those cultures exposed to 4 µg Dedalo Elite/ml ($p < 0.05$). In cultures exposed to 6 and 10 µg Dedalo Elite/ml, an evident diminution in the proportion of viable cells was found which did not allow monitoring the proportion of nucleoids. The results showed that the proportion of damaged cells increased in a positive concentration-dependent manner in cultures that were treated with either 2,4-D ($r = 0.93$, $p < 0.001$) or Dedalo Elite ($r = 0.92$, $p < 0.001$) (Table 2).

Values obtained from the NRU are shown in Fig. 1. A significant induction of cytotoxicity was observed in EtOH-treated cultures (positive control) in comparison with negative controls ($p < 0.001$). Lysosomal activity was not significantly altered by any of the 2,4-D concentrations assayed ($p > 0.05$). Contrarily, experiments demonstrated a significant diminution of the lysosomal activity when cells were treated with 4–100 µg Dedalo Elite/ml ($p < 0.001$). The lysosomal activity diminished in a concentration-related manner following exposure to Dedalo Elite titrated into cultures ($r = -0.60$, $p < 0.001$). Overall, the NRU revealed that Dedalo Elite induced a higher cytotoxicity than 2,4-D did.

Values obtained from the MTT experiments are depicted in Fig. 2. EtOH-treated cultures induced a significant diminution in the energetic cell metabolism in CHO-K1 cells compared with the negative control ($p < 0.001$). Mitochondrial activity of CHO-K1 cells treated with 10 and 100 µg 2,4-D/ml was diminished in regard to control values ($p < 0$ .05). Dedalo Elite-treated cells showed an inhibition of mitochondrial activity in the exposure range of 4–100 µg/ml ($p < 0.001$). A correlation analysis revealed that the energetic cell metabolism diminished as a negative function of the concentration of 2,4-D ($r = -0.91$, $p < 0.001$) and Dedalo Elite ($r = -0.57$, $p < 0.05$). Overall, both the NRU and MTT bioassays showed that the commercial microformulation product Dedalo Elite induced a higher cytotoxicity than the active ingredient 2,4-D in cells exposed within the 0.1–100 µg/ml concentration range (Figs. 1 and 2).

Flow cytometry analyses of cells exposed to 4 and 10 µg/ml 2,4-D and Dedalo Elite are depicted in Fig. 3, respectively. The proportions of alive, early and late apoptotic as well as necrotic cells evidenced in the ACTN-treated cultures were consistent with the observed frequency for the negative control cultures, and thus pooled data are presented for the control cultures. Both 4 and 10 µg 2,4-D/ml concentrations increased in a significant manner the proportions of early and late apoptotic cells ($p < 0.001$) (Fig. 3). Similarly, when 4 and 10 µg Dedalo Elite/ml concentrations were employed, a statistically significant enhancement in early and late apoptosis was achieved ($p < 0.001$) (Fig. 3). Both compounds, regardless of the concentration, were unable to exert an increment in the proportion of necrotic cells compared to the negative control values ($p > 0.05$) (Fig. 3). A correlation analysis showed that the proportion of apoptogenic cells increased as a function of the concentration of 2,4-D or Dedalo Elite ($r = 0.99$, $p < 0.001$) (Fig. 3).

4. Discussion

We comparatively analyzed the *in vitro* cytotoxic and genotoxic properties exerted by the pure 2,4-D and its technical microformulation Dedalo Elite on CHO-K1 mammalian cells after an acute 24 h treatment. CBMN-cyt and SCGE bioassays were selected to estimate the level of DNA lesions exerted by both compounds. In addition, NDI, NRU and MTT bioassays as well as the apoptogenic process were further quantitatively determined.

Results revealed that both the pure herbicide and the 2,4-D-based microformulated product augmented the frequency of MNs at 10 and 2 µg/ml, respectively. Furthermore, higher concentrations of Dedalo Elite, namely 1–10 µg/ml, resulted in cytotoxicity revealed by a reduction in NDI as well as a serious impairment in mitochondrial and lysosomal cellular activities. Whereas increased GDI in CHO-K1 cells was observed when 6 and 10 µg 2,4-D/ml was assayed, GDI was increased in treatments employing 4 and 6 µg Dedalo Elite/ml. Finally, the results show that both the 2,4-D and its microformulated product were able to enhance apoptotic cell death after a period of 24 h treatment.

