Comparative Evaluation *In vitro* of the Herbicide Flurochloridone by Cytokinesis-block Micronucleus Cytome and Comet Assays

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ABSTRACT: The *in-vitro* effects of flurochloridone and its formulations Twin Pack Gold® (25% a.i.) and Rainbow® (25% a.i.) were evaluated in Chinese Hamster Ovary K1 (CHO-K1) cells. The cytokinesis-block micronucleus cytome (CBMN-cyt) and single-cell gel electrophoresis (SCGE) assays were used. The activities were tested within the range of final concentrations of 0.25–15 μg flurochloridone/mL. The results demonstrated that both the flurochloridone and Rainbow® were not able to induce micronuclei (MN). On the other hand, Twin Pack Gold® only increased the frequency of MN at 5 μg/mL. Furthermore, 10 and 15 μg/mL of both formulations resulted in a cellular cytotoxicity demonstrated by alterations in the nuclear division index and cellular death. SCGE assay appeared to be a more sensitive bioassay for detecting primary DNA strand breaks at lower concentrations of flurochloridone than MN did. A marked increase in the genetic damage index was observed when 5 and 15 μg/mL of both flurochloridone and Rainbow® but only when 15 μg/mL of Twin Pack Gold® were used. This is the first report demonstrating that flurochloridone and its two commercial formulations are able to induce single-strand DNA breaks *in vitro* on mammalian cells. © 2012 Wiley Periodicals, Inc. Environ Toxicol 29: 884–892, 2014.

Keywords: flurochloridone; CBMN-cyt assay; DNA-single strands breaks; CHO-K1 cells; commercial formulations

INTRODUCTION

According to the FAO (2006), the global agricultural production is growing and it must increase about 70% percent by 2050. Consequently, a large number of pesticides are introduced daily into the environment worldwide which requires predictive, rapid, and practical techniques for toxicity assessment, especially those concerning to their genotoxic and cytotoxic aspects. The single-cell gel electrophoresis (SCGE) and cytokinesis-block micronucleus cytome (CBMN-cyt) assays are widely used to identify the genotoxic properties of a variety of xenobiotics in different mammalian cells (Grover et al., 2010; Igarashi et al., 2010; Minozzo et al., 2010; Ali et al., 2011). Both assays are simple, sensitive, and fast procedures that detect genotoxicity in any eukaryotic cell type after xenobiotic exposure and usually used to identify short-lived DNA and chromosomal damage, respectively (Singh et al., 1988; Fenech, 2000, 2007; González et al., 2003, 2007; Corvi et al., 2008; Molinari et al., 2009; Vera Candioti et al., 2010). 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 (OECD, 1997, 2007; ICH, 2011).

Flurochloridone is a selective herbicide widely applied around the world, including in Argentina, and used to

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control many broadleaf weeds and annual grasses in several crops. Flurochloridone interferes with carotenoid biosynthesis, causing bleaching of the leaves (Lay and Niland, 1983; Klíčová et al., 2002). According the European Food Safety Authority (EFSA), flurochloridone has no genotoxic, carcinogenic, or neurotoxic potential and it is unlikely to be genotoxic (EFSA, 2010). However, flurochloridone causes adverse effects in male reproductive functions and hormonal system alterations (EFSA, 2010). Furthermore, when flurochloridone is administered orally, dermally, or by inhalation to rats, it induces low or moderate acute toxicity (EFSA, 2010). In addition, flurochloridone induced abnormal cell-cycle progression, cellular mitodepressive activity and chromosomal abnormalities in *Allium cepa* root meristematic cells (Yüzbasioglu et al., 2003). In a recent investigation, we reported the genotoxic and cytotoxic effects of the pure herbicide and its formulations Twin Pack Gold® and Rainbow® using several end-points in Chinese Hamster Ovary K1 (CHO-K1) cells (Nikoloff et al., 2012). 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., 2012).

