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Assessment of DNA damage, cytotoxicity, and apoptosis in human hepatoma (HepG2) cells after flurochloridone herbicide exposure

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ABSTRACT

In vitro effects of flurochloridone (FLC) and its formulations Twin Pack Gold® [25% active ingredient (a.i.)] and Rainbow® (25% a.i.) were evaluated in HepG2 cells. Whereas cytokinesis-blocked micronucleus cytome (CBMN-cyt) and single-cell gel electrophoresis (SCGE) assays were employed for genotoxicity, MTT, neutral red, and apoptosis detections were used for cytotoxicity evaluation. Activities were tested within the concentration range of 0.25–15 μg/ml FLC. Results demonstrated that neither FLC nor Rainbow® was able to induce MNs. On the other hand, 5 μg/ml Twin Pack Gold® only increased MN frequency. Furthermore, 10 and 15 μg/ml of both formulations resulted in cellular cytotoxicity demonstrated by alterations in the nuclear division index and cellular death. A marked increase in the genetic damage index was observed after treatment with all compounds. SCGE assay appeared to be more sensitive bioassay for detecting primary DNA strand breaks at lower concentrations of FLC than did MN. Our results reveal that FLC and its two formulations trigger apoptosis on HepG2 cells. The results represent the first experimental evidence of the *in vitro* apoptogenic role exerted on mammalian cells by FLC and the FLC-based formulations Rainbow® and Twin Pack Gold®, at least on HepG2 cells.

1. Introduction

Flurochloridone (FLC) is a pyrrolidone selective herbicide applied worldwide, including in Argentina, to control many broad-leaf weeds and annual grasses in several crops. FLC interferes with carotenoid biosynthesis, causing bleaching of the leaves (Klíčová et al., 2002; Lay et al., 1985). The exact mechanism(s) of action of the herbicide are not totally clarified. However, it has been recently demonstrated that FLC exposure caused important biochemical changes on leaves of *Vicia sativa*, increasing the activities of glutathione-*S*-transferases and the content of tripeptide glutathione and a reduction of glutathione reductase (Kaya and Yigit, 2012). Accordingly, the involvement of reactive oxygen species (ROS) in the mechanism(s) of action has been suggested (Kaya and Yigit, 2012).

According the European Food Safety Authority, FLC has no genotoxic, carcinogenic or neurotoxic potential, and it is unlikely to be genotoxic (EFSA, 2013). However, while considerable information is accessible about the environmental and ecological effects of FLC (EFSA, 2013; WHO, 1990, 2009), scarce information on genotoxicity and/or cytotoxicity has been reported. Yüzbasioglu et al. (2003) reported previously that FLC induced abnormal cell-cycle progression and cellular mitodepressive activity in *Allium cepa* root meristematic cells. c-Metaphase, multipolarity, polyploidy, chromosome lagging, chromosomal stickiness, chromosome breaks, bridges, fragments, sister union, and micronucleus (MN) were the most frequently observed alterations after herbicide exposure (Yüzbasioglu et al., 2003). Recently, we reported the genotoxic and cytotoxic effects of the pure herbicide and its formulations Twin Pack Gold® and Rainbow® employing several end points in hamster CHO-K1 cells (Nikoloff et al., 2012b). For all compounds, our observations revealed a significant increase in sister chromatid exchange frequencies, a delay in cell-cycle progression, alterations in mitotic activity, as well as cellular growth inhibition, measured by a reduction in mitochondrial activity after 24 h of continuous exposure (Nikoloff et al., 2012b). Furthermore, by using the same *in vitro* cellular system, we recently demonstrated the ability of FLC to induce DNA single-strand breaks measured by the comet assay (Nikoloff et al., 2012b).

Induction of DNA single-strand breaks and DNA macrolesions evaluated by single-cell gel electrophoresis (SCGE) and cytokinesis-blocked micronucleus cytome (CBMN-cyt) bioassays,

Abbreviations: CP, cyclophosphamide; CBMN-cyt, cytokinesis-blocked micronucleus cytome assay; ETOH, ethanol; FLC, flurochloridone; GDI, genetic damage index; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; NDI, nuclear division index; MN, micronucleus; NR, neutral red; SCGE, single cell gel electrophoresis assay.

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respectively, are widely used end points to identify in different eukaryotic cells the genotoxic properties of a variety of xenobiotics, including agrochemicals (Ali et al., 2011; Fenech, 2007; González et al., 2003, 2007; Molinari et al., 2009; Nikoloff et al., 2012b). Furthermore, the MN test is a required end point by regulatory agencies and has emerged as one of the preferred methods for the assessment of both clastogenic and aneugenic damage as well as a valid alternative methodology for chromosomal aberration analysis (ICH, 2011; OECD, 1997, 2007).

However, the above-mentioned assays cannot give an indication of the mechanism of cytotoxicity. It is well known that toxic effects of environmental chemicals can lead to passive cell death or necrosis, or otherwise result in the active mechanism via apoptotic cell signaling. Apoptotic cell death can be induced by a variety of stimuli, e.g., ligation of cell surface receptors, starvation, growth factor/survival factor deprivation, heat shock, hypoxia, or DNA damage (Circu and Aw, 2010). The dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions, such as immunodeficiency, autoimmune diseases, neurodegenerative diseases, and cancer, among others (Circu and Aw, 2010).

One of the major problems of *in vitro* genotoxicity and cytotoxicity bioassays is the lack of a drug-metabolizing system in the cell lines currently used for routine testing of xenobiotics. Besides, exogenous activation mixtures isolated from rodent livers only partly mimic the biotransformation of a test compound in the *in vivo* condition. Several xenobiotics require metabolic activation to react with DNA; thus the use of *in vitro* cells possessing endogenous biotransforming activity can avoid the use of exogenous activation mixtures. Human hepatoma cell lines, HepG2 being the most promising cell line among them (Aden et al., 1979), appear to be a practical and suitable alternative for assessing genotoxicity. HepG2 cells are easy to handle and contain several enzymes responsible for the activation of various xenobiotics, including phase I and II enzymes (Knasmüller et al., 2004).

Since few studies have reported the effects of FLC on mammalian cells, the objective of this report is to investigate the genotoxicity and cytotoxicity exerted by the herbicide as an active ingredient and two of its commercial formulations most commonly used in Argentina, Rainbow® [25% active ingredient (a.i.)] and Twin Pack Gold® (25% a.i.) in HepG2 cells by the CBMN-cyt assay, SCGE, neutral red (NR), and MTT end points. Furthermore, the cellular response of HepG2 cells in terms of the induction of the apoptosis process by the herbicide was also assayed.