In accordance with the U.S. EPA (2019), 2,4-D and its related compounds are at present undergoing a registration review process to re-evaluate their safety for humans and the biota. However, it has been ranked by the WHO (2009) as a moderately hazardous (Class II)

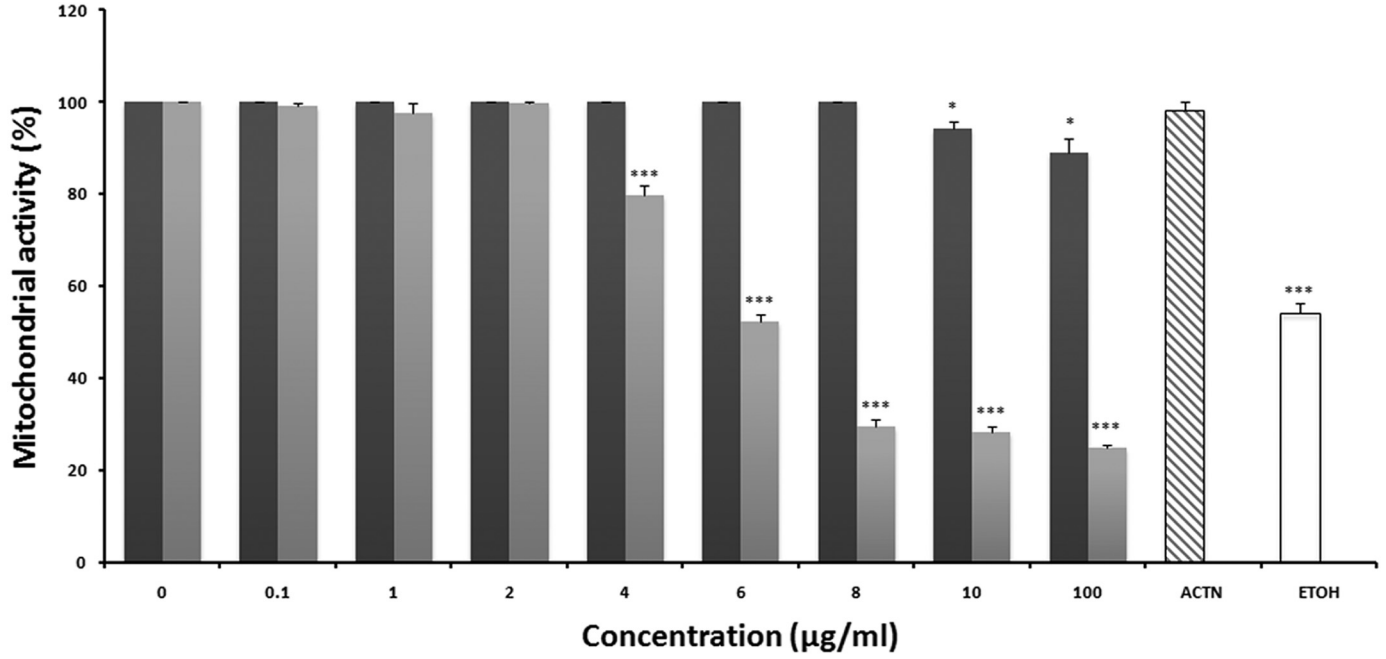

Fig. 2. Cellular metabolism inhibition evaluated by MTT assay in 2,4-D- (dark grey bars) and Dedalo Elite- (light grey bars) treated CHO-K1 cells. 5% ethanol-treated (white bar) and 0.1% acetone-treated (striped bar) cells were used as positive and solvent controls, respectively. * $p < 0.05$; *** $p < 0.001$; significant differences with respect to control values.

compound being incorporated in Category II-III by the U.S. EPA (2005). Concerning its carcinogenic properties, 2,4-D has been positioned by the U.S. EPA into Group D, "agents without adequate data either to support or refute human carcinogenicity" (USEPA, 2005). However, recently the International Agency for Research on Cancer (IARC) ranked the herbicide into Group 2B highlighting the herbicide as a "possibly carcinogenic compound to humans" (IARC, 2018). While considerable data are accessible about the environmental and ecological profile of 2,4-D (USEPA, 2019), information on 2,4-D-induced genotoxicity and/or cytotoxicity remains insufficient or even contradictory, and thus its health risks are still not totally understood (USEPA, 2019). Knowledge of the early biological response associated with DNA-damaging compounds is crucial, since potential mutagens/carcinogens could be related with increased cancer susceptibility through diverse mechanisms of action (Gagné, 2014) as IARC clearly highlighted (IARC, 2018). It is well known that the determination of the genotoxic profile of pesticides is crucial to estimate the carcinogenic/mutagenic potential for target and non-target exposed organisms. Our results demonstrate that concentrations of 10 µg 2,4-D/ml as well as 2 µg Dedalo Elite/ml increased MNs on mammalian CHO-K1 cells. In our study, the concentration range employed includes the 4 µg 2,4-D/ml concentration that represents the lowest-observed-adverse-effect level estimated in both rats and mice after 2-year oral administration according to the United Nations reports (FAO, 1988; WHO, 2003).