In this study, we use the *in vitro* CBMN-cyt and SCGE assays in CHO-K1 cells to characterize the genotoxicity and cytotoxicity exerted by the herbicide flurochloridone 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.). Results of this investigation could provide data for hazard assessments related to the worldwide employ of flurochloridone herbicide.

MATERIALS AND METHODS

Chemicals

Flurochloridone [3-chloro-4-(chloromethyl)-1-[3-(trifluoromethyl) phenyl]-2-pyrrolidone; CAS 61213-25-0] and cytochalasin B from *Dreschslera dematioidea* (CAS 14930-96-2) were obtained from Sigma Chemical (St. Louis, MO). 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 was purchased from Merck KGaA (Darmstadt, Germany). Bleomycin (Blocamycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina).

Cell Cultures and Herbicide Treatment for the CBMN-cyt Assay

CHO-K1 cells were grown in Ham's F-10 medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 10 μg/mL streptomycin (all from Gibco, Grand Island, NY) at 37°C in a 5% CO_2 atmosphere. Experiments were set up with cultures at the log phase of growth. The cells were seeded onto precleaned 22 mm × 22 mm cover slips in 35 mm Petri dishes at a density of 1.2×10^4 cells in a final media volume of 3 mL per dish. Herbicide treatments with the test compounds were performed 24 h after plating as recommended previously (González et al., 2006, 2007; Molinari et al., 2009; Soloneski and Larramendy, 2010). Prior to use, flurochloridone was first dissolved in acetone 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 flurochloridone to reach the required concentration ranges of 0.25–15 μg/mL. The concentration range used were selected according our previous genotoxic data (Nikoloff et al., 2012). The final solvent concentration was less than 1% for all treatments. Immediately after treatments, cytochalasin B at a final concentration of 3 μg/mL was added into cultures, and then the cells were incubated at 37°C in a 5% CO_2 atmosphere for an additional 24 h until harvesting. Negative controls (untreated cells and solvent vehicle-treated cells) 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.

CBMN-cyt Assay

At the end of the culture period, 1000 μL of methanol/acetic acid (6:1) was directly added to each dish for a 15 min prefixation period. Afterward, the supernatant was removed by pipetting and the cells were then fixed with methanol/acetic acid (6:1) for 15 min in the same Petri dish, and finally stained with 3% aqueous Giemsa solution after air dried as reported elsewhere (González et al., 2011). 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 MN was determined in binucleated cytokinesis-blocked cells following the examination criteria reported by Fenech (2007). Necrotic or apoptotic cells were not included in the scoring of binucleated cells according to criteria proposed 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).

Nucleoplasmic bridges and nuclear buds were blind-scored from 1000 binucleated cells per experimental point from each experiment at 1000× magnification. Examination criteria followed those established previously (Fenech, 2007).

Cell Cultures and Herbicide Treatment for SCGE

Prior to herbicide treatment, exponentially growing CHO-K1 cell were detached with a rubber policeman, centrifuged, and then resuspended in complete culture medium. Afterward, aliquots containing 3.5×10^4 cells/mL were incubated for 80 min at 37°C in a 5% CO_2 atmosphere in a final media volume of 3 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 (1.0 μg/mL bleomycin) 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 an 80 min short treatment period. Each experiment was repeated three times and the cultures were performed in duplicate for each experimental and time point.

SCGE Assay

The SCGE assay was performed following the alkaline procedure described by Singh et al. (1988) with minor modifications. Briefly, a solution containing 0.5% normal-melting agarose in Ca^{2+}/Mg^{2+}-free PBS was prepared. Normal-melting agarose (200 μL) was transferred onto 100% ethanol precleaned slide, spread evenly, and placed at 37°C for 20–30 min. Two solutions containing 0.5% normal-melting agarose and 1% low-melting agarose solution in Ca^{2+}/Mg^{2+}-free PBS were prepared. Afterward, 150 μL of 1% low-melting agarose together with 7.0×10^3 cells (50 μL cell suspension plus 150 μL low-melting agarose) was applied, covered with a coverslip, and placed at 4°C for 10 min. After this layer had solidified, slides were immersed in ice-cold freshly prepared lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, and 10% DMSO) and then lysed in darkness for a period of 1 h (4°C). When this period concluded, slides were placed in an electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 25 min at 4°C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 30 min at 25 V and 250 mA. Finally, the alkali condition in the gels was neutralized with a solution comprising 0.4 M Tris-HCl, pH 7.5, stained with 4′-6′-diamino-2-phenylindole (DAPI) (Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA). 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 50 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 (Cavaş and Konen, 2007). 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. In addition, 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).

