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Genotoxic and cytotoxic evaluation of the herbicide flurochloridone on Chinese hamster ovary (CHO-K1) cells

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ABSTRACT

The *in vitro* effects of flurochloridone (FLC) and its formulations Twin Gold Pack® (25% a.i.) and Rainbow® (25% a.i.) were evaluated on Chinese hamster ovary (CHO-K1) cells by genotoxicity [sister chromatid exchange (SCE)] and cytotoxicity [cell-cycle progression, proliferative rate index (PRI), mitotic index (MI), MTT, and neutral red] end points. Cells were treated for 24 h within the 0.25–15 μg/ml concentration range. FLC and Twin Pack Gold® induced a significant and equivalent increase in SCEs regardless of the concentration. Rainbow®-induced SCEs at concentrations higher than 2.5 μg/ml; however, the increases were always lower than those induced by FLC and Twin Pack Gold®. For all compounds, the PRI decreased as a function of the concentration titrated into cultures. Whereas only the highest FLC and Twin Pack Gold® concentrations induced a significant reduction of the MI, all tested Rainbow® concentrations induced MI inhibition. Overall, the results demonstrated that although all compounds were not able to reduce the lysosomal activity, the mitochondrial activity was diminished when the highest concentrations were employed. These observations represent the first study analyzing the genotoxic and cytotoxic effects exerted by FLC and two formulated products on mammalian cells *in vitro*, at least on CHO-K1 cells.

1. Introduction

In recent years, agricultural practices have evolved in the context of a quickly changing and globalizing food economy and also as a result of the concerns and responsibilities of a broad range of participants about food production and security, food safety and quality, and the environmental sustainability of agriculture (Goodland, 1997). Although the benefits of conventional agricultural practices have been immense, they utilize levels of pesticides and fertilizers that can result in a negative impact on the environment (WHO, 1990).

Genotoxicity and cytotoxicity studies have been conducted to test numerous agrochemical compounds using several end points on different test systems (Ergene et al., 2007; Lin and Garry, 2000; Rakitsky et al., 2000; Soloneski et al., 2007, 2008; Soloneski and Larramendy, 2010; Zeljezic et al., 2006). The use of *in vitro* cell cultures for genotoxic and cytotoxic evaluation is a valuable and very well-known tool for the early and sensitive detection or estimation of chemical exposure (DiPaolo et al., 1981). Among them, one of most used systems for clastogenic and/or aneugenic screening is the cultured mammalian cells, in which sister chromatid exchanges (SCEs), chromosomal aberrations, micronuclei, and cell proliferation kinetics have been widely employed as cytogenetic end points (Aouadene et al., 2008; Brezden et al., 1997; Soloneski et al., 2002, 2008; Soloneski and Larramendy, 2010; Zwanenburg et al., 1984).

Herbicides constitute a heterogeneous category of chemicals particularly designed for the control of weeds. They are the most widely applied agrochemicals around the world, significantly increasing the agricultural productivity and crop yields (Bolognesi, 2003). Their application is still the most effective and accepted method for plant protection from weeds, with the environment consequently and inevitably being exposed to these chemicals.

Flurochloridone (FLC) is a pre-emergence herbicide used to control a range of weeds in umbelliferous, cereal, sunflower, and potato crops, among others. It is a selective compound, absorbed by roots and stem, causing bleaching of the leaves by interference with biosynthesis of carotenoid, chlorophyll, and abscisic acid metabolites (Klíčová et al., 2002; Lay et al., 1985; Lay and Niland, 1983).

Toxicological information for FLC has been poorly documented. So far, it has been reported that the herbicide does not reveal genotoxic, carcinogenic, or neurotoxic potential in rodents (EFSA, 2010). FLC induces low or moderate acute toxicity in rats when the herbicide is administered by oral, dermal, or inhalational routes (EFSA, 2010). However, FLC causes adverse effects in male reproductive functions and hormonal system alterations (EFSA, 2010). When other models were employed, e.g., birds, fish, and aquatic invertebrates, the level of acute toxicity exerted by FLC was found to be moderate (EFSA, 2010). However, in toxicological studies using

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aquatic plants and algae, moderate and high toxicity have been reported, respectively (EFSA, 2010). Accessible information on the genotoxic properties of FLC is scarce. To the best of our knowledge, a single report is available. When root meristematic cells of *Allium cepa* were exposed to the herbicide, abnormal cell-cycle progression and cellular mitodepressive activity were found (Yüzbasioglu et al., 2003). The most frequently observed abnormalities were c-metaphase, multipolarity, polyploidy, and chromosome lagging. In addition, chromosomal stickiness, chromosome breaks, bridges, fragments, sister union, and micronuclei were also observed after FLC exposure (Yüzbasioglu et al., 2003).