2. Materials and methods

2.1. Chemicals

Flurochloridone [3-chloro-4-(chloromethyl)-1-[3-(trifluoromethyl) phenyl]-2-pyrrolidone; CAS 61213-25-0] was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Twin Pack Gold® (25% a.i.) and Rainbow® (25% a.i.) were kindly provided by Syngenta Agro S.A. (Buenos Aires, Argentina) and Magan Argentina S.A. (Buenos Aires, Argentina), respectively. Acetone (ACTN) and hydrogen peroxide were purchased from Merck KGaA (Darmstadt, Germany). Cytochalasin B from *Dreschslera dematioidea* (CAS 14930-96-2), cyclophosphamide (CP; CAS 6055-19-2), ethanol (CAS 64-17-5), neutral red (CAS 553-24-2), MTT (CAS 57360-69-7), and propidium iodide (CAS 25535-16-4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC was purchased from Invitrogen Molecular Probes® (Carlsbad, CA, USA).

2.2. Cell cultures and herbicide treatment for the cytokinesis-blocked micronucleus cytome (CBMN-cyt) assay

HepG2 (HB-8065; American Type Culture Collection, Rockville, MD) cells were grown in MEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin (all from Gibco, Grand Island, NY) at 37 °C in a 5% CO_2 atmosphere. The MN assay was performed following the protocol of Chiang et al. (2011) with minor modifications. Experiments were set up with cultures at the log phase of growth. The cells were seeded onto precleaned 22 × 22 mm cover slips in 35 mm Petri dishes at a density of 1.5×10^5 cells in a final media volume of 3 ml per dish for 24 h. Cells were treated with FLC, Twin Pack Gold® and Rainbow® within the concentration range of 0.25–15 μg/ml. Prior to use, FLC was first dissolved in ACTN and then diluted in culture medium, whereas Twin Pack Gold® and Rainbow® were diluted directly in culture medium. Both compounds were diluted so that addition of 100 μl into 2.9 ml of cultures would allow herbicides to reach the required concentration ranges. Cells were cultured for 24 h as recommended previously (Chiang et al., 2011) and then washed twice with PBS, treated with cytochalasin B (3 μg/ml), and incubated at 37 °C in a 5% CO_2 atmosphere for an additional 16 h period until harvesting. The final solvent concentration was less than 1% for all treatments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (0.2 mg/ml CP) were run simultaneously with herbicide-treated cultures. None of the treatments produced pH changes in the culture medium (range, 7.2–7.4). Each experiment was repeated three times, and the cultures were performed in duplicate for each experimental and time point. The same batches of culture medium, serum, and reagents were used throughout the study. At the end of the culture period, the cells were treated with a cold hypotonic solution (KCl 0.075 M, 4 °C, 5 min), prefixed with methanol at −20 °C for 20 min, and fixed with methanol at −20 °C for 20 min. Afterward, slides were stained with 3% aqueous Giemsa solution for 10 min. The cover slips were air dried and then placed down onto precleaned slides using Depex mounting medium. For the MN assay, at least 1000 binucleated cells per experimental point from each experiment were blind-scored at 1000× magnification according to our previous report (González et al., 2011). The number of binucleated cells with zero, one, two, or three MNs was determined in binucleated cytokinesis-blocked cells following the examination criteria reported by Fenech (2007). A minimum of 500 viable cells per experimental point were scored to determine the percentage of cells with one, two, and three or more nuclei, and the nuclear division index (NDI) was calculated for each experimental point according to the method of Eastmond and Tucker (1989).

2.3. Cell cultures and herbicide treatment for SCGE assay

Prior to herbicide treatment, exponentially growing HepG2 cells were detached with a rubber policeman, centrifuged, and then resuspended in complete culture medium. Afterward, aliquots containing 2×10^5 cells/ml were incubated for 2 h at 37 °C in a 5% CO_2 atmosphere in a final media volume of 1 ml containing the test compounds. All compounds were used at a final concentration between 1 and 15 μg/ml. The final solvent concentration was <1% for all the treatments in all experiments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (0.2 mg/ml CP) were run simultaneously with herbicide-treated cultures. None of the treatments produced pH changes in the culture medium (pH 7.2–7.4). The SCGE and cell viability assays were performed immediately after 2 h short treatment period. Each experiment was repeated three times, and the cultures were performed in duplicate for each experimental and time point. The SCGE assay was performed following the alkaline procedure described by Singh et al. (1988) with minor modifications as reported elsewhere (Nikoloff et al., 2012a). Analysis of the slides was performed under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The extent of DNA damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and nonoverlapping cells. DNA damage was classified in four classes (I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavas and Konen, 2007) (Fig. 1). Results are expressed as the mean number of damaged nucleoids (sum of classes II, III, and IV) and the mean comet score for each treatment group. Additionally, a genetic damage index (GDI) was calculated for each test compound using the formula $GDI = [(I) + 2(II) + 3(III) + 4(IV)]/N_{(I–IV)}$, where I–IV represent the nucleoid type, and N_I–N_{IV} represent the total number of nucleoids scored (Pitarque et al., 1999).

2.4. Cell viability assay

At the end of the culture period, cell viability was determined using the ethidium bromide/acridine orange assay (McGahon et al., 1995). Briefly, 10 μl of a 1:1 freshly prepared mixture of ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml) was mixed with 10 μl of the cell suspension under study. Afterward, the cells were analyzed using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. Each experiment was repeated three times, and the cultures were performed in duplicate for each experimental and time point. The cell viability was monitored at 2 h. At least 500 cells were counted per experimental point, and results are expressed as the percentage of viable cells among all cells.