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, 5 μL of a 1:1 freshly prepared mixture of ethidium bromide (100 μg/mL) and acridine orange (100 μg/mL) was mixed with 50 μL of the cell suspension 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 80 min and 24 h after herbicide treatment. At least 500 cells were counted per experimental point, and results are expressed as the percentage of viable cells among all cells.

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 NDI, nucleoplasmic bridges, nuclear buds, GDI, and viability in treated and control cells were evaluated by χ^2 test. To check for a dose-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.

RESULTS

Table I shows the results of the analysis of herbicide-induced MN in binucleated cytokinesis-blocked cells. An increased frequency of MN was observed in those

TABLE I. Micronucleus (MN) induction, nuclear division index (NDI), and viability values in control and flurochloridone (FLC)-, Twin Pack Gold®-, and Rainbow®- treated in binucleated cytokinesis-blocked Chinese hamster ovary (CHO-K1) cells[a]

Compound	Concentration (μg/mL)	MN frequencies[b]	Micronucleated Cell Numbers[c] 1 MN	2 MN	3 MN	NDI[d]	Viability (%)[d]
Control		39.7 ± 2.9	32.7 ± 3.3	3.0 ± 0.6	0.5 ± 0.3	2.1 ± 0.0	100.0 ± 0.0
FLC	0.25	40.7 ± 5.0	32.0 ± 2.1	1.7 ± 0.9	1.3 ± 0.3	2.2 ± 0.1	99.3 ± 0.6
	1	38.3 ± 3.2	29.3 ± 0.9	2.7 ± 0.7	1.0 ± 0.6	2.2 ± 0.1	97.0 ± 2.5
	5	38.0 ± 3.8	33.3 ± 3.7	1.7 ± 0.7	0.7 ± 0.7	2.0 ± 0.1	99.0 ± 0.3
	10	44.0 ± 1.2	34.3 ± 2.1	3.0 ± 0.0	1.3 ± 0.3	1.9 ± 0.1	99.0 ± 0.3
	15	42.7 ± 0.9	34.3 ± 4.1	2.7 ± 1.2	1.0 ± 0.6	1.8 ± 0.1*	97.0 ± 0.3
Control		35.7 ± 3.0	28.0 ± 1.7	3.3 ± 0.7	0.6 ± 0.2	2.1 ± 0.0	99.0 ± 0.3
Twin Pack Gold®	0.25	31.3 ± 1.5	26.3 ± 1.5	2.0 ± 1.2	0.3 ± 0.3	2.2 ± 0.1	100.0 ± 0.0
	1	41.0 ± 4.5	30.3 ± 4.2	4.3 ± 0.3	1.0 ± 0.6	2.0 ± 0.1	99.0 ± 1.3
	5	66.3 ± 5.6***	58.3 ± 4.4***	3.0 ± 0.6	0.7 ± 0.7	1.7 ± 0.1**	99.0 ± 2.5
	10	ND	ND	ND	ND	ND	95.0 ± 0.3
	15	ND	ND	ND	ND	ND	0.0 ± 0.0***
Control		41.0 ± 5.3	34.0 ± 3.4	2.0 ± 0.6	1.0 ± 0.3	2.1 ± 0.0	100.0 ± 0.0
Rainbow®	0.25	44.1 ± 5.8	33.0 ± 4.2	4.0 ± 1.2	1.0 ± 0.3	2.1 ± 0.0	100.0 ± 1.6
	1	37.3 ± 4.4	33.0 ± 4.8	2.0 ± 0.9	0.0 ± 0.0	2.1 ± 0.1	99.5 ± 0.5
	5	47.0 ± 5.5	41.3 ± 4.9	3.0 ± 0.7	0.0 ± 0.0	1.9 ± 0.1	89.6 ± 6.0
	10	ND	ND	ND	ND	ND	90.0 ± 6.0
	15	ND	ND	ND	ND	ND	65.3 ± 6.0***
ACTN[e]		40.0 ± 6.0	30.7 ± 5.2	2.6 ± 0.3	1.3 ± 1.3	2.1 ± 0.1	99.0 ± 0.6
BLM[f]		62.3 ± 1.8***	48.3 ± 1.4***	5.0 ± 0.0*	1.0 ± 0.8***	2.1 ± 0.1	97.0 ± 0.2

