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Analysis of possible genotoxicity of the herbicide flurochloridone and its commercial formulations: Endo III and Fpg alkaline comet assays in Chinese hamster ovary (CHO-K1) cells

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ABSTRACT

Cytotoxic and genotoxic effects of flurochloridone (FLC) and its formulations Twin Pack Gold® and Rainbow® were evaluated in CHO-K1 cells. Using the alkaline single-cell gel electrophoresis (SCGE) assay, we observed that FLC (15 μg/ml), Twin Pack Gold® or Rainbow® induced primary DNA damage, increasing the frequency of damaged nucleoids. Vitamin E pretreatment did not modify the effect. Decreased cell viability was observed only in Twin Pack Gold®-treated cultures and was significantly ameliorated by vitamin E. Post-treatment of herbicide-damaged CHO-K1 cells with the enzymes Endo III or Fpg did not increase FLC-, Twin Pack Gold®-, or Rainbow®-induced DNA damage. These results demonstrate that neither FLC nor FLC-based formulations induce DNA damage through hydroxyl radical or lipid alkoxyl radical production, and that the induced DNA lesions were not related to oxidative damage at the purine/pyrimidine level. Our observations strongly suggest that the cytotoxic effects observed after Twin Pack Gold® exposure are due to the excipients contained within the technical formulation rather than FLC itself.

1. Introduction

Herbicide use is an effective and economical strategy for controlling weeds [1]. Use of herbicides is increasing, regardless of the development of herbicide-tolerant crops, environmental contamination, hazards to non-target species, including humans [2,3].

Flurochloridone (FLC), [3-chloro-4-(chloromethyl)-1-[3-(trifluoromethyl)phenyl]-2-pyrrolidone], is a pyrrolidone herbicide applied worldwide, including in Argentina, to control broadleaf weeds and annual grasses among several crops, including grains, umbelliferous crops, sunflowers, and potatoes. It is a selective pesticide, absorbed by roots and stem, causing bleaching of the leaves by interference with biosynthesis of carotenoid, chlorophyll, and abscisic acid metabolites [4,5]. According to the European Food Safety Authority [6], FLC has no genotoxic, carcinogenic, or neurotoxic potential. However, while considerable information is available about the environmental and ecological effects of this herbicide [6–8], little information on its genotoxicity and/or cytotoxicity has been reported. Yüzbasioglu et al. [9] observed that the FLC-based commercial formulation Racer® induced abnormal cell-cycle progression and cellular mitodepressive activity in *Allium cepa* root meristematic cells. Various alterations were found in the meristematic root-tip cells after herbicide exposure, including c-metaphase, multipolarity, polyploidy, chromosome lagging, chromosomal stickiness, chromosome breaks, bridges, fragments, sister union, and micronuclei (MNs). The percentage of total abnormalities increased gradually with extent of treatment [9].

Recently, we reported the effects of the pure herbicide and its formulations Twin Pack Gold® and Rainbow®, examining several endpoints for genotoxicity and cytotoxicity in Chinese hamster ovary (CHO-K1) cells [10,11]. For all compounds, we saw significant increases in sister chromatid exchange frequencies and DNA single-strand breaks, delay in cell-cycle progression, alterations in mitotic activity, and cellular growth inhibition after *in vitro* exposure [10,11]. The herbicide not only exerted cytotoxicity and genotoxicity, but also triggered apoptosis in liver hepatocellular carcinoma HepG2 cells [12].

Oxidative stress has been defined as disturbance in the prooxidant–antioxidant steady-state, resulting in potential cell damage [13]. Antioxidants protect cells against the deleterious effects of many toxicants [14–16]. Antioxidant vitamins can inactivate free radicals generated during metabolism [17–19]. Vitamin E

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(vitE) is a family of ten lipid-soluble compounds that include tocopherols and tocotrienols. Of the many forms of vitE, α-tocopherol is the most biologically active [20]. The protective effects of vitE have been examined in various biological models [18,19,21]. Furthermore, vitE was found to protect against pesticides both *in vitro* and *in vivo* [22–25].