2.5. Neutral red and MTT assays

Briefly, 1×10^5 HepG2 cells/ml were cultured in MEM complete culture medium on 96-well microplates for 24 h. Afterward, the culture medium was removed, and the cells were treated with FLC, Twin Pack Gold®, or Rainbow® within the 0.25–15 μg/ml concentration range for 24 h. Five percent ethanol-treated and

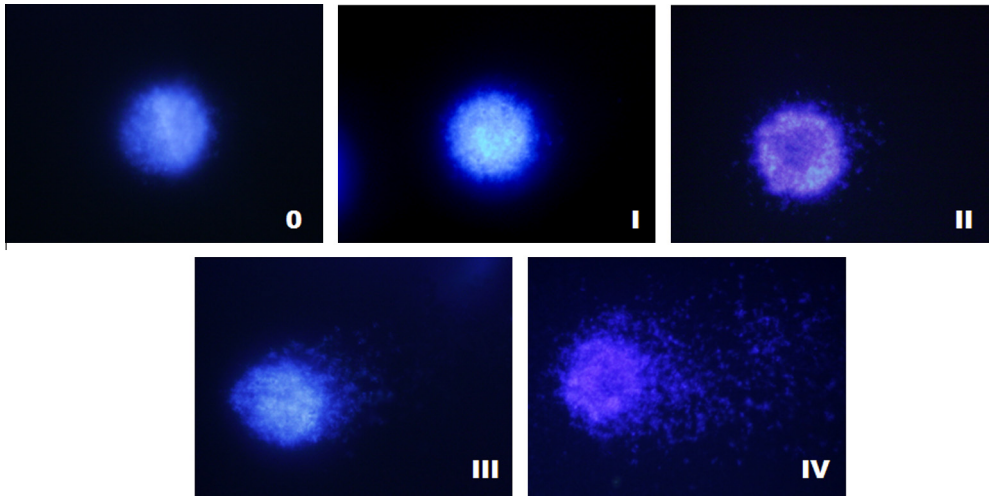

Fig. 1. Digitized comet images showing undamaged (0–I) and damaged nucleoids (II–IV) of flurochloridone-treated HepG2 cells. They represent classes 0–IV as used for visual scoring (0–I: undamaged, II: minimum damage, III: medium damage, IV: maximum damage). Cells were stained with DAPI and capture at fluorescent microscope. Magnification: 1000×.

0.075% ACTN-treated cells were used as positive and vehicle controls, respectively. The protocol described by Borenfreund and Puerner (1985) was employed for the NR assay. Following exposure with test compounds, cells were incubated for an additional 3 h period in the presence of 100 μg/ml neutral red dye dissolved in PBS. Then the cells were washed with PBS and the dye was extracted in each well. The procedure of MTT assay was performed following the techniques described by Kosmider et al. (2004). Following exposure with tested compounds, 20 μl of MTT was added for an additional 3 h period. Then the formazan crystals were dissolved in 100 μl of DMSO. Absorbance at 550 nm was measured with a microplate spectrophotometer (Sunrise Absorbance Reader, Tecan Austria GmbH, Salzburg, Austria). Results were expressed as the mean percentage of lysosomal and mitochondrial activities from three independent experiments performed in parallel.

2.6. Apoptosis detection by annexin V affinity assay

Prior to herbicide treatment, exponentially growing HepG2 cells were detached with a rubber policeman, centrifuged, and then resuspended in complete culture medium. Afterward, aliquots containing 5×10^5 cells/ml were incubated at 37 °C in a 5% CO_2 atmosphere in a final media volume of 1 ml after treatments with FLC, Twin Pack Gold®, and Rainbow® (15 μg/ml, 2 and 24 h). The final solvent concentration was <1% in all experiments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls were run simultaneously with herbicide-treated cultures. None of the treatments produced pH changes in the culture medium (pH 7.2–7.4). At the end of the treatment period, cells were washed in PBS, resuspended in 250 μl of 1× binding buffer (10 mm HEPES, 140 mm NaCl, and 2.5 mm $CaCl_2$) and exposed to annexin V-FITC (1 μl, 15 min, room temperature). Afterward, cells were washed with binding buffer, and 3 μl of propidium iodide (PI; stock solution, 10 μg/ml) was added to each sample. Samples were analyzed under an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. The cell population was visually classified following the examination criteria reported by Pläsier et al. (1999), and the proportions of alive (annexin V negative/PI negative), early apoptotic (annexin V positive/PI negative), late apoptotic (annexin V positive/PI positive), and necrotic cells (annexin V negative/PI positive) were determined. Each experiment was repeated three times, and cultures were performed in duplicate for each experimental point. At least 400 cells were scored per experimental point, and the results are expressed as the mean percentages of alive, early apoptotic, late apoptotic, and necrotic cells among all cells from three independent experiments performed in parallel (Pläsier et al., 1999).

2.7. Statistical analysis

The data were analyzed using Statgraphics 5.1 Plus software. MN and SCGE data 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, viability, and apoptosis in treated and control cells were evaluated by χ^2 test. The two-tailed Student's *t* test was used to compare MTT and NR data between treated and 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 shows the results of the analysis of herbicide-induced MNs in binucleated cytokinesis-blocked cells. An increased frequency of MNs was observed in those CP-treated cultures (positive control) compared to control values ($p < 0.001$). On the other hand, ACTN treatment (FLC solvent control) did not modify MN frequency compared with negative control values ($p > 0.05$). The frequency of MNs was not significantly increased when HepG2 cells were exposed to either FLC or Rainbow®, regardless of the herbicide concentration added to cultures ($p > 0.05$). Similarly, no MN induction was observed when cells were treated with 0.25–1 μg/ml Twin Pack Gold® ($p > 0.05$). On the other hand, Twin Pack Gold® increased the frequency of MNs compared to control cultures only in those 5 μg/ml-treated cells ($p < 0.05$; Table 1). When the cultures were treated with 10 and 15 μg/ml of both Twin Pack Gold® and Rainbow®, evident alteration in cell morphology was induced, which did not allow monitoring of MN frequencies.

The NDI values induced by FLC, Twin Pack Gold® and Rainbow® treatments are also presented in Table 1. A significant delay in cell-cycle progression and a concomitant significant reduction of the NDI were observed only in 10 μg/ml Twin Pack Gold®- or Rainbow®-treated cultures ($p < 0.001$). A correlation analysis showed that the NDI decreased in a concentration-dependent manner when Twin Pack Gold® ($r = -0.97$, $p < 0.001$) or Rainbow® ($r = -0.97$, $p < 0.001$) but not when FLC ($r = 0.17$, $p > 0.05$) were added to cultures (Table 1).

Cell viability was assessed in FLC-, Twin Pack Gold®-, and Rainbow®-treated cultures. The results are presented in Table 1. Regardless of the concentration, no significant alterations in cell viability were found in cultures treated with FLC compared to controls ($p > 0.05$). On the other hand, a significant decrease in cell viability were observed in those cultures treated with 10–15 μg/ml Twin Pack Gold® or Rainbow®, respectively ($p < 0.001$). Furthermore, the decrease in cell viability reached values as high as 97% and 95% when the highest concentrations of Twin Pack Gold® and Rainbow® were assayed, respectively.