For weed control, an herbicide is not used as a single active ingredient, but instead as a complex commercial formulation. Inert ingredients such as surfactants, humectants, and dispersants, which in some cases comprise more than 90% of the volume of pesticide formulations (Cox and Surgan, 2006), increase the penetration of the active ingredient into the cells, and they are not required to be identified on the product labels (Freeman and Rayburn, 2006; Haefs et al., 2002). Several reports have shown that the toxicity of several commercial formulations is higher than that of the active ingredients. Hence, additional genotoxic and cytotoxic effects exerted by inert ingredients must be taken into consideration for risk assessment, as suggested previously (Lin and Garry, 2000; Mann and Bidwell, 1999; Rayburn et al., 2005; Soloneski et al., 2001, 2002, 2003, 2007, 2008; Soloneski and Larramendy, 2010; Sorensen et al., 2003; Zeljezic et al., 2006).

The study presented here aims to contribute information about the comparative genotoxic and cytotoxic effects of the herbicide FLC as an active ingredient and two of its formulation products employed in Argentinean crops, Rainbow® (25% a.i.) and Twin Gold Pack® (25% a.i.). In this report, we employed SCEs, cell-cycle progression analysis, mitotic index (MI), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and neutral red (NR) bioassays as different end points on mammalian Chinese hamster ovary (CHO-K1) cells.

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., respectively. Colchicine (CAS 64-86-8), ethanol (CAS 64-17-5), dimethyl sulfoxide (DMSO; CAS 67-68-5), neutral red dye (CAS 553-24-2), 5-bromo-2-deoxiuridine (BrdU; CAS 59-14-3), and MTT (CAS 57360-69-7) were purchased from Sigma Chemical Co. Acetone (ACTN) was purchased from Merck KGaA (Darmstadt, Germany). Bleomycin (BLM; Blocamycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina).

2.2. Cell cultures and herbicide treatment for SCE and cell-cycle progression assays

CHO-K1 cells were grown in Ham's F10 medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY, USA), 100 units/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco) at 37 °C in a 5% CO_2 atmosphere. Experiments were set up with cultures in the long phase of growth. The cells were seeded in T25 flasks at a density of 3.5×10^5 cells per flask. Treatments with the test compounds were performed 24 h after plating as previously recommended (González et al., 2006, 2007; Molinari et al., 2009; Soloneski and Larramendy, 2010). Prior to use, FLC was first dissolved in ACTN and then diluted in culture medium, whereas Twin Pack Gold® and Rainbow® were directly diluted in cultured medium. Test compounds were diluted so that addition of 100 μl into cultures allowed them to reach the required concentrations specified in Section 3 within the range of 0–15 μg/ml. The final solvent concentration was <1% for all treatments in the different experiments. Negative controls (untreated cells and solvent vehicle-treated cells) and positive controls (1 μg/ml BLM) were run simultaneously with pesticide-treated cultures. BLM was selected due to its capacity of introduce both single- and double-strand breaks into DNA and to induce chromosomal damage in *in vitro* mammalian cells (Bolzán and Bianchi, 2004; Bolzán et al., 1992; Sánchez et al., 2009; Soloneski and Larramendy, 2010). None of the treatments produced significant pH changes in the culture medium. Afterward, 10 μg/ml BrdU was incorporated into cultures, and cells were incubated at 37 °C in a 5% CO_2 atmosphere under a safety light for an additional 24 h period until harvesting. Cultures were duplicated for each experimental point, in at least three independent experiments. The same batches of culture medium, sera, and reagents were used throughout the study.

2.2.1. Chromosome preparations

During the last 3 h of culture, the cells were treated with 1 μg/ml colchicine. Cells were detached with a rubber policeman, collected by centrifugation, hypotonically shocked (0.075 M KCL, 37 °C, 10 min), and fixed in methanol/acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique (Larramendy and Knuutila, 1990).