Regarding MN induction, so far the available literature shows inconclusive results. Years ago, Holland et al. (2002) revealed the induction of MNs in peripheral human lymphocytes when incubated with 2,4-D and a 2,4-D-based product with and without S9 mix complex. Similarly, Zeljezic and Garaj-Vrhovac (2004) also reported that concentrations of 0.4 and 4 µg 2,4-D/ml contained in the commercial presentation Deherban A® augmented the frequencies of chromatid and chromosome breaks, MNs and nuclear buds in *in vitro* circulating human lymphocytes with and without S9 mix complex. However, Mustonen et al. (1986) revealed that whereas 2,4-D did not generate chromosomal instability, concentrations of 100 µg/ml of the 2,4-D-based formulated product Vesakontuho Tasku, augmented the proportion of chromosome breaks in *in vitro* peripheral human lymphocytes. Gollapudi et al. (1999) found that 2,4-D was neither clastogenic nor mutagenic in primary cultures of rat lymphocytes and CHO cells. Finally, Galloway et al. (1987) and Turkula and Jalal (1985) described 2,4-D induced SCEs in mammalian cells such as human peripheral lymphocytes and CHO cells incubated with S9 mix. Our present results reveal the capability of the auxinic herbicide to increase the proportion of MNs *in vitro* and corroborate the weak genotoxicity of pure 2,4-D previously reported by other authors. In our current experimental design, MN induction was exerted by treatment at low concentrations of Dedalo Elite but not when exposed to the same equal concentration of the active ingredient. Additionally, the MN assay approach showed that higher concentrations of 4 µg Dedalo Elite/ml induced remarkable cytotoxicity demonstrated by alterations in the NDI. This result suggests that carrier solvents present in the trade herbicide may be responsible for the genomic instability pattern also observed by other authors when employing comparatively pure compounds and formulated products in genotoxicity studies (González et al., 2005; Molinari et al., 2009; Nikoloff et al., 2012; Soloneski et al., 2007; Zeljezic and Garaj-Vrhovac, 2004).

Despite the extensive use of 2,4-D, the capability of this chemical to exert DNA damage estimated by SCGE bioassay is not completely documented. The results presented here revealed that both 2,4-D and its microformulated product exerted a statistically significant augment in primary DNA damage in CHO-K1 cells. We observed that whereas 2,4-D gave rise to an increase in the proportion of damaged cells when 6 and 10 µg/ml were employed, a concentration range of 2–6 µg/ml 2,4-D-based formulated product provoked a statistically significant increment in the DNA damage. In concordance with the genotoxicity pattern found in our current investigation, González et al. (2005) reported the capability of 2,4-D to extend the ratio of damaged CHO-K1 cells over control values. González et al. (2005) showed that both the a.i. as well as the commercial amine variant 2,4-D DMA induced comparable levels of DNA lesions following a 90 min pulse exposition for all concentrations within the range of 2–10 µg/ml. Moreover, the authors demonstrated that regardless of the concentration, even in the negative controls, the percentage of damaged cells was always higher than those shown in the current study. The difference between our present findings