Data of the SCGE assay obtained in HepG2 cells exposed during 2 h with different concentrations of FLC, Twin Pack Gold®, and Rainbow® are presented in Table 2 and Fig. 1. The proportion of damaged nucleoids, genetic damage index (GDI), as well as viability values are showing in Table 2. CP treatment (positive control) induced an enhancement in the frequency of damaged cells as well

Table 1
Micronucleus (MN) induction, nuclear division index (NDI), and viability values for control, flurochloridone (FLC)-, Twin Pack Gold®- and Rainbow®- treated in binucleated cytokinesis-blocked HepG2 cells.[a]

Compound	Concentration (μg/ml)	MN frequencies [b]	Micronucleated cell numbers [c]			NDI	Viability (%)
			1 MN	2 MNs	3 MNs		
Negative control	0	30.78 ± 1.88	26.56 ± 1.23	1.78 ± 0.49	0.22 ± 0.22	1.69 ± 0.02	97.05 ± 0.03
ACTN[d]		29.50 ± 3.50	25.00 ± 4.00	1.50 ± 0.50	0.50 ± 0.50	1.71 ± 0.03	97.25 ± 0.35
FLC	0.25	33.00 ± 5.77	29.00 ± 4.62	2.00 ± 0.58	0.00 ± 0.00	1.70 ± 0.01	95.75 ± 3.87
	1	28.00 ± 5.29	26.67 ± 4.67	0.67 ± 0.33	0.00 ± 0.00	1.79 ± 0.02	97.40 ± 2.26
	5	29.33 ± 2.60	27.00 ± 1.15	0.67 ± 0.33	0.33 ± 0.33	1.75 ± 0.03	93.22 ± 1.52
	10	31.33 ± 2.67	26.33 ± 1.45	3.33 ± 2.33	0.33 ± 0.33	1.74 ± 0.01	96.27 ± 1.79
	15	35.50 ± 6.94	26.00 ± 4.90	2.50 ± 0.41	1.50 ± 0.41	1.76 ± 0.01	96.50 ± 0.70
Twin Pack Gold®	0.25	27.50 ± 3.67	26.50 ± 2.85	0.50 ± 0.40	0.00 ± 0.00	1.69 ± 0.03	95.25 ± 1.25
	1	33.30 ± 4.84	29.00 ± 1.73	1.67 ± 1.20	0.33 ± 0.33	1.73 ± 0.02	97.62 ± 1.85
	5	37.30 ± 5.78*	31.33 ± 5.23	3.00 ± 1.52	0.00 ± 0.00	1.54 ± 0.04	70.23 ± 2.98
	10	ND	ND	ND	ND	1.11 ± 0.09***	22.19 ± 3.69***
	15	ND	ND	ND	ND	ND	2.36 ± 0.86***
Rainbow®	0.25	24.67 ± 2.40	20.33 ± 3.28	1.67 ± 0.88	1.00 ± 3.28	1.64 ± 0.03	96.50 ± 1.50
	1	24.67 ± 0.88	22.67 ± 0.33	1.00 ± 0.58	0.00 ± 0.00	1.58 ± 0.04	89.18 ± 4.88
	5	27.00 ± 4.08	24.00 ± 4.90	1.50 ± 0.41	0.00 ± 0.00	1.53 ± 0.02	65.04 ± 5.17
	10	ND	ND	ND	ND	1.29 ± 0.02***	20.38 ± 4.07***
	15	ND	ND	ND	ND	ND	5.42 ± 3.42***
CP[e]		51.50 ± 4.50***	39.50 ± 3.50**	4.50 ± 0.50	1.00 ± 0.00	1.68 ± 0.02	97.00 ± 0.60

ND. Not determined.
[a] HepG2 cells were treated 24 h after seeding with test compounds and 16 h with cytochalasin B before harvested.
[b] Results are presented as mean MNs/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/3000 binucleated cells of pooled data from three independent experiments ± S.E. of the mean.
[d] Acetone (ACTN, 0.1%) was used as solvent control.
[e] Cyclophosphamide (CP, 0.2 mg/ml) was used as positive control.
* $p < 0.05$ values in regards to control values.
** $p < 0.01$ values in regards to control values.
*** $p < 0.001$ values in regards to control values.

Table 2
Analysis of DNA damage measured by comet assay in HepG2 cells exposed during 2 h to flurochloridone (FLC), Twin Pack Gold®, or Rainbow®.

Compound	Concentration (μg/ml)	Proportion of damaged nucleoids (%)[a]				DNA damage (%)[b] (II + III + IV)	GDI[c]	Viablility (%)[b]
		Type I	Type II	Type III	Type IV			
Negative control		79.00	14.00	3.00	4.00	21.00 ± 3.61	1.34	90.66 ± 0.67
ACTN[d]		80.50	12.00	4.50	3.00	19.50 ± 4.50	1.30	88.50 ± 6.94
FLC	1	37.00**	24.00	17.00*	22.00*	63.00 ± 6.00***	2.24***	88.33 ± 4.18
	5	41.33**	22.67	11.33	24.67**	58.67 ± 5.00***	2.19***	87.00 ± 6.35
	15	51.00	27.67	7.67	13.66	49.00 ± 4.35***	1.84***	91.33 ± 2.33
Twin Pack Gold®	1	63.33	22.67	5.67	8.33	36.67 ± 6.76**	1.59*	91.66 ± 1.20
	5	29.33*	21.33	15.67	33.67*	70.67 ± 6.00***	2.54***	88.16 ± 3.00
	15	54.00	21.67	7.00	17.33	46.00 ± 4.50***	1.88***	80.66 ± 3.00
Rainbow®	1	63.33	20.00	3.67	13.00	36.67 ± 2.96**	1.66**	88.16 ± 5.53
	5	40.33**	19.33	10.67	29.67*	59.67 ± 4.27***	2.30***	99.00 ± 4.09
	15	64.67	19.00	5.33	11.00	35.33 ± 4.33**	1.63*	86.00 ± 4.51
CP[e]		30.50**	23.50	14.00	32.00 **	69.50 ± 5.50***	2.48***	88.00 ± 2.33

[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] Cyclophosphamide (CP, 0.2 mg/ml) was used as positive control.
* $p < 0.05$ values in regard to control values.
** $p < 0.01$ values in regard to control values.
*** $p < 0.001$ values in regard to control values.