2.2.2. Fluorescence plus Giemsa method for sister chromatid differentiation

Chromosome spreads were stained using the fluorescence plus Giemsa technique for sister chromatid differentiation as described previously (Larramendy and Knuutila, 1990). Slides were coded and scored blind by one researcher.

2.2.3. Cell-cycle progression and mitotic index

A minimum of 100 metaphase cells per sample were scored to determine the percentage of cells that had undergone one (M_1) and two (M_2) mitoses. The proliferative rate index (PRI) was calculated for each experimental point as described previously (Lamberti et al., 1983). The MI was determined by scoring 1000 cells from each experimental point and expressed as the number of mitoses among 1000 nuclei. Changes in the MI were expressed as a factor (f) of the mean MI from treated cultures (MI_t) over the mean MI from controls (MI_c) ($f = MI_t/MI_c$) (Miller and Adler, 1989).

2.2.4. Sister chromatid exchange analysis

A total of 25 well-spread diploid M_2 cell metaphases were scored per experimental point from each treatment. The data were expressed as the mean number of SCEs per cell ± SE from 75 pooled cells scored per test-compound concentration.

2.3. Neutral red assay

The NR assay is based on the protocol described by Borenfreund and Puerner (1985). Briefly, 1×10^5 CHO-K1 cells/ml were cultured in Ham's F10 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 0.075% ACTN-treated cells were used as positive and negative controls, respectively. 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 serum-free medium. 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 cell growth inhibition from three independent experiments performed in parallel.

2.4. MTT assay

MTT is a yellow dye that is converted into formazan, a violet compound, by the activity of the enzyme succinate dehydrogenase of mitochondria. Since the conversion takes place in living cells, the amount of formazan produced is directly correlated with the number of viable cells. The procedure was performed following the technique described by Kosmider et al. (2004) with slight modification. Briefly, CHO-K1 cells (10^5 cells/ml) were seeded in Ham's F10 complete culture medium for 24 h on 96-well microplates. Afterward, the culture medium was removed, and the cells were treated with FLC, Twin Gold Pack®, or Rainbow® within a range of 0.25–15 μg/ml for 24 h. Five percent ethanol-treated and 0.075% ACTN-treated cells were used as positive and negative controls, respectively. 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 cell growth inhibition from three independent experiment performed in parallel.

2.5. Statistical analysis

The two-tailed Student's *t* test was used to compare SCE frequencies and MTT and NR data between treated and control groups, whereas a χ^2 test was employed for cell-cycle progression and MI data. Unless indicated otherwise, the level of significance chosen was 0.05.

3. Results

Since no differences in cell-cycle progression, SCEs, and PRI values were observed between untreated and ACTN-treated cells, pooled data are presented for control cultures.

Fig. 1 shows the results of SCE analysis in CHO-K1 cells treated during 24 h with different concentrations of FLC, Twin Pack Gold®, or Rainbow® obtained from three independent experiments. Results revealed statistically significant differences between negative (untreated and ACTN-treated cells) and positive controls ($p < 0.001$). The SCE frequencies observed in FLC- and Twin Pack Gold®-treated cultures were significantly higher than those of control cultures when treated within the 0.25–10 and 0.25–5 μg/ml concentration ranges, respectively ($p < 0.001$). When Rainbow® was assayed, an increase in SCE frequency was found in those cultures treated within the 2.5–10 μg/ml concentration range ($0.05 > p < 0.001$), whereas no SCE induction was achieved when cells were treated with lower concentrations (0.25–1 μg/ml; $p > 0.05$). In those 15 μg/ml FLC- or Rainbow®-treated as well as 10–15 μg/ml Twin Pack Gold®-treated cultures, we were unable to determine the frequency of SCEs because the frequency of M_1 cells reached values as high as 88–100% of the cell population (Fig. 1). A correlation analysis revealed that SCE frequencies induced by FLC and Twin Pack Gold® increased in a manner independent of the concentration of the test compounds titrated into cultures (FLC, $r = 0.62$, $p > 0.05$; Twin Pack Gold®, $r = 0.42$, $p > 0.05$). On the other hand, a significant concentration-dependent increase of SCEs was observed in Rainbow®-treated cultures ($r = 0.97$, $p < 0.001$). Overall, Fig. 1 also depicts that, for a given concentration, the capability of Rainbow® to induce SCEs was always lower than that of FLC or Twin Pack Gold®, and that of Twin Pack Gold® was higher than that of FLC (Fig. 1).