[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 mean number of cells carrying 1, 2, or 3 MN among 1000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.

[d] Results are presented as mean values of pooled data from three independent experiments ± S.E. of the mean.

[e] Acetone (ACTN, 0.1%) was used as flurochloridone-solvent control.

[f] Bleomycin (BLM, 1.0 μg/mL) was used as positive control.

$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to control values. ND, not determined.

bleomycin-treated cultures (positive control) compared to control values ($p < 0.001$). The frequency of MN was not significantly increased when cells were exposed to either flurochloridone or Rainbow®, regardless of the herbicide concentration added to cultures ($p > 0.05$). A similar scenario was observed for all concentrations of Twin Pack Gold® added to cultures except one. Twin Pack Gold® increased the frequency of MN compared to control cultures only in 5 μg/mL-treated cells ($p < 0.001$), whereas no MN induction was achieved when cells were treated with lower concentrations (0.25–1 μg/mL; $p > 0.05$) (Table I). The highest tested of Twin Pack Gold® and Rainbow® concentrations (10 and 15 μg/mL) produced evident alterations in cell morphology that did not allow monitoring of MN frequencies. Overall, results indicate that, for a given concentration, the capability of Twin Pack Gold® to induce MN was higher than that of flurochloridone and Rainbow®.

The NDI values induced by flurochloridone, Twin Pack Gold®, and Rainbow® treatments are presented in Table I. A significant delay in cell-cycle progression and a concomitant significant reduction of the NDI were observed only in 15 μg/mL flurochloridone-treated and in 5 μg/mL Twin Pack Gold®-treated cultures ($p < 0.05$ and $p < 0.01$, respectively). A correlation analysis showed that the NDI decreased in a concentration-dependent manner when flurochloridone ($r = -0.99$, $p < 0.01$) or Twin Pack Gold® ($r = -0.98$, $p < 0.05$) were added to cultures (Table I).

Cell viability was assessed in flurochloridone-, Twin Pack Gold®-, and Rainbow®-treated cultures (Table I). Regardless of the concentration, no significant alterations in cell viability were found in cultures treated with flurochloridone compared to controls ($p > 0.05$). On the other hand, a total loss of cell viability and a significant decrease of about 35% in cell viability were observed in those cultures treated with either 15 μg/mL Twin Pack Gold® or Rainbow®, respectively ($p < 0.001$).

When the frequencies of nucleoplasmic bridges were analyzed, flurochloridone, Twin Pack Gold®, and Rainbow® treatments, regardless of the concentration, did not