as of the GDI compared to the negative control ($p < 0.001$) by increasing mostly the frequency of grade IV comets (Table 2). On the other hand, ACTN treatment did not alter the frequency of damaged nucleoids compared with that of negative control cultures ($p > 0.05$). FLC treatments induced an enhancement of GDI values with all concentrations assayed ($p < 0.001$). Statistical analyses demonstrated that the GDI increase induced by 1 μg/ml was due to an enhancement in the frequency of type III and IV comets ($p < 0.05$) and a concomitant decrease of type I comets ($p < 0.01$; Table 2 and Fig. 1). Similarly, the increase in GDI ratio induced by 5 μg/ml was due to an increase in the frequency of type IV comets ($p < 0.01$) and decrease of type I comets ($p < 0.01$; Table 2 and Fig. 1). Finally, no statistical difference was observed in the proportion of different nucleoid types between 15 μg/ml FLC-treated cells and negative controls ($p > 0.05$). Twin Pack Gold® treatments induced an enhancement of the GDI with all concentrations assayed

($p < 0.05$ and $p < 0.001$ for 1 and 5–15 μg/ml, respectively). Similar results were observed after all Rainbow® treatments ($p < 0.05$ for 1 μg/ml and $p < 0.001$ for 5 or 15 μg/ml). When the proportion of different damaged nucleoids was analyzed only when 5 μg/ml of both commercial formulations was assayed, an increase in the frequency of type IV comets ($p < 0.05$) and decrease in the frequency of type I comets ($p < 0.05$ and $p < 0.01$ for Twin Pack Gold® or Rainbow®, respectively) was achieved (Table 2). A regression analysis demonstrated that the total number of damaged cells induced by FLC, Twin Pack Gold® and Rainbow® increased as an independent function of the concentration of the test compounds titrated into cultures (FLC, $r = 0.21$, $p > 0.05$; Twin Pack Gold®, $r = 0.37$, $p > 0.05$; Rainbow®, $r = 0.38$, $p > 0.05$). Similarly, when the analysis was performed between GDI rates and herbicide concentrations, the results revealed that GDI increased as an independent function of the concentration of the test compounds added into cultures (FLC, $r = 0.82$, $p > 0.05$; Twin Pack Gold®, $r = 0.35$, $p > 0.05$; Rainbow®, $r = 0.12$, $p > 0.05$).

Results from the cell viability assays, which were run before the SCGE, are presented in Table 2. Regardless of the concentration, no significant alterations in cell viability were found in cultures treated with FLC, Twin Pack Gold®, or Rainbow® compared to negative controls ($p > 0.05$).

The values of alterations of lysosomal activity assay obtained are presented in Fig. 2. Statistically significant loss of lysosomal activity was observed between negative (untreated and ACTN-treated cells) and positive controls ($p < 0.001$; Fig. 2). The data revealed a significant cell growth inhibition when HepG2 cells were exposed to 2.5–15 μg/ml Twin Pack Gold® ($0.01 > p < 0.001$) and 5-15 μg/ml Rainbow® ($p < 0.001$). No statistical alteration in the lysosomal activity was observed when HepG2 cells were exposed to all assayed concentrations of FLC ($p > 0.05$). A regression test showed that lysosomal activity decreased as a function of the concentration of Twin Pack Gold® ($r = -0.93$, $p < 0.001$) or Rainbow® ($r = -0.98$, $p < 0.001$) titrated into cultures. Overall, the NR assay demonstrated that Twin Pack Gold® exerted a more cytotoxic effect than Rainbow® in the HepG2 cell line within the 2.5–15 μg/ml concentration range.

The results of the alterations in the energetic cell metabolism induced by FLC, Twin Pack Gold®, and Rainbow® are depicted in Fig. 3. Ethanol-treated cultures (positive controls) produced a statistically significant toxicity in HepG2 cells compared with negative and ACTN-treated cultures ($p < 0.001$). Data presented in Fig. 3 show cellular metabolism inhibition when HepG2 cells were exposed to 5–15 μg/ml Twin Pack Gold® ($p < 0.001$) and 15 μg/ml Rainbow® ($p < 0.001$), whereas no statistical alteration in the

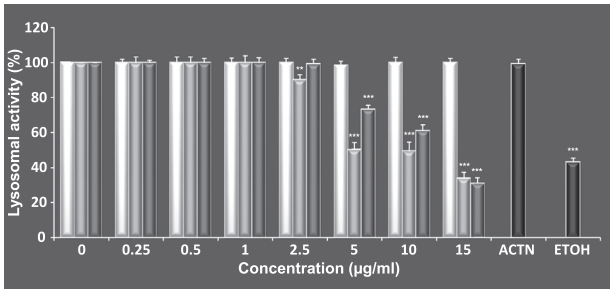

Fig. 2. Lysosomal activity assessed with the NR assay in FLC- (white bars), Twin Pack Gold®- (light grey bars), and Rainbow®-treated (dark grey bars) HepG2 cells. Cultures were incubated for 3 h with NR dye after 24 h treatment. Results are expressed as the mean percentage of lysosomal activity from three independent experiments performed in parallel (*y*-axis) and plotted against the herbicide concentration (0–15 μg/ml concentration range; *x*-axis). Five percent ethanol-treated (black bar) and 0.075% ACTN-treated (black bar) cells were used as positive and solvent controls, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values.

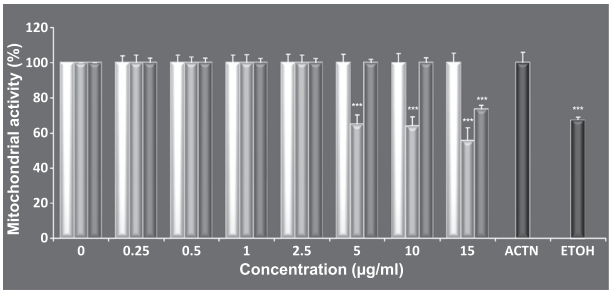

Fig. 3. Cellular metabolism inhibition evaluated by MTT assay in FLC- (white bars), Twin Pack Gold®- (light grey bars), and Rainbow®- (dark grey bars) treated HepG2 cells. Cultures were incubated for 3 h with MTT after 24 h of herbicide treatment. Results are expressed as the mean percentage of mitochondrial activity from three independent experiments performed in parallel (*y*-axis) and plotted against the herbicide concentration (0–15 μg/ml concentration range; *x*-axis). Five percent ethanol-treated (black bar) and 0.075% ACTN-treated (black bar) cells were used as positive and solvent controls, respectively. $^{***}p < 0.001$; significant differences with respect to control values.

mitochondrial activity was observed when HepG2 cells were exposed to all assayed concentrations of FLC ($p > 0.05$). A regression test showed that the inhibition decreased as a function of the concentration of Twin Pack Gold® ($r = -0.89$, $p < 0.01$) or Rainbow® ($r = -0.79$, $p < 0.05$) titrated into cultures. Overall, the MTT assay demonstrated that Twin Pack Gold® exerted a more cytotoxic effect than Rainbow® in the HepG2 cell line within the 5–15 μg/ml concentration range.