The mechanism by which FLC induces genotoxic and cytotoxic damage has not been elucidated. It may be associated with reactive oxygen species (ROS) generation [26] but no data are available concerning possible ameliorating effects of antioxidant compounds or radical scavengers. The single-cell gel electrophoresis (SCGE) assay is a simple, sensitive, and fast procedure that detects primary DNA lesions in any eukaryotic cell type after xenobiotic exposure, and can be used to identify and quantify short-lived DNA damage [27,28]. To achieve more specific information about the type of DNA damage evaluated by the SCGE assay, a modification of the method was developed that incorporates digestion with lesion-specific enzymes. The endonucleases most commonly used in this manner are the bacterial enzymes endonuclease III (Endo III, also known as Nth) and formamidopyrimidine DNA-glycosylase (Fpg). Endo III recognizes oxidized pyrimidines, including thymine glycol and uracil glycol [29,30]. Fpg is a glycosylase involved in the first step of base excision repair. The enzyme recognizes and removes a wide range of oxidized purines from damaged DNA, including 8-oxo-7,8-dihydroguanine (8-oxo-Gua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 4,6-diamino-5-formamidopyridine (FapyAde); its AP lyase activity leaves an APsite that can be detected by the comet assay [29,30].

The aim of this study was to investigate, using the alkaline SCGE assay, the mechanisms that underlie FLC-induced cytotoxicity and genotoxicity in CHO-K1 cells. The ability of vitE to modulate the DNA-damaging effect exerted by this herbicide was examined. To assess the role of oxidative DNA damage in FLC genotoxicity, we employed Endo III and Fpg in combination with the SCGE assay.

2. Materials and methods

2.1. Chemicals

Flurochloridone (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 and hydrogen peroxide were purchased from Merck KGaA (Darmstadt, Germany), and α-tocopherol (vitE; CAS 10191-41-0) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Endo III and Fpg were purchased from New England Biolabs (Ipswich, MA, USA). Bleomycin (BLM; Blocamycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina). All other chemicals and solvents of analytical grade were purchased from Sigma Chemical Co.

2.2. Cell cultures and herbicide treatment for the viability and alkaline single cell gel electrophoresis assays

CHO-K1 (CCL-61; American Type Culture Collection, Rockville, MD, USA) cells were grown in Ham's F-10 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 10 μg/ml streptomycin (all from Invitrogen, Grand Island, NY, USA) at 37 °C in a 5% CO_2 atmosphere. The cells were seeded in 35 mm Petri dishes at 3.5×10^5 cells/ml in final medium volume = 3 ml. Cells were treated with an equimolar concentration of 15 μg/ml of FLC, Twin Pack Gold®, or Rainbow® for 80 min. The concentration employed in this study was selected from our previous investigation, employing 1–15 μg/ml FLC, considering that 15 μg/ml did induce significant DNA damage after 80 min of exposure in less than 50% of the nucleoids analyzed by the comet assay [11]. Prior to use, FLC was dissolved in acetone and diluted in culture medium, whereas Twin Pack Gold® and Rainbow® were diluted directly in culture medium. The final solvent concentration was <1% for all treatments. 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 cultures were performed in duplicate for each experimental point. To evaluate the effect of vitE, CHO-K1 cells were preincubated with antioxidant following the protocol of Lazarová and Slameňová [21] with minor modifications. Accordingly, when required, samples were preincubated with vitE (50 μg/ml, 2 h), whereas control samples were kept in fresh culture medium without vitE. VitE was used at a nontoxic concentration and was first dissolved in ethanol. The final concentration of ethanol in the experimental treatments and control sets was 0.1%.

Immediately after the 2 h preincubation period in the presence of vitE and the 80 min pulse herbicide treatment, the SCGE assay was performed. Cells were detached with a rubber policeman, centrifuged, and then resuspended in complete culture medium. The SCGE assay was performed following the alkaline procedure described by Singh [31] with minor modifications as reported elsewhere [11]. The slides were immersed in ice-cold freshly prepared lysis solution for 1 h in darkness (1% sodium sarcosinate, 2.5 m NaCl, 100 mm Na_2EDTA, 10 mm Tris, pH 10.0, 1% Triton X-100, and 10% DMSO). 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 (0.8 V/cm). Finally, the slides were neutralized with a solution comprising 0.4 m Tris–HCl, pH 7.5, stained with DAPI (4′,6′-diamino-2-phenylindole; 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 100 randomly selected and non-overlapping nucleoids, as suggested previously [32]. DNA damage was classified in five classes (0–I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously [33]. Results are expressed as the mean number of damaged nucleoids (sum of classes II–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 [34].