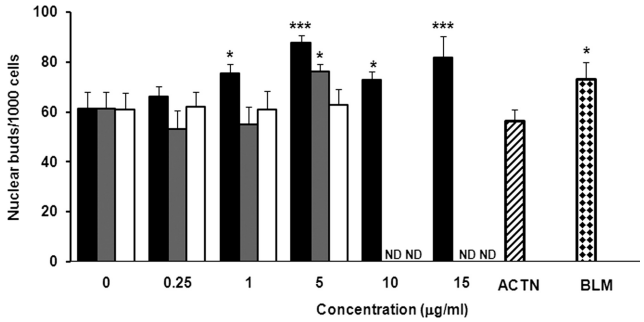

Fig. 1. Effect of flurochloridone (black bars) and its commercial formulations Twin Pack Gold® (gray bars) and Rainbow® (white bars) on nuclear buds induction in binucleated cytokinesis-blocked CHO-K1 cells. Cells were treated 24 h after seeding with test compounds and cytochalasin B and harvested 24 h later. Results are presented as mean of nuclear buds per 1000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean. Acetone (ACTN, 0.1%) was used as flurochloridone-solvent control. Bleomycin (BLM 1.0 μg/mL) was used as positive control. ND, not determined. $^{*}p < 0.05$; $^{***}p < 0.001$; significant differences with respect to control values.

modified the frequency of nucleoplasmic bridges compared to control values ($p > 0.05$) (data not shown).

An increased frequency of nuclear buds was observed in those bleomycin-treated cultures (positive control) compared to controls ($p < 0.05$) (Fig. 1). No nuclear bud induction was observed after Twin Pack Gold® and Rainbow® treatments within the 0.25–1 and 0.25–5 μg/mL concentration ranges, respectively ($p > 0.05$). A significant enhancement of the frequency of nuclear buds was observed both in 1–15 μg/mL flurochloridone-treated and 5 μg/mL Twin Pack Gold®-treated cultures ($p < 0.001$ to $p < 0.05$ and $p < 0.05$, for flurochloridone and Twin Pack Gold®, respectively) (Fig. 1). A regression test showed that the increase observed with flurochloridone treatments was independent of the added concentration ($r = 0.56$, $p > 0.05$) (Fig. 1). The highest tested of Twin Pack Gold® and Rainbow® concentrations (10 and 15 μg/mL) produced evident alterations in cell morphology that did not allow monitoring nuclear buds.

Results of the SCGE assay obtained in CHO-K1 cells exposed during 80 min with different concentrations of flurochloridone, Twin Pack Gold®, and Rainbow® are presented in Table II showing the proportion of damaged nuclei and the GDI values. When the proportion of damaged nuclei was analyzed, an increase in the frequency of damaged nucleoids ($p < 0.001$) by increasing mostly the frequencies of grade III comets within the 1–15 μg/mL concentration range ($p < 0.01$) (Table II) was observed. Twin Pack Gold® treatment produced an enhancement in the frequency of type IV damaged cells ($p < 0.001$) only when 15 μg/mL was added to cultures. When Rainbow® was assayed, an enhancement in the frequency of damaged cells ($p < 0.05$ and $p < 0.001$) (Table II) was found when both 5 and 15 μg/mL concentrations were added to cultures. The latter was achieved by decreasing and increasing the frequencies of type I ($p < 0.05$) and type IV ($p < 0.001$) comets, respectively (Table II). A regression analysis demonstrated that the total number of damaged cells was directly correlated with the concentration of Rainbow® ($r = 0.96$, $p < 0.05$), but not with those of flurochloridone ($r = 0.86$, $p > 0.05$) and Twin Pack Gold® ($r = 0.93$, $p > 0.05$), added to cultures. Overall, the comet assay demonstrated that flurochloridone and Rainbow® were more prone to induce genotoxic effects than Twin Pack Gold®.

The GDI values induced by flurochloridone, Twin Pack Gold®, and Rainbow® treatments are presented in Table II. Flurochloridone treatments induced an enhancement of GDI values when concentrations of 1 ($p < 0.05$), 5 ($p < 0.001$), and 15 μg/mL ($p < 0.001$) were added to cultures. Twin Pack Gold®-treatment produced an enhancement of the GDI only at concentration of 15 μg/mL ($p < 0.001$), whereas Rainbow®-treatment increased the GDI when 5 μg/mL and 15 μg/ml were employed ($p < 0.001$). When an analysis was performed between GDI rates and herbicide concentrations, the results revealed that DNA damage was directly correlated with the concentrations of Twin Pack Gold® ($r = 0.99$, $p > 0.001$) and Rainbow® ($r = 0.96$, $p < 0.05$), but not with that of flurochloridone ($r = 0.78$, $p > 0.05$) added to cultures.