Data of the apoptosis detection by annexin V affinity assay for cells harvested 2 and 24 h after treatment are presented in Table 3. Results revealed an induction of apoptotic cells after both H_2O_2 and ETOH treatment ($p < 0.001$) by increasing the frequency of late ($p < 0.001$) and early and late apoptotic cells ($p < 0.01$), respectively. Neither H_2O_2 nor ETOH modified the frequencies of necrotic cells ($p > 0.05$). Results demonstrated that ACTN treatment did not modify the frequencies of alive, early apoptotic, late apoptotic, or necrotic cells, after either 2 or 24 h of exposure ($p > 0.05$). Results indicate that FLC, Twin Pack Gold®, and Rainbow® after 2 h of treatment were not able to induce significant alterations in the frequencies of either apoptotic (early and late) or necrotic cells ($p > 0.05$). When treatments lasted for 24 h, an enhancement of necrotic ($0.05 > p < 0.001$; Fig. 4C–C′) and apoptotic cells due to an increased frequency of early apoptotic cells ($p < 0.001$; Fig. 4A–A′) was observed in those FLC-, Twin Pack Gold®-, and Rainbow®-exposed cultures. In addition, a concomitant decrease in the frequency of alive cells (Fig. 4A–A′ and C–C′) was observed in those cultures treated with both Twin Pack Gold® and Rainbow® ($0.01 > p < 0.001$). No induction of late apoptosis was observed in cultures treated with FLC and FLC-based formulations when exposed during 24 h ($p > 0.05$; Fig. 4B–B′ and Table 3).

4. Discussion

In the present study, the CBMN-cyt and SCGE assays were employed to analyze the damage inflicted by the herbicide FLC and its commercial formulations Twin Pack Gold® and Rainbow® on the DNA from of HepG2 cells. Additionally, the NDI, NR and MTT bioassays as well as apoptosis detection were further analyzed to quantitatively determine whether treatment with the xenobiotics were able to induce cytotoxicity. The results demonstrated that both the active ingredient and the commercial formulations, but the lowest concentration of Twin Pack Gold® (5 μg/ml), were unable to induce MNs. Furthermore, higher concentrations (10 and 15 μg/ml) of Twin Pack Gold® and Rainbow® resulted in a cellular cytotoxicity clearly demonstrated by alterations in the NDI and

Table 3
Analysis of apoptosis measured by annexin V-FITC in HepG2 cells exposed during 2 and 24 h to 15 mg/ml of flurochloridone (FLC), Twin Pack Gold®, or Rainbow®.

Compound	Time exposure (h)	Cells (%)[a]				
		Alive	Early apoptotic	Late apoptotic	Total apoptotic[d]	Necrotic
Negative control	2	91.25 ± 4.22	2.33 ± 0.94	3.54 ± 1.37	5.87 ± 2.31	2.88 ± 0.95
ACTN[b]	2	93.50 ± 2.00	2.51 ± 0.32	2.31 ± 0.20	4.82 ± 0.24	2.68 ± 0.10
FLC	2	90.91 ± 0.61	1.97 ± 0.20	4.82 ± 1.07	6.80 ± 0.90	2.30 ± 0.51
Twin Pack Gold®	2	87.85 ± 2.17	1.11 ± 0.20	6.80 ± 2.90	7.92 ± 2.38	4.24 ± 0.56
Rainbow®	2	88.04 ± 1.87	2.05 ± 1.04	5.71 ± 1.68	7.76 ± 1.36	4.20 ± 0.67
H_2O_2[c]	2	77.67 ± 2.05	2.23 ± 0.21	17.07 ± 2.58***	19.30 ± 2.78***	3.03 ± 0.73
Negative control	24	87.10 ± 3.42	4.85 ± 0.82	4.55 ± 1.32	9.41 ± 1.09	3.49 ± 1.21
ACTN[b]	24	87.82 ± 2.00	0.00 ± 0.00	10.00 ± 1.00	10.00 ± 0.24	2.17 ± 0.10
FLC	24	72.53 ± 2.23	5.46 ± 0.26***	11.68 ± 1.95	17.15 ± 1.45*	10.32 ± 2.16***
Twin Pack Gold®	24	57.32 ± 3.77**	28.17 ± 3.53***	7.09 ± 1.04	35.26 ± 2.59***	7.42 ± 0.97*
Rainbow®	24	38.60 ± 2.95***	44.60 ± 3.60***	7.19 ± 0.89	51.80 ± 1.47***	9.59 ± 1.32**
ETOH[e]	24	73.14 ± 2.18	11.71 ± 2.18**	10.33 ± 1.65**	22.04 ± 2.77***	4.82 ± 1.69

[a] Results are presented as mean values of pooled data from three independent experiments ± S.E. of the mean.
[b] Acetone (ACTN, 0.1%) was used as solvent control.
[c] H_2O_2 (125 μM) was used as positive control.
[d] Total apoptotic = (early apoptotic + late apoptotic).
[e] ETOH (5%) was used as positive control.
* $p < 0.05$ values in regard to control values.
** $p < 0.01$ values in regard to control values.
*** $p < 0.001$ values in regard to control values.