Results of cell-cycle progression and MI analyses after FLC, Twin Pack Gold®, and Rainbow® treatments are summarized in Table 1. BLM induced a significant inhibition of the cell-cycle progression and MI compared with the corresponding negative control value ($p < 0.001$). ACTN treatment did not modify either the PRI or MI compared with the corresponding negative control value ($p > 0.05$). A significant reduction of the PRI was observed only in those cultures treated with 15 μg/ml FLC ($p < 0.001$). The cultures showed a significant reduction of PRI ratio when 5–15 μg/ml Twin Pack Gold® ($0.05 > p < 0.001$) or Rainbow® ($p < 0.001$) was employed. A regression test showed that the PRI decreased as a function of the concentration of FLC ($r = -0.91$, $p < 0.01$), Twin Pack Gold® ($r = -0.98$, $p < 0.001$), or Rainbow® ($r = -0.97$, $p < 0.001$) titrated into cultures. Overall, Table 1 also shows that, for a given

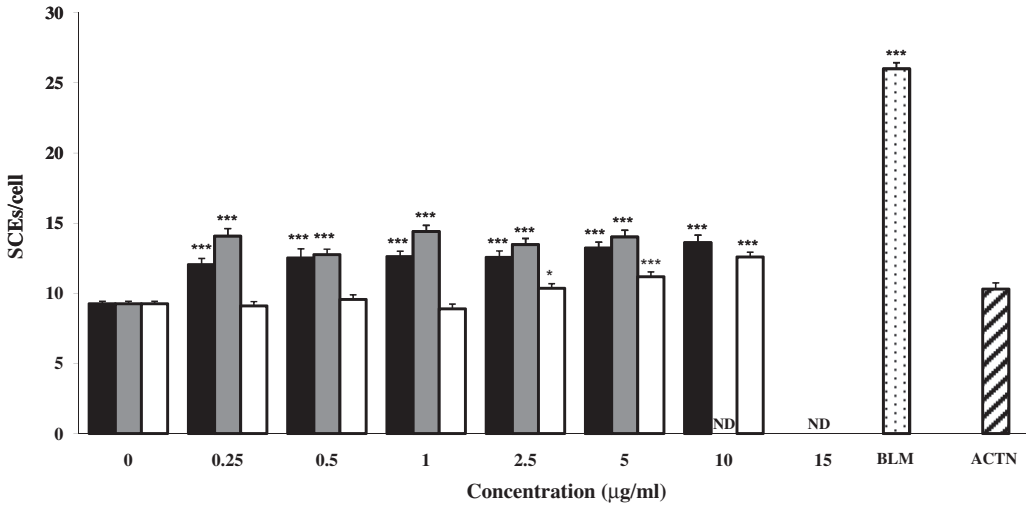

Fig. 1. Effect on *in vitro* treatment with flurochloridone (black bars), Twin Pack Gold® (gray bars), and Rainbow® (white bars) on SCE frequency of CHO-K1 cells. Cultures were harvested after 24 h treatment, and the frequencies of SCEs were determined in 75 M_2 mitoses for each experimental point. For each test compound, data are expressed as mean SCE ± SE (*y*-axis) from three independent experiments and plotted against the herbicide concentration (0–15 μg/ml concentration range; *x*-axis). BLM (1 μg/ml) and ACTN (0.5%) were used as positive (dotted bar) and solvent (stripped bar) controls, respectively. ND, not determined. $^{*}p < 0.05$; $^{***}p < 0.001$.

Table 1
Proliferative rate index (PRI), mitotic index (MI), and mitotic factor (*f*) values in control and flurochloridone-, Twin Pack Gold®-, and Rainbow®-treated CHO-K1 cells[a].