Results from the cell viability assays, which were run before the SCGE, are presented in Table II. While flurochloridone and Rainbow® treatments did not alter cell viability within the 5–15 μg/mL concentration range ($p > 0.05$), a significant decrease in the cellular viability of cultures was achieved when 15 μg/mL Twin Pack Gold® was employed ($p < 0.05$). Overall, the viability assay demonstrated that Twin Pack Gold® exerted more cytotoxic effects than flurochloridone and Rainbow® in CHO-K1 cells even after an 80 min treatment.

DISCUSSION

The purpose of this study was to further investigate the *in vitro* genotoxicity and cytotoxicity effects exerted by the herbicide flurochloridone and two of its commercial formulations, Twin Pack Gold® and Rainbow®. The activities of all compounds were tested within the 0.25–15 μg/mL concentration range in CHO-K1 cells employing the CBMN-cyt and SCGE assays as genotoxic biomarkers. The results demonstrate that both the active ingredient and Rainbow® were not able to induce MN. On the other hand, Twin Pack Gold® increased the frequency of MN at 5 μg/mL. Furthermore, higher concentrations (10 and 15 μg/mL) of Twin Pack Gold® and Rainbow® resulted in a cellular cytotoxicity clearly demonstrated by alterations in NDI and cellular death. In addition, the SCGE assay appeared to be a more

TABLE II. Analysis of DNA damage as measured by comet assay in CHO-K1 cells exposed 80 min to Flurochloridone (FLC), Twin Pack Gold® and Rainbow®

Compound	Concentration (μg/mL)	Proportion of Damaged Nuclei (%)[a]				% of Damaged Cells (II + III + IV)	GDI[b]	Viability (%)[c]
		Type I	Type II	Type III	Type IV			
Control		80 ± 0.96	13 ± 0.64	4 ± 0.51	3 ± 0.44	20	1.35	100 ± 0.33
FLC	1	72 ± 5.85	14 ± 3.51	13 ± 2.02**	1 ± 0.66	28	1.64*	100 ± 0.33
	5	61 ± 2.40	20 ± 3.78	15 ± 3.17**	4 ± 1.15	39***	1.89***	99 ± 1.33
	15	57 ± 3.17*	26 ± 2.08	15 ± 2.60**	2 ± 0.57	43***	1.92***	99 ± 0.88
Twin Pack Gold®	1	83 ± 1.45	9 ± 0.33	5 ± 0.88	3 ± 1.20	17	1.34	99 ± 0.58
	5	81 ± 1.33	8 ± 0.67	5 ± 0.67	6 ± 1.15	19	1.43	96 ± 3.00
	15	67 ± 2.33	12 ± 2.00	7 ± 0.33	14 ± 0.58***	33**	1.79***	85 ± 3.00*
Rainbow®	1	80 ± 1.52	12 ± 0.57	7 ± 0.66	1 ± 0.66	20	1.38	100 ± 0.33
	5	67 ± 2.33	15 ± 1.33	11 ± 1.20	7 ± 0.33	33**	1.76***	99 ± 1.00
	15	57 ± 1.85*	15 ± 1.85	10 ± 0.00	18 ± 3.21***	43***	2.09***	91 ± 3.06
ACTN[c]		83 ± 1.85	12 ± 2.00	3 ± 0.33	2 ± 0.33	17	1.16	99 ± 0.67
BLM[d]		58 ± 1.53**	17 ± 1.45*	12 ± 1.20*	13 ± 0.33***	42***	2.05***	98 ± 1.00

[a,c]Results are presented as mean values of pooled data from three independent experiments ± S.E. of the mean.
[a]I-IV indicate grades of DNA damage.
[b]Genetic damage index (GDI).
[c]Acetone (ACTN, 0.1%) was used as flurochloridone-solvent control.
[d]Bleomycin (BLM, 1.0 μg/mL) was used as positive control.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significant differences with respect to control values.