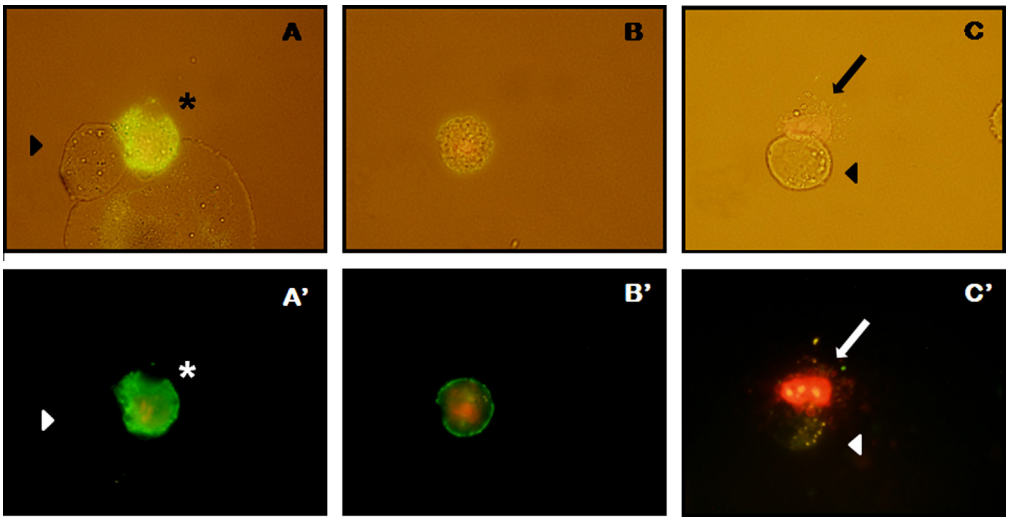

Fig. 4. Representative images using annexin V-FITC and propidium iodide staining in HepG2 cells *in vitro*. (A–C) Light microscopy images correspond to the fluorescence images A′–C′ of annexin V-FITC and propidium iodide staining, respectively. The cell population was classified using the combined microscopic images obtained through light and fluorescence microscopy images. (A and A′) Early apoptotic cell. (B and B′) Late apoptotic (asterisk) and alive (arrow head) cells. (C and C′) Alive (arrow head) and necrotic (arrow) cells.

cellular death. However, when the SCGE assay was employed as another end point for genotoxicity, a marked increase in the GDI was observed for FLC, Twin Pack Gold®, and Rainbow®, regardless of the concentrations incorporated into cultures within the 1–15 μg/ml range. Overall, the results demonstrated that only the formulated products were able to reduce both lysosomal and mitochondrial activities when concentrations higher than 2.5 or 5 μg/ml were employed in Twin Pack Gold®- and Rainbow®-treated cultures, respectively. Finally, the results indicate that both pure technical and formulated products were able to induce a significant increase in the frequency of both apoptotic as well as necrotic cells after 24 h of treatment. Therefore, the results represent the first evidence that the pure active herbicide FLC is able to introduce DNA single-strand breaks as revealed by the comet assay but not a clear cytotoxic effect. However, the formulated based-FLC products, at least Twin Pack Gold® and Rainbow®, were able to induce both genotoxicity and cytotoxicity in HepG2 cells but not the FLC. The partial differences of the genotoxicity potential obtained with the pure herbicide and the commercial formulated products indicate that they may contain additional unsafe compounds with genotoxic and cytotoxic potential, and then, they could represent a significant hazard source both to the health of neighbouring populations and, potentially, to the environment itself. Furthermore, taking into account not only the origin but also the biochemical characteristics of the cells we employed, FLC could be considered, then, as a compound with likely hepatotoxic potential. In agreement with this suggestion, FLC has been classified as a slightly hazardous compound (class III) by the World Health Organization (2009). Furthermore, FLC causes adverse effects in male reproductive functions, hormonal system alterations, and low to moderate acute toxicity in rats (EFSA, 2013).

The *in vitro* CBMN-cyt assay has been widely used in genotoxicity testing, especially as a screening tool for genotoxicity evaluation in many classes of environmental pollutants, including pesticides (Dearfield and Moore, 2005; Ergene et al., 2007; González et al., 2011). In the present study, FLC and Rainbow®

appear as non-MN inducing agents on HepG2 cells. However, the formulated product Twin Pack Gold® increased MN frequency only in 5 μg/ml-treated cells and cellular growth inhibition in HepG2 cultures, at least when estimated by the CBMN-cyt bioassay. We observed previously that neither FLC nor Rainbow® was able to induce MNs. However, a positive result was found when CHO-K1 cells were exposed to only 5 μg/ml of Twin Pack Gold® (Nikoloff et al., 2012b). Furthermore, we have also observed that treatment of CHO-K1 cells with Twin Pack Gold® and Rainbow® concentrations higher than 10 μg/ml resulted in a cellular cytotoxicity clearly revealed by alterations in the nuclear division index and cellular death (Nikoloff et al., 2012b). The current findings are in total accordance with our study performed on CHO-K1 cells (Nikoloff et al., 2012b). Several studies described that the toxicity of nonactive ingredients present in pesticide formulations, e.g., carrier substances and solvents that improve the pesticide absorption (WHO, 1990), could be more genotoxic than the pesticide active itself, and could affect the overall toxicity of the product (González et al., 2006, 2007; Molinari et al., 2009, 2010; Soloneski et al., 2001, 2008; Zeljezic et al., 2006). Although additive compounds frequently comprise part of a commercial pesticide formulation, they are not usually included in any discussion of the effects on living cells, and their adverse effects may exceed those of the active ingredients. Our current observations could suggest that the deleterious effect induced by Twin Pack Gold® is most probably due to the presence of xenobiotic(s) included in the excipient of the FLC-based herbicide leading to MN formation and cytotoxicity. Unfortunately, the origin, identity, and purity of the nonactive ingredients in the commercial formulation Twin Pack Gold® were not made available to us by the producer. It should be mentioned that according to our Argentinean administration, the excipients present in any agrochemical are not required to be listed on the agrochemical data sheet and can be kept as a "trade secret." Years ago, USEPA (1982) claimed that the acute toxicity of a technical active ingredient can differ significantly from that of the end-use formulation containing that active ingredient.

It its known that the difference between results in the SCGE and CBMN-cyt assays is essentially due to variations in the types of DNA alterations that the assays detect: whereas the SCGE assay detects DNA primary lesions that are often repairable, the CBMN-cyt test detects irreparable lesions. Several studies describing combined experiments where SCGE and CBMN-cyt assays have comparatively been performed showed that the CBMN-cyt method seems to be less sensitive than SCGE assay for assessing DNA damage potential (Goethem et al., 1997; He et al., 2000; Severin et al., 2010; Tafazoli and Volders, 1996). Our current observations agree well with this concept. Furthermore, the results showed that a concentration as low as 1 μg/ml of all compounds induced DNA single-strand breaks in HepG2 cells but not MNs. Accordingly, it seems evident that FLC at low concentrations can only induce single-strand DNA breaks but not any other type of DNA macrolessions leading to MN production.