Concentration (μg/ml)		PRI	MI	*f*
	0	1.97 ± 0.88	85 ± 5.93	1.00 ± 0.00
Flurochloridone	0.25	1.94 ± 0.67	61 ± 2.65*	0.86 ± 0.06
	0.5	1.91 ± 1.00	74 ± 4.26	0.87 ± 0.10
	1	1.91 ± 1.20	72 ± 5.04	0.85 ± 0.06
	2.5	1.91 ± 1.19	66 ± 7.00*	0.78 ± 0.08
	5	1.86 ± 1.76	66 ± 8.00*	0.78 ± 0.11
	10	1.70 ± 1.33	53 ± 7.26***	0.63 ± 0.04
	15	1.03 ± 0.85***	39 ± 7.17***	0.46 ± 0.06
Twin Pack Gold®	0.25	1.93 ± 0.33	75 ± 3.33	0.88 ± 0.09
	0.5	1.91 ± 1.33	83 ± 6.67	0.98 ± 0.12
	1	1.90 ± 0.93	79 ± 8.37	0.94 ± 0.10
	2.5	1.84 ± 0.96	76 ± 2.96	0.89 ± 0.08
	5	1.68 ± 1.01*	67 ± 3.71*	0.79 ± 0.08
	10	1.12 ± 0.53***	56 ± 2.60***	0.65 ± 0.03
	15	1.00 ± 0.33***	43 ± 7.33***	0.51 ± 0.08
Rainbow®	0.25	1.84 ± 0.86	57 ± 3.71**	0.67 ± 0.02
	0.5	1.71 ± 0.19	53 ± 3.28***	0.62 ± 0.06
	1	1.78 ± 0.19	62 ± 2.08*	0.73 ± 0.03
	2.5	1.77 ± 0.19	52 ± 2.91 **	0.62 ± 0.08
	5	1.60 ± 1.03***	46 ± 2.60***	0.55 ± 0.07
	10	1.21 ± 0.88***	51 ± 2.31***	0.60 ± 0.06
	15	1.06 ± 1.03***	52 ± 2.52***	0.61 ± 0.07
BLM[b]	1	1.60 ± 0.02***	35 ± 0.30***	0.35 ± 0.03***
ACTN[c]		1.86 ± 0.33	82 ± 4.17	0.96 ± 0.03

[a] CHO-K1 cells were treated with flurochloridone, Twin Pack Gold® (25% a.i.), and Rainbow® (25% a.i.) and harvested 24 h later.
[b] Bleomycin (BLM, 1 μg/ml) was used as positive control.
[c] Acetone (ACTN, 0.5%) was used as solvent control.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ significant differences with respect to control values.

concentration, the ability of both FLC-containing technical formulations to induce a delay in cell-cycle progression was always higher than that exerted by FLC, and that both Twin Pack Gold® and Rainbow® were able to induce an equivalent PRI reduction (Table 1). When the MI was analyzed in FLC- and Twin Pack Gold®-treated CHO-K1 cells, a significant decrease in the mitotic activity was found. Doses higher than 2.5 μg/ml for FLC or 5 μg/ml for Twin Pack Gold® applied into the culture system revealed a significant inhibition of the MI value ($p < 0.05$ and $p < 0.001$, respectively). On the other hand, the commercial formulation, Rainbow®, induced a significant decrease in the MI with all concentrations assayed ($0.01 > p < 0.001$) (Table 1). A regression test showed that the MI decreased as a function of the concentration of FLC ($r = -0.96$, $p < 0.01$) or Twin Pack Gold® ($r = -0.98$, $p < 0.001$), but not Rainbow® ($r = -0.44$, $p > 0.05$) titrated into cultures (Table 1). Overall, Table 1 also shows that, for a given concentration, the capability of the technical formulation Rainbow® to induce an inhibition of the mitotic activity was higher than that of FLC or Twin Pack Gold® applied into the culture system. The MI of cultures decreased over control values ($f = 1.00$) by means of $f = 0.46 \pm 0.06$, 0.50 ± 0.08, and 0.61 ± 0.07 when 15 μg/ml of FLC, Twin Pack Gold®, and Rainbow® were employed, respectively (Table 1).

Statistically significant loss of lysosomal activity was observed between negative (untreated and ACTN-treated cells) and positive controls ($p < 0.001$) (Fig. 2). While no statistical alteration in the lysosomal activity was observed when CHO-K1 cells were exposed to all assayed concentrations of FLC and Twin Pack Gold® ($p > 0.05$), a significant cell growth inhibition was achieved only in those 10 and 15 μg/ml Rainbow®-treated cultures ($p < 0.05$). A regression test showed that Rainbow®-induced lysosomal activity decreased as a function of the herbicide concentration ($r = -0.88$, $p < 0.01$). Overall, the NR assay demonstrated that Rainbow® exerted more cytotoxic effect than FLC or Twin Pack Gold® (Fig. 2).