sensitive bioassay for detecting primary DNA strand breaks at lower concentrations of flurochloridone than MN did. Furthermore, a marked increase in the GDI was observed when all flurochloridone concentrations, 15 μg/mL Twin Pack Gold®, and 5–15 μg/mL Rainbow® were added to cultures. Although the *in vitro* flurochloridone treatments in this study covered a wide range of concentration, the 0.25–15 μg/mL range represents the relatively high end of the threshold value of 0.1 μg flurochloridone/L found in ground, surface and drinking water reported by EFSA (2010). Accordingly, the range of concentrations used in this investigation would be expected to be rare in the environment, perhaps only being observed when specific events could be occur, e.g., a direct application adjacent to surface waters by accidental discharge/spills or when humans are exposure by occupational working, among others.

Although considerable information is accessible about the environmental and ecological effects of flurochloridone (WHO, 1990, 2009; EFSA, 2010), available information on flurochloridone-induced genotoxicity and/or cytotoxicity is scarce. However, flurochloridone is a pyrrolidone herbicide classified as a slightly hazardous compound (class III) by the World Health Organization (2009). In regard to genotoxicity, to the best of our knowledge, two reports can be mentioned. Yüzbasioglu et al. (2003) have reported genotoxic effects in *A. cepa* meristematic root tips. They observed the induction of different types of chromosomal abnormalities such as chromosomal stickiness, chromosome breaks, bridges, fragments, sister union, and MN (Yüzbasioglu et al., 2003). Recently, we demonstrated that both flurochloridone and its formulations Twin Pack Gold® and Rainbow® are DNA-damaging agents, since an enhancement of the frequency of sister chromatid exchanges, alterations in lysosomal and mitochondrial activities, a delay in the cell-cycle progression, as well as a decrease of the mitotic activity were observed to occur in *in vitro* treated mammalian cells (Nikoloff et al., 2012). Accordingly, our current results represent the first evidence demonstrating the ability of flurochloridone to induce single-strand DNA breaks. However, only the herbicide formulation Twin Pack Gold® significantly increase the MN frequency at least in CHO-K1 cells.

The CBMN-cyt assay has been used in cytogenetic studies to detect small chromosomal fragments like acentric chromosome/chromatid fragments in interphasic cells induced by clastogens or chromosome lagging at anaphase produced by aneugens (OECD, 1997). It is a sensitive and valuable technique characterized by restricting the analysis to cells that have passed through their first division (Fenech, 2007). In the present study, flurochloridone and Rainbow® showed no clastogenic effect on CHO-K1 cells cultures during a 24 h exposure. However, the formulated product Twin Pack Gold® increased the MN frequency in binucleated cells when higher concentrations were tested. It is well known that, in addition to the active ingredient(s), pesticide formulations include carrier substances and solvents that improve the pesticide absorption (WHO, 1990). Although the additive compounds frequently comprise a large 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 (WHO, 1990). Several investigations

have demonstrated that the additive compounds present in pesticide commercial formulations have the ability to induce cellular damage by themselves, separate from the active ingredient (Kaya et al., 1999; Zeljezic et al., 2006; González et al., 2006, 2007; Soloneski et al., 2001, 2008; Molinari et al., 2009, 2010). Unfortunately, the identities of the additive compounds present in the commercial formulation Twin Pack Gold® were not made available to us by the manufacturers. Our results could suggest that the MN induction exerted by Twin Pack Gold® is most probably due to a deleterious effect(s) induced by xenobiotics included in the excipient of the flurochloridone-based herbicide.

When chromatin instabilities were included in the study, the induction of nuclear buds but not nucleoplasmic bridges was significantly enhanced in flurochloridone- and Twin Pack Gold®-treated cells. Several reports have shown that the content of the nuclear buds is diverse, but the genesis of the nuclear buds remains to be understood (Fenech, 2007; Dutra et al., 2010). However, recent investigations showed that nuclear buds could be detached from the original nucleus before reaching apoptosis (Dutra et al., 2010). Further studies are required to understand the formation of nuclear buds in flurochloridone-treated mammalian cells and to determine whether nuclear buds induction is a step in the apoptosis pathway, as suggested elsewhere (Dutra et al., 2010).