Results of the SCGE assay also demonstrated that when HepG2 cells were treated with 15 μg/ml of FLC, Twin Pack Gold® and Rainbow® presented a lower increment in DNA damage than the maximum DNA migration found when exposed to a lower concentration, i.e., 5 μg/ml. This observation might indicate that the decrease in the frequency of DNA damage estimated by a reduction in the tailed comets could be mostly related to several aspects of cell cytotoxicity. Among them can be included the induction of a selective FLC-mediated cell death of the most damaged cells, leaving only a reduced proportion of cells able to be included in the SCGE score. However, the results of cell viability revealed no alteration in the frequency of alive/dead cells among FLC-treated cell cultures in those SCGE experiments. Thus, this possibility could be ruled out. Another plausible explanation could be related to the induction of an apoptotic process that could also lead to a reduction of the frequency of damaged cells included in the final count. However, our results demonstrated that FLC as well as the commercial formulations Twin Pack Gold® and Rainbow® are able to enhance the frequency not only of necrotic but also apoptotic cells only when treatments lasted for 24 h, but not after 2 h of exposure, the incubation time employed in the SCGE protocol. Then, this latter explanation can also be discarded as a causal effect. Finally, the possibility that HepG2 cells could be able to repair through an SOS mechanism the induced damage when cells reach a threshold level compatible with cell survival cannot be dismissed. In agreement, the SCGE assay revealed a decrease of the frequency of type III and IV comets with a concomitant increased frequency of normal nucleoids in FLC cells treated with 15 μg/ml when compared with those treated with 5 μg/ml. Rodriguez-Ferreiro et al. (2001) suggested a similar explanation for human lymphocytes when treated *in vitro* with the antiparasiticide tinidazole.

The present study demonstrated that FLC induced the formation of DNA fragmentation and increased the apoptotic index in HepG2 cells. These results could make it possible to draw some conclusions with respect to the mechanism(s) involved in its geno- and cytotoxicity. The action of FLC can be attributed to a clastogenic effect, considering that the SCGE assay detects single-strand breaks and alkaline labile sites (Singh et al., 1988), lesions that are often repairable DNA ones. Both types of alterations may result from the action of reactive oxygen species delivered by the bleaching herbicide. So far, the exact mechanism(s) of mutagenicity by which FLC causes DNA damage in mammalian cells has not been totally clarified. However, it has been reported that another bleaching herbicide like amitrole causes *in vitro* oxidative DNA damage by the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf thymus DNA (Furukawa et al., 2010), and genotoxicity when the *Drosophila* wing spot test is employed (Kaya et al., 2000). The results obtained in our study are in good agreement with those reported previously (Furukawa et al., 2010; Kaya et al., 2000); thus a similar genotoxic effect of these chemically related bleaching herbicides could be suggested. Further studies are required to solve this possibility.

Numerous xenobiotics, including pesticides, can produce ROS via several mechanisms, e.g., inactivation of antioxidant enzymes, depletion of nonenzymatic antioxidants, and membrane lipid peroxidation, among others (Kaya and Yigit, 2012). It is currently believed that FLC could mediate detrimental effects on several cellular targets associated with ROS generation (Kaya and Yigit, 2012). It is well known that glutathione is one of the crucial metabolites that are considered the most important intracellular defence against ROS-induced oxidative damage. Recently, it was reported that FLC was able to induce biochemical changes by the generation of ROS in leaves of *V. sativa*, increasing the glutathione content, glutathione-*S*-transferase, and glutathione reductase activities (Kaya and Yigit, 2012). Imbalances in the cellular redox state may regulate cell survival, and, thus, high glutathione levels may provide resistance to oxidative stress-induced apoptosis (Alberts et al., 2004; Kirkland and Franklin, 2001). Based on the evidence that the cellular redox steady state has an influence on the apoptosis mechanism in mammalian cells, it may also play a revealing role in the response of these cells to xenobiotics such as the herbicide FLC. In the present investigation, apoptosis was detected by the annexin V binding method, where annexin V specifically binds to the negatively charged phosphatidylserine that is translocated form the inner leaflet of the cell membrane to the cell surface during the early apoptosis (Alberts et al., 2004). Our current observations reveal that while both FLC-based formulations induced a strong increase in the level of early apoptotic cells, a slight increase in this type of apoptosis was achieved after an equivalent treatment with the active principle FLC. Overall, our biological assay data demonstrate that the

commercial formulations Twin Pack Gold® and Rainbow® are more toxic than the active ingredient FLC, with Twin Pack Gold® having stronger effects than Rainbow® in terms of cell growth inhibition and with Rainbow® having stronger effects than Twin Pack Gold® in terms of apoptogenic effect. In particular, both commercial formulations induced a statistically significant cell growth inhibition compared to the active ingredient, which did not have any effect. Moreover, the commercial formulations induced severe apoptosis, consistent with the MTT data, whereas the active ingredient induced only mild apoptosis. Percentages of apoptosis after 24 h treatment were mostly an indication of early apoptotic cell population. Furthermore, a mild increase in the percentage of necrotic cells was observed after treatment with either the active ingredient or the commercial formulations. These observations represent the first evidence of the apoptotic effect exerted by FLC and two FLC-based formulated products on mammalian cells *in vitro*, at least on HepG2 cells.

Previous studies have reported that FLC is extensively metabolised in animals by oxidation, hydrolysis, and glutathione conjugation, yielding numerous metabolites in urine and faeces in rats (EFSA, 2013). The most relevant metabolite is R42819 [(4*RS*)-4-(chloromethyl)-1-[3-(trifluoromethyl)phenyl] pyrrolidin-2-one]. However, no toxicological information is available on this metabolite, which may be found in groundwater at levels above the threshold value of 0.1 μg/L, according to environmental models (EFSA, 2013). Several reports indicate the relevance of this metabolite in groundwater because it may cause harm to unborn children and possible risk of impaired fertility (EFSA, 2013). At present, it seems evident that more studies are required to determine the origin of DNA damage exerted by FLC to understand whether the metabolite R42819 is related to the genotoxicity and cytotoxicity of this xenobiotic when HepG2 cells are employed as the target.

According to the Organisation for Economic Co-operation and Development, the herbicide FLC represents a key compound in relation to toxicity studies because a data gap was identified in its toxicology section (EFSA, 2013). Accordingly, the institution has proposed the realization of several studies to obtain more evidence in relation to FLC risk assessment in nontarget organisms. Moreover, further ecotoxicological studies are required highlighting the relevance of the ground water metabolite R42819 and the potential endocrine-disrupting properties in nontarget organisms such as birds, fish, and amphibians, among others.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency documents associated with this article can be found in the online version.

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