Fig. 3 summarizes the alterations in the energetic cell metabolism induced by FLC, Twin Pack Gold®, and Rainbow®. The results demonstrated a statistically significant depression in the MTT dye reduction to an insoluble violet formazan product in those ethanol-treated cultures (positive controls) compared with negative cultures ($p < 0.001$). Data presented in Fig. 3 show a cellular metabolism inhibition when CHO-K1 cells were exposed to 5–15 μg/ml FLC ($0.05 > p < 0.001$) or 2.5–15 μg/ml Twin Pack Gold® or Rainbow® ($0.01 > p < 0.001$). A regression test showed that the inhibition decreased as a function of the concentration of FLC ($r = -0.76$, $p < 0.05$), Twin Pack Gold® ($r = -0.87$, $p < 0.01$), or Rainbow® ($r = -0.97$, $p < 0.001$) titrated into cultures (Fig. 3). Overall, the MTT assay demonstrated that Twin Pack Gold® and Rainbow® exerted more cytotoxic effects than FLC.

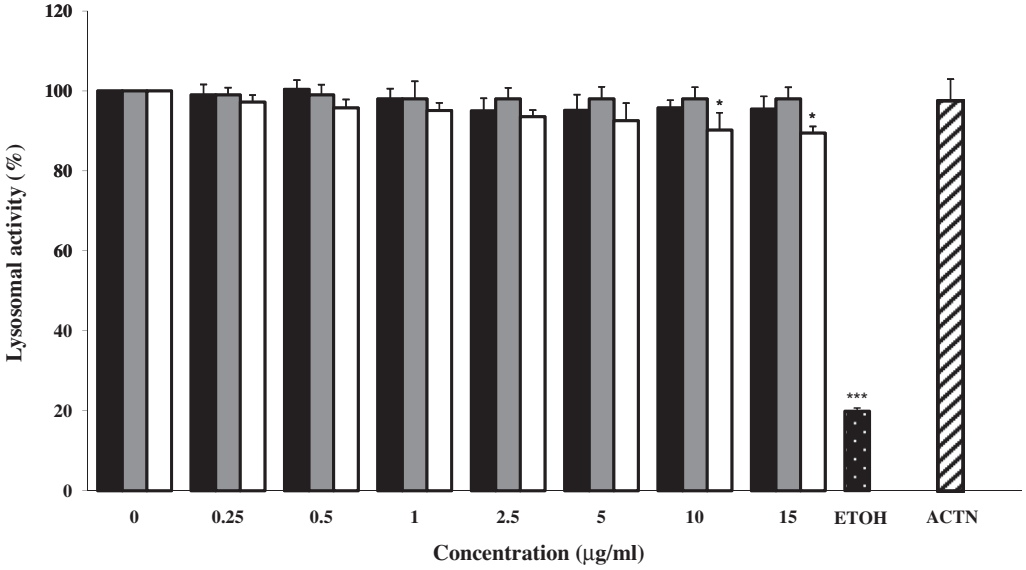

Fig. 2. Lysosomal activity assessed with the NR assay in flurochloridone- (black bars), Twin Pack Gold®- (gray bars), and Rainbow®-treated (white bars) CHO-K1 cells. Cultures were incubated for 3 h with NR dye after 24 h treatment. Results are expressed as the mean percentage of cell growth inhibition 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 (dotted bar) and 0.075% ACTN-treated (stripped bar) cells were used as positive and solvent controls, respectively. *$p < 0.05$; ***$p < 0.001$.