2.3. Cell viability assay

At the end of the culture period, cell viability was determined using the ethidium bromide/acridine orange assay [35]. Briefly, a 1:1 freshly prepared mixture of ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml), 10 μl, was mixed with the cell suspension (10 μl). 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. At least 500 cells were counted per experimental point, and results are expressed as the percentage of viable cells among all cells.

2.4. Cell cultures and herbicide treatment for the modified alkaline single cell gel electrophoresis assay

Normal-melting agarose 1% (200 μl) was transferred onto 100% ethanol precleaned slides and placed at 37 °C for 20–30 min. Afterward, 2×10^4 CHO-K1 cells resuspended in 150 μl 1% low-melting agarose was applied, covered with a coverslip, and placed at 4 °C for 10 min. Then, the slides were treated with FLC, Twin Pack Gold®, or Rainbow®, 50 μl, 15 μg/ml, for 80 min at room temperature in a humid atmosphere. Negative controls (PBS and solvent vehicle-treated cells) and positive controls (25 μm H_2O_2, 5 min, 4 °C) were run simultaneously with herbicide-treated cultures. Each experiment was repeated three times, and the cultures were performed in duplicate for each experimental point.

The modified comet assay was performed following the procedure described by Collins et al. [36,37]. After lysis, the slides were washed three times in an enzyme buffer (40 mm HEPES, 1 m KCL, 0.5 mm EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0) for 5 min each at room temperature. Then they were drained and exposed to Endo III or Fpg diluted 1:1000 or 1:3000, respectively, following recommendations of the enzyme supplier. Briefly, slides were incubated with 50 μl Endo III (0.5 U) or Fpg (0.13 U), as suggested elsewhere [38,39]. Control cells were treated with a 50 μl aliquot of the corresponding enzyme buffer. After incubation for 30 or 45 min at 37 °C for Endo III- or Fpg-exposed samples, respectively, the slides were processed following the conventional alkaline SCGE protocol (Section 2.4). Analysis of the slides was performed as described in Section 2.4. Net oxidative DNA damage (OD) was obtained for each treatment using the formula OD = [(%GDI buffer + enzyme + herbicide) − (% GDI buffer + herbicide)], as indicated previously [40–43].

2.5. Statistical analysis

The data were analyzed using Statgraphics 5.1 Plus software. 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 and viability in treated and control cells were evaluated by χ^2 test. The chosen level of significance was 0.05 unless indicated otherwise.

3. Results

Table 1 shows the results of the analysis of DNA damage and viability bioassays in CHO-K1 cells treated for 80 min with FLC, Twin Pack Gold®, or Rainbow®, in the presence or absence of vitE. An increased frequency of damaged nucleoids was observed in BLM-treated cultures (positive control) compared to negative control values ($p < 0.001$). Acetone treatment (FLC solvent control) did not alter the frequency of damaged nucleoids, compared with negative control values ($p > 0.05$). An enhancement in the frequency of damaged nucleoids was observed in cultures treated with equimolar concentration (15 μg/ml) of FLC, Twin Pack Gold®, or Rainbow® compared with negative control cultures ($p < 0.001$).

The effect of pretreatment of the cells with vitE prior to xenobiotic exposure was studied. Table 1 shows that pretreatment with vitE did not alter the basal level of damaged nucleoids in negative control or acetone-treated cultures ($p > 0.05$). On the other hand, vitE significantly reduced the level of DNA damage induced by BLM (positive control; $p < 0.05$). Data from Table 1 also demonstrate that preincubation with vitE prior to FLC, Twin Pack Gold®, or Rainbow®

Table 1
Analysis of DNA damage and viability measured on CHO-K1 cells exposed for 80 min to 15 μg/ml of Flurochloridone (FLC), Twin Pack Gold® and Rainbow® with and without vitamin E.