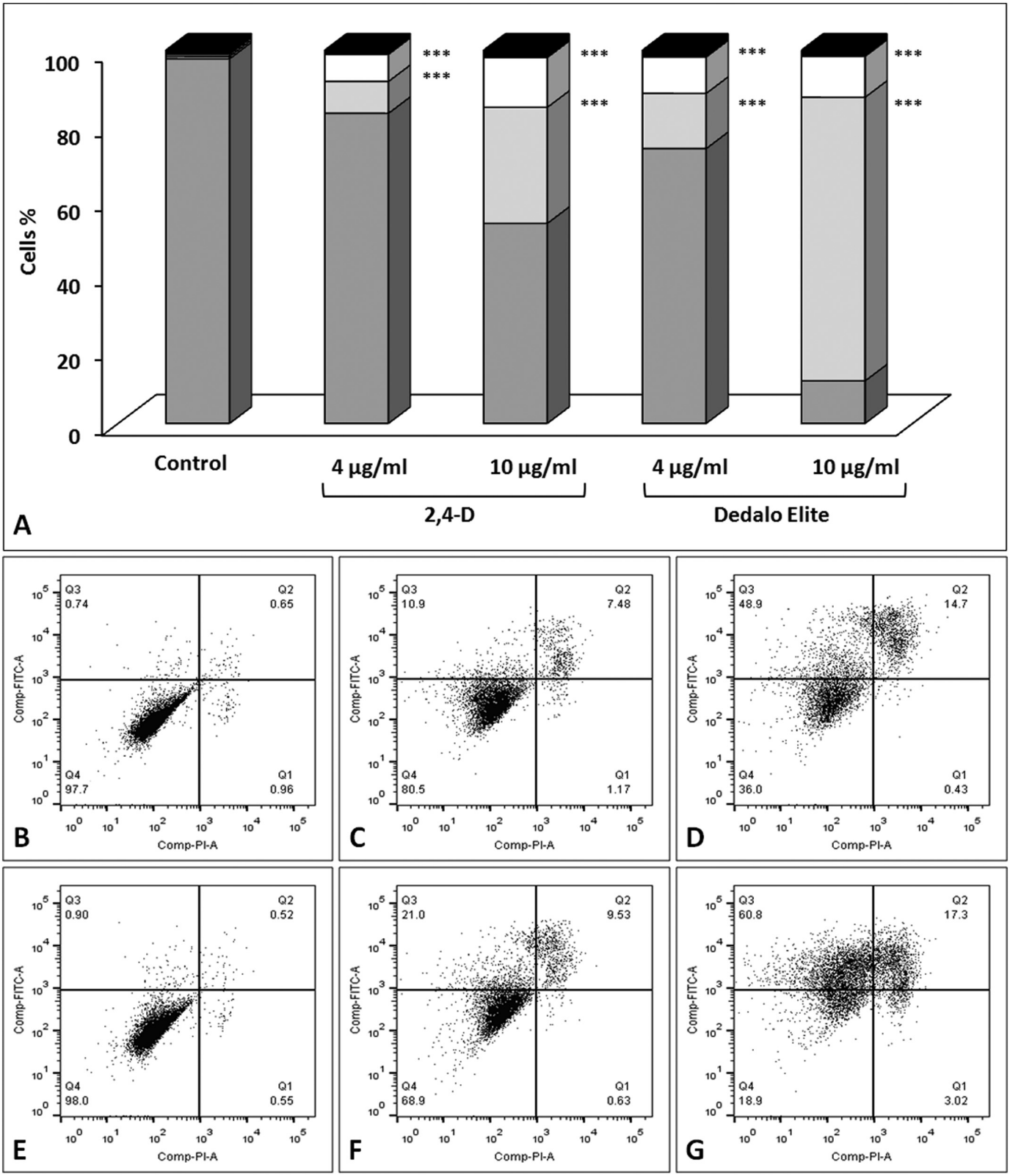

Fig. 3. 2,4-D and the 2,4-D-based commercial formulation Dedalo Elite induce apoptosis in CHO-K1 cells. Cells were treated with the test compounds and processed 24 h later with Annexin V-FITC/PI staining for quantification of the incidence of apoptosis by flow cytometry. The percentages of cells from the three independent experiments after flow cytometry analysis are shown (A). Live (dark grey bar sections), early apoptotic (light grey bar sections), late apoptotic (white bar sections), and necrotic cells (black bar section) among all cells from 2,4-D- and Dedalo Elite-treated cell cultures are shown. Representative dot plot analyses showing (B, E) control, (C) 4 µg/ml 2,4-D-treated, (D) 10 µg/ml 2,4-D-treated, (F) 4 µg/ml Dedalo Elite-treated, (G) 10 µg/ml Dedalo Elite-treated cultures. $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values.