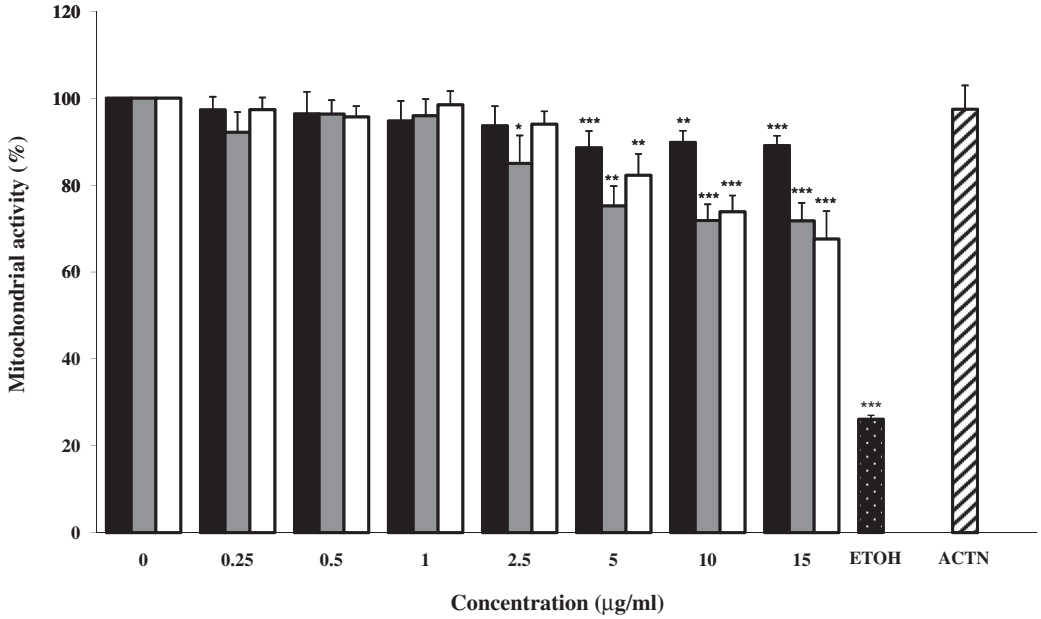

Fig. 3. Cellular metabolism inhibition evaluated by MTT assay in flurochloridone- (black bars), Twin Pack Gold®- (gray bars), and Rainbow®- (white bars) treated CHO-K1 cells. Cultures were incubated for 3 h with MTT after 24 h of herbicide treatment. Results are expressed as the mean percentage of cell growth inhibition 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 (dotted bar) and 0.075% ACTN-treated (stripped bar) cells were used as positive and solvent controls, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Discussion

In the current study, we evaluated the genotoxicity and cytotoxicity of the herbicide FLC as an active ingredient and the two commercial formulations, Twin Pack Gold® and Rainbow®, on CHO-K1 cells by analyzing different end points, i.e., SCE frequency, PRI, MI, MTT, and NR assays. FLC and Twin Pack Gold® induced a significant and equivalent increases in SCEs regardless of the concentration titrated into cultures (range, 0.25–15 μg/ml). However, the ability of Rainbow® to induce SCE was found only at concentrations higher than 2.5 μg/ml, and was lower than that of FLC and Twin Pack Gold®. For all compounds, the PRI decreased as a function of the concentration titrated into cultures. Whereas the highest concentrations of FLC (2.5–15 μg/ml) or Twin Pack Gold® (5–15 μg/ml) induced a significant mitodepressive activity, all concentrations of Rainbow® tested revealed a significant inhibition of the mitotic activity. Overall, the results demonstrated that while the compounds were not able to reduce the lysosomal activity, the mitochondrial succinate dehydrogenase activity was considerably diminished when the highest concentrations of the three compounds were employed (2.5–15 μg/ml). Therefore, the results demonstrated that all *in vitro* bioassays were sensitive enough to detect both genotoxic and cytotoxic properties exerted by the herbicide FLC and its two formulated products, at least on CHO-K1 cells.

FLC is a novel pyrrolidone herbicide classified as a slightly hazardous compound (class III) by the WHO (2009). Available data on FLC-induced genotoxicity and/or cytotoxicity are scarce. So far, only Yüzbasioglu et al. (2003) have reported its 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 micronuclei after FLC exposure (Yüzbasioglu et al., 2003).