Compound	DNA damage (%) (II + III + IV)[a]						Viability (%)					
	Exp. 1		Exp. 2		Exp. 3		Pooled data[b]		Exp. 1		Exp. 2		Exp. 3		Pooled data[b]	
	(−) VitE	(+) VitE[d]	(−) VitE	(+) VitE[d]	(−) VitE	(+) VitE[d]	(−) VitE	(+) VitE[d]	(−) VitE	(+) VitE[d]	(−) VitE	(+) VitE[d]	(−) VitE	(+) VitE[d]	(−) VitE	(+) VitE[d]
Negative control	24	26	20	24	19	22	21 ± 1.28	24 ± 0.62	98.50	99.00	100.00	99.50	99.50	95.50	99.40 ± 0.49	98.60 ± 1.56
ACTN[c]	24	18	18	30	20	25	21 ± 1.76	24 ± 3.48	98.50	98.50	99.50	99.50	97.50	97.00	98.50 ± 1.15	98.33 ± 1.45
FLC	32	30	24	40	50	52	35 ± 7.69***	41 ± 7.33***	99.50	97.50	99.00	95.00	100.00	99.50	99.50 ± 0.57*	97.33 ± 2.60
Twin Pack Gold®	34	22	40	48	48	44	41 ± 4.06***	38 ± 8.08***	83.80	97.50	78.12	96.05	75.00	95.71	83.45 ± 8.50*	97.17 ± 1.50#
Rainbow®	44	60	46	46	66	42	52 ± 7.02***	49 ± 5.46***	98.50	99.00	98.00	99.00	96.00	97.50	97.37 ± 2.66	98.12 ± 1.03
Positive control[e]	83	63	81	67	82	65	82 ± 0.91***	65 ± 1.86***#	99.50	98.50	98.50	98.50	99.00	98.50	99.00 ± 0.50	98.5 ± 0.00

[a] II–IV indicate grades of DNA damage estimated by comet assay.
[b] Results are presented as mean values of pooled data from three independent experiments ± S.E. of the mean.
[c] Acetone (ACTN, 0.1%) was used as FLC-solvent control.
[d] VitE (α-tocopherol, 50 μg/ml) was used as antioxidant compound.
[e] Bleomycin (10 μg/ml) was used as positive control.
* $p < 0.05$.
*** $p < 0.001$, compared with respective control values.
# $p < 0.05$; differences between VitE treated- and VitE non-treated samples.

Table 2
Analysis of DNA damage measured by comet assay after treatment of CHO-K1 cells with 15 μg/ml of Flurochloridone (FLC), Twin Pack Gold® and Rainbow® with or without incubation with lesion-specific endonuclease Endo III and Fpg.

Compound	Treatment	Proportion of damaged nucleoids (%)[a]				DNA damage (%)[b]	GDI[c]	OD[d]
		Type I	Type II	Type III	Type IV	(II + III + IV)		
Negative control		90.00	9.00	1.00	0.00	10.00 ± 0.88	1.11	
	Endo III buffer	74.00	20.00	5.00	1.00	26.00 ± 7.67	1.35*	
	Endo III	80.00	11.00	6.00	3.00	20.00 ± 6.65	1.32*	0.03
	Fpg buffer	82.00	13.00	3.00	2.00	18.00 ± 5.35	1.25	
	Fpg	77.00	15.00	8.00	1.00	23.00 ± 5.36	1.33*	0.08
ACTN[e]		90.67	8.67	0.33	0.33	9.33 ± 0.88	1.10	
	Endo III buffer	41.00	51.00	6.00	2.00	59.00 ± 4.00	1.69**	
	Endo III	45.00	37.50	12.00	5.50	55.00 ± 4.18	1.78***	0.09
	Fpg buffer	65.33	18.67	6.33	9.67	34.67 ± 3.52	1.60**	–
	Fpg	66.33	15.33	7.33	11.00	33.67 ± 4.91	1.63**	0.03
FLC		67.00	31.00	1.00	1.00	33.00 ± 4.25	1.37**	
	Endo III buffer	36.00	45.33	15.00	3.67	64.00 ± 3.78	1.86***	–
	Endo III	31.00	42.67	17.33	9.00	69.00 ± 5.29	2.04***	0.18
	Fpg buffer	54.00	21.00	10.67	14.33	46.00 ± 6.65	1.85***	–
	Fpg	54.67	23.00	10.67	11.67	45.33 ± 4.33	1.79***	0.06
Twin Pack Gold®		49.83	33.78	8.36	8.03	50.17 ± 1.02	1.74***	
	Endo III buffer	38.16	44.74	10.53	6.58	61.85 ± 2.93	1.86***	–
	Endo III	37.22	42.39	9.06	11.33	62.78 ± 3.23	1.94***	0.08
	Fpg buffer	38.89	45.10	8.17	7.84	61.11 ± 1.18	1.85***	–
	Fpg	38.87	44.20	8.15	8.78	62.13 ± 1.50	1.87***	0.02
Rainbow®		63.33	25.67	5.00	6.00	36.67 ± 7.83	1.54***	
	Endo III buffer	56.67	26.00	12.67	4.66	43.33 ± 4.37	1.65**	–
	Endo III	52.33	38.34	9.33	0.00	47.67 ± 5.66	1.57*	0.08
	Fpg buffer	57.00	25.33	8.00	9.67	43.00 ± 4.00	1.70***	–
	Fpg	52.33	30.33	8.33	9.00	47.67 ± 4.91	1.74***	0.04
Positive control[f]		42.00	28.00	20.00	10.00	58.00 ± 1.15	1.98***	
	Endo III buffer	21.00	32.50	26.00	20.50	79.00 ± 1.00	2.46***	–
	Endo III	9.50	34.50	28.00	28.00	90.50 ± 1.50	2.75***	0.29#
	Fpg buffer	41.00	40.00	12.00	7.00	59.00 ± 4.04	1.85***	–
	Fpg	17.33	62.00	15.33	5.33	82.67 ± 5.36	2.09***	0.24#