and those of González et al. (2005) may be related to the different comet grading for analyzing DNA damage and the use of a different 2,4-D-based formulated product. In the previous study, cellular nucleoids were analyzed taking into account the width and length of the comets in 50 randomly selected nucleoids per experimental point and graded into three categories of damage according to Lebailly et al. (1997). In our investigation we analyzed the proportions of five categories according to the DNA migration of nucleoids as suggested by Cavaş and Könen (2007). Finally, the different level of DNA-damaging activity observed when employing the SCGE methodology between the two different commercial formulations employed could be attributable to the different composition of the products. Whereas González et al. (2005) employed 2,4-D as dimethylamine salt, in our present study we employ the acid form of 2,4-D contained in the Dedalo Elite microformulation. These results indicate the differential level of toxicity exerted by the two herbicide formulations, highlighting the necessity to focus on the safety profile of commercial pesticides rather than active ingredients in agreement with the recommendations of the U.S. EPA (2010).

In this study, we observed that the alkaline version of the SCGE was more sensitive than the MN bioassay. For the SCGE assay, the lowest-observed-adverse-effect levels were 6 and 2 μg/ml for 2,4-D and Dedalo Elite, respectively, whereas 10 μg 2,4-D/ml and 2 μg Dedalo Elite/ml were required to exert an increase of MN in CHO-K1 cells. In agreement, several reports have suggested that, in general terms, SCGE assay is more sensitive than MN bioassay for estimating DNA alterations exerted by genotoxic compounds, including herbicides (Nikoloff et al., 2012), insecticides (Feng et al., 2005), cobalt powder, tungsten carbide and cobalt-tungsten carbide (Goethem et al., 1997), waste disposal sites (Hartmann et al., 1998) and antibiotics (Leandro et al., 2013), among others. Our observations could be explained by considering the nature of the bioassay employed. The different sensitivity between the SCGE and the MN tests could be related to the different level of DNA damage that the SCGE and cytogenetic bioassay identify. Whereas the SCGE bioassay recognizes essentially direct DNA single-strand breaks and alkali-labile sites, the MN test identifies fixed structural and/or numerical chromosomal modifications that persist throughout the subsequent mitosis (Gagné, 2014). However, another plausible explanation for our observation could also be suggested. In order for a MN to be detected, cells must undergo at least one complete cell cycle after DNA injure. Thus, the possibility that DNA lesions could experience a repair process to ensure genome stability during the 24 h exposure period we employed in the MN assay could not be discarded, a process that cannot be achieved during the short 90 min pulse treatment employed for SCGE assay.

Cytotoxic tests have proved the most valuable methodologies to elucidate the cellular biochemical or metabolic pattern concerned with the mechanisms of action of numerous compounds such as genotoxic, cytotoxic and oxidative stressors, among others (Mazzotti et al., 2001). We analyze the early cytotoxic response exerted by 2,4-D and its microformulated product by NRU and MTT bioassays. Overall, our results show that Dedalo Elite causes a similar cytotoxic pattern in CHO-K1 cells by increasing lysosomal damage and reducing mitochondrial metabolism. However, the MTT but the NRU bioassay was able to reveal cytotoxicity in 2,4-D-treated-CHO-K1 cells after herbicide exposure when the highest doses (10 and 100 μg/ml) were assayed. It can be suggested that this result may be due to a higher sensitivity in the mitochondrial functionality and/or by decreasing lysosomal activity. Years ago, Tuschl and Schwab (2003) reported cytotoxic effects by disruption of the mitochondrial membrane potential when human hepatoma HepG2 cells were treated with the pure 2,4-D. Our results support this observation.