The apparent lack of sensitivity of the CBMN-cyt assay toward the genotoxicity of the herbicide flurochloridone compared to the SCGE assay might be attributable to the generation of a particular type of damage. Comparative investigations between SCGE and CBMN-cyt assays employing several compounds have been performed previously, showing that the CBMN-cyt technique seems to be less sensitive than SCGE assay for assessing DNA damage potential (Tafazoli and Volders, 1996; Goethem et al., 1997; He et al., 2000; Severin et al., 2010). In this study, the DNA damage induced by 1 μg/mL flurochloridone was enough to be detected by the SCGE assay but not by MN frequency. The difference we observed in the sensitivity between these two end-points may be due to the type of flurochloridone-induced lesions differentially estimated by these assays. Whereas the SCGE assay can detect single-strand DNA breaks, the CBMN assay only detects MN that come from chromatid or chromosomal fragments and vagrant chromosomes. Accordingly, at low concentration, it could be suggested that flurochloridone can only induce single-strand DNA breaks but not any other type of macrolesions leading to MN production. Furthermore, the possibility that CHO-K1 cells could be able to repair the damage induced by flurochloridone cannot be ruled out. Similarly, the possibility that the most severely damaged cells were unable to proliferate after injury and enter into mitosis could be ruled out, since any concentration of flurochloridone and its commercial formulation did not alter cell viability in CHO-K1 cultures.

So far, the exact mechanism by which flurochloridone causes DNA damage in mammalian cells has not been previously reported. However, several herbicides, including flurtamone, amitrole, norflurazon, and flurochloridone have been found to interfere with the capability of conversion of phytoene into colored carotenoids, thus inducing bleaching herbicides (Di Baccio et al., 2002). Because of the striking similarity in flurochloridone mode of action and that observed for another herbicide, namely amitrole, it could be suggested that former may cause its toxic effects in a similar manner than that amitrole does. Recently, it has been reported that amitrole caused *in vitro* oxidative DNA damage by the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf thymus DNA (Furukawa et al., 2010). Furthermore, Meteroja and collaborators (1976) reported that amitrole interfered with lymphoblast transformation and inhibited cell growth on cultured human leukocytes showing a positive response in carcinogenesis studies. In *in vivo* mutation systems, amitrole was found to be positive when the Drosophila wing spot test was used revealing the genotoxic effect of this herbicide (Kaya et al., 2000). The results obtained in our study are in good agreement with those previously reported by others could suggest a similar genotoxic effects of these chemically related bleaching herbicides. Further studies are required to solve this possibility.

Years ago, Moretti et al. (2002) tested *in vitro* the genotoxicity of the *s*-triazine herbicide terbutryn on stimulated human peripheral blood lymphocytes. They found that while the herbicide induced primary DNA damage revealed by the comet assay, it failed to produce a significant increase in MN frequency, either in the absence or presence of S9-mix. Furthermore, these authors suggested that the cells were able to induce either the DNA lesions evaluated by the comet assay or a selective elimination of heavily damaged cells that could not survive long enough to contribute to MN formation (Moretti et al., 2002). Our results accord well with this concept.

Finally, in agreement with our previous observations (Nikoloff et al., 2012), the current findings are in accord with the genotoxic profile shown by *s*-triazine herbicides like terbutryn (Moretti et al., 2002) and atrazine (Kligerman et al., 2000; Malik et al., 2004; Zeljezic et al., 2006). Several studies have reported that these herbicides show a suspected endocrine activity (Simić et al., 1991; Stoker et al., 2000). The mechanism of flurochloridone action has not yet been determined, but a similar endocrine activity could be suggested. Although the underlying mechanism of action is still beyond our knowledge, the genotoxicity of the herbicide require further study.

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