[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] Net oxidative damage (OD).
[e] Acetone (ACTN, 0.1%) was used as solvent control.
[f] Hydrogen peroxide (H_2O_2, 25 μM) was used as positive control.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$; compared with respective control.
# $p < 0.05$, compared with buffer enzyme treated-cells.

treatment did not alter the frequency of damaged nucleoids compared with control cultures ($p > 0.05$).

Cell viability was assessed in FLC-, Twin Pack Gold®-, and Rainbow®-treated CHO cells with and without preincubation with vitE. The results are presented in Table 1. Whereas FLC and Rainbow® did not alter cell viability ($p > 0.05$), a significant decrease in the cellular viability was achieved in Twin Pack Gold®-treated cultures ($p < 0.05$). Finally, when the cells were pretreated with vitE, the results demonstrated that the viability was significantly ameliorated only in cultures treated with Twin Pack Gold® ($p < 0.05$), but not in cultures treated with FLC or Rainbow® ($p > 0.5$; Table 1).

Data from the Endo III- and Fpg-modified comet assays obtained in CHO-K1 cells exposed during 80 min with FLC, Twin Pack Gold®, or Rainbow® are presented in Table 2, and the levels of net oxidative DNA damage are presented in Fig. 1. To verify the ability of Endo III and Fpg to recognize oxidized bases in our test system, cells were incubated with 25 μm H_2O_2 and employed as positive controls. H_2O_2 treatment induced an enhancement in the frequency of damaged cells ($p < 0.001$), the GDI ($p < 0.001$), and the net oxidative DNA damage ($p < 0.05$) values in cells treated with both Endo III and Fpg compared with the cells not treated with these enzymes (Table 2 and Fig. 1). The treatment with both Endo III and Fpg endonucleases did not produce any significant difference in the level of DNA damage and GDI in cells exposed to FLC, Twin Pack Gold®, or Rainbow® compared with cells without treatment with the enzymes (enzyme buffer-treated cells) ($p > 0.05$; Table 2 and Fig. 1).

4. Discussion

Herbicide use is increasing in many countries where tillage for weed control is being minimized to conserve natural resources and improve environmental conditions. However, the consumption of herbicides is of concern for human health and environmental risks. FLC is a pyrrolidone-selective herbicide used worldwide as a complement to glyphosate in crops, due to the increasing prevalence of glyphosate-resistant weeds, to control many broadleaf weeds and annual grasses [44]. In Argentina, according to the last record reported by the National Food Safety and Quality Service, annual importation of FLC into the country has increased to values as high as 390,140 kg [45]. Despite its generalized use, toxicological information for FLC has been poorly documented.