To our knowledge, we have conducted the first *in vitro* genotoxic and cytotoxic evaluation of the herbicide FLC and two of its commercial formulations, Twin Pack Gold® and Rainbow®, on mammalian cells. As stated previously, our results reveal a significant induction of SCEs, but the level of genotoxicity, although significant with respect to the negative control, remained weak with all concentrations employed. This finding is in agreement with the genotoxic profile showed by other herbicides, e.g., atrazine. Genotoxicity effects exerted by atrazine have been studied in different cellular systems. Human lymphocyte cultures exposed to atrazine in the presence or absence of the S9 fraction revealed a weak genotoxic effect estimated by SCEs, chromosome aberrations, and micronuclei frequencies (Ribas et al., 1998). Similarly, other studies reported that atrazine did not appear to be genotoxic in *in vitro* human lymphocytes when either the comet and the diffusion assays (Zeljezic et al., 2006) or chromosome-level end points were performed (Kligerman et al., 2000; Malik et al., 2004). However, studies in other cellular processes pointed out atrazine as an endocrine disruptor. This latter effect has been observed in fish (Spano et al., 2004), amphibians (Hayes et al., 2002), reptiles (Crain et al., 1997), and mammals (Simić et al., 1991; Stoker et al., 2000). Usually, it is accepted that an endocrine disruptor plays a role in a variety of adverse health effects in an organism or its descendants as a consequence of changes in the endocrine system (Choia et al., 2004). Primary toxic side effects of endocrine disruptors were reported to be related to infertility, teratogenicity, carcinogenicity, and mutagenicity, among others (Choia et al., 2004). The mechanism of FLC action has not been yet determined, but some studies have shown that FLC induces Sertoli cell vacuolation in rats (EFSA, 2010). An alteration in the cellular metabolism of the latter cell type could result in a disruption in the hormonal control, thus showing a similar toxic effect than that of atrazine. Whether this hypothesis would be corroborated, a potential endocrine disruptor with activity similar to that of atrazine could then be suggested for FLC. Nevertheless, there are no further data allowing us to confirm or to discard this putative assumption.

With regard to cytotoxicity, all compounds showed a significant toxic response compared to their concurrent negative control when tested on CHO-K1 cells. The order of declining toxicity of the PRI and the MI values for each compound was FLC > Twin Gold Pack® > Rainbow®. Therefore, our results confirm previous reports indicating that FLC is able to exert cytotoxic effects (Yüzbasioglu et al., 2003). Yüzbasioglu et al. (2003) observed that the herbicide induced cytotoxicity when meristematic root cells of *A. cepa* were

exposed due to alterations in the normal cell-cycle progression and mitodepressive activity. Several investigations have reported that most agrochemicals have the ability to alter the cell cycle of eukaryotic cells (González et al., 2006, 2007; Soloneski et al., 2001, 2008; Soloneski and Larramendy, 2010). Although both protein and DNA synthesis are included as the major prerequisites for cell division (Alberts et al., 2004), there is no available information allowing us to suggest whether alterations in these processes are involved in FLC-induced cell-cycle delay.

When considering the eventual response of the cytotoxicity, measured by the NR uptake, often very low decreases in the proportion of viable CHO-K1 cells were noticed after FLC, Twin Pack Gold®, or Rainbow® exposure. It is accepted that a reduction in lysosomal activity reflects enhanced instability of the cell membranes (Borenfreund and Puerner, 1985; Jafari et al., in press; Molinari et al., 2009, 2010; Sharma et al., 2011). According to our current results, it can be claimed that the herbicide's deleterious effect on the integrity of mammalian cell membranes is negligible, at least in CHO-K1 cells.

As revealed by the MTT assay, the incubation of CHO-K1 cells with FLC, Twin Pack Gold®, or Rainbow® resulted in a significant reduction in the mitochondrial activity, particularly when higher concentrations were tested. Further investigations are required to obtain comprehensive knowledge of the possible mechanism(s) through which the agrochemical exerts this cytotoxic effect. Furthermore, our results highlight the importance of employing multiple end points for the determination of cytotoxicity in *in vitro* systems.

In our results, the genotoxic and cytotoxic comparisons were made at the same active ingredient concentrations, regardless of the added excipients. Overall, Twin Pack Gold® gave similar results to the active ingredient at the same concentration. Interestingly, and on the other hand, Rainbow® resulted in a marked cytotoxic effect compared with both FLC and Twin Pack Gold®. Numerous reports have revealed that the additive compounds present in a pesticide commercial formulation have the ability to induce cellular damage by themselves (González et al., 2006, 2007; Kaya et al., 1999; Molinari et al., 2009, 2010; Soloneski et al., 2001, 2008; Zeljezic et al., 2006). Unfortunately, the identities of the additive compounds present in the commercial formulations, Twin Pack Gold® and Rainbow®, were not made available to us by the manufacturers.

Finally, the present study has shown for the first time that FLC by itself and its two formulations, Twin Pack Gold® and Rainbow®, are genotoxic in mammalian cells *in vitro*, at least in CHO-K1 cells. Overall, Rainbow® was more cytotoxic than FLC and Twin Pack Gold®. Although the underlying mechanism of action is still beyond our knowledge, the genotoxicity and the undoubted cytotoxicity of the herbicide suggest the need for further studies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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