The present study revealed that 2,4-D and its commercial microformulation Dedalo Elite enhance the apoptotic occurrence. An augment in both early and late apoptogenic cells for both compounds was observed in CHO-K1 cells when the auxinic herbicide was employed at both 4 and 10 μg/ml. Our results reveal for the first time an apoptogenic induction when CHO-K1 cells were exposed to either 2,4-D or its commercial microformulation Dedalo Elite. After 4 μg 2,4-D/ml exposure, apoptotic cells (both early and late) increased approximately 15% over control values whereas a 24% increase of apoptogenic cells was evidenced following 10 μg 2,4-D/ml exposure. However, when 10 μg Dedalo Elite/ml was assayed, apoptotic cells (both early and late) increased approximately 44% over control values, whereas an 87% increment of apoptogenic cells was observed after 10 μg/ml exposure. Finally, our results show that the microformulated product Dedalo Elite was more toxic than the same concentration of the pure ingredient 2,4-D. Overall, our results reveal an approximately 1.55- and 1.95-fold increase in apoptotic cell frequencies after 4 and 10 μg Dedalo Elite/ml treatment, respectively, compared to that induced by the active ingredient 2,4-D. Thus, it could be suggested that the impossibility of quantifying DNA lesions by the comet assay due to the marked cytotoxicity found in cells exposed to 6 and 10 μg Dedalo Elite/ml could be explained by the high level of apoptotic cells present in the culture. Our results demonstrating 2,4-D-iduced apoptosis are in agreement with those of Tuschl and Schwab (2003). The authors reported an apoptotic effect of 2,4-D by disruption of the mitochondrial membrane potential in HepG2 cells exposed to 4–8 mM. Similarly, Di Paolo et al. (2001) reported apoptogenic induction exerted by 2,4-D in mitochondrion from rat exposed hepatocytes. Kaioumova et al. (2001) have also reported apoptotic induction exerted by 2,4-D by disruption of the mitochondrial transmembrane potential in *in vitro* human lymphocytes. Furthermore, whereas very high concentrations of the pure herbicide (from 2 to 4 mM) were necessary to promote a low level of apoptosis in SHE cells, no apoptogenic effect was observed at 2,4-D concentrations exerting morphological cellular transformation in the same cell line (Maire et al., 2007). Finally, taking into account that 2,4-D exerts cytotoxicity by affecting the mitochondrial functionality as previously stated, our investigation could suggest that a mitochondrial pathway could be involved in the apoptogenic process exerted by 2,4-D.

We focused our study on assaying both the a.i. and the commercial microformulated presentation taking into account that workers, using different types of agrochemicals, are mostly exposed to commercial formulations rather than the pure herbicides. Our results clearly point out that regardless of the bioassay we employed, the microformulated product Dedalo Elite was shown to be more toxic than the pure ingredient 2,4-D at an equivalent dose. Numerous studies report that the toxicity of excipients such as, *e.g.*, solvents, surfactants and reactants, among others, contained in the marketed products could exert additional toxicity than the a.i. itself, and could influence, then, the overall toxicity of the manufactured herbicide (González et al., 2006; Molinari et al., 2009; Soloneski et al., 2007; Soloneski and Larramendy, 2010). Our current observations are in total concordance with this concept.

Although the employment of pesticide-based microformulations is actually considered much more safe in plant and food protection applications, some reports have shown that nano- and micro-pesticides are able to induce higher toxicity levels than conventional products (Gonzalez et al., 2008). At present, there are limited data indicating whether or not nano- and micro-pesticide-based formulations increase toxicity. However, some studies dealing with the toxicity of pesticide-based microformulation properties have been reported. Studies reported by the Pesticide Action Network Database (PPDB, 2007) reported greater toxicity exerted by the pre-emergent herbicide pendimethalin in nanoscale hydrogel carriers. Similarly, it has been revealed that nano-imidacloprid exerts greater toxicity than conventional insecticide on the medicinal insect *Martianus dermestoides* (Coleoptera, Tenebrionidae) (Guan et al., 2008). When γ-cyhalothrin was delivered in nanosized capsules to the freshwater macroinvertebrate *Ceriodaphnia dubia* (Cladocera, Daphniidae), Slattery et al. (2019) reported that the encapsulated pesticide influences the toxicity on the branchiopod in a size-dependent manner. In genotoxic studies employing *Drosophila melanogaster* as biotic matrix, Demir et al. (2015) reported significant

increases in the DNA strand breaks estimated by SCGE bioassay after exposure to synthetic amorphous silica.

Today, newly synthesized pesticide formulations have to pass several examination processes before they can be commercially employed in their safe form. The results we obtained clearly highlight that the active ingredient 2,4-D and its technical microformulation Dedalo Elite increase the genomic instability revealed by SCGE assay and MN formation in CHO-K1 mammalian cells. In addition, 2,4-D and Dedalo Elite were found to be cytotoxic by diminishing the NDI as well as by enhancing the apoptotic process. Our results suggest that a more exhaustive evaluation of the toxicity of commercially microformulated pesticide must be carried out in order to explain the toxicity of this new generation of compounds. Finally, due to the worldwide extensive employment of the herbicide 2,4-D for weed management in modern agriculture production, it remains essential to improve the current understanding of 2,4-D toxicity properties towards the biota and human health, factors that may lead to a more comprehensive 2,4-D pesticide regulation.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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