In the present study, the comet assay was used to analyze the damage inflicted on the DNA from of CHO-K1 cells by FLC and two of its commercial formulations most commonly employed in Argentina, namely, Twin Pack Gold® and Rainbow®. The results demonstrated that both the active ingredient and the commer-

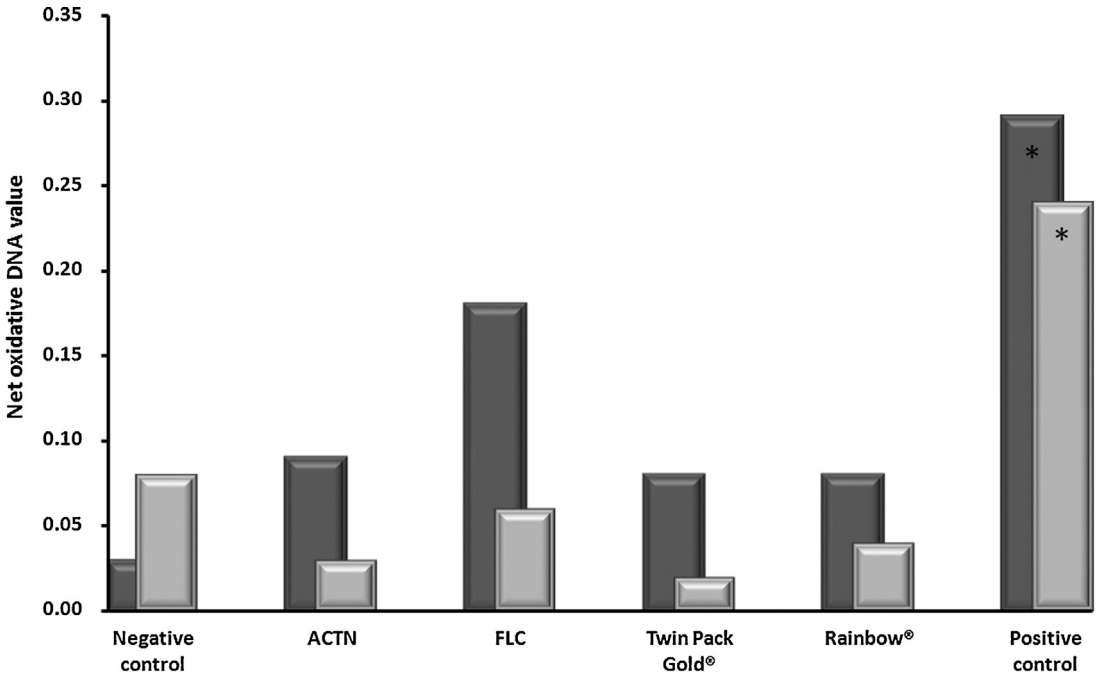

Fig. 1. FLC-, Twin Pack Gold-®, and Rainbow®-induced DNA damage in CHO-K1 cells as evaluated by the modified comet assay using Endo III (dark gray bars) and Fpg (light gray bars) enzymes. Net oxidative DNA damage was calculated as the difference between the scores obtained after incubation with the respective enzymes and with the buffer. Hydrogen peroxide and acetone were used as positive and solvent controls, respectively. *, $p < 0.05$; **, $p < 0.01$; significant differences with respect to control values.

cial formulations were able to induce primary DNA lesions at an equimolar concentration of 15 μg/ml after a short (80 min) pulse treatment. The results obtained in our study are in agreement with our previously reported observations. We recently demonstrated that FLC, Twin Pack Gold®, and Rainbow® induced DNA damage evaluated by comet assay in CHO-K1 and HepG2 cells exposed to 1–15 μg/ml concentration range [11,12]. The effects of the commercial formulations were also studied *in vivo* in anuran circulating erythrocytes of *Rhinella arenarum* tadpoles. We observed that whereas both Twin Pack Gold® and Rainbow® increased the frequency of primary DNA lesions, estimated by alkaline comet assay, only the latter FLC-based formulation induced DNA damage at chromosomal level, estimated by an increased frequency of micronucleated erythrocytes [46].

We also studied the ability of FLC to induce DNA damage and cytotoxicity in the presence of vitE. Several reports demonstrate that this antioxidant prevents reactive oxygen species-induced genotoxic and cytotoxic damage [18,21–23,47]. In our study, vitE was able to reverse the cytotoxic effect exerted by Twin Pack Gold® on CHO-K1 cells. In contrast, we did not find any protective effect of vitE against the genotoxic effect exerted by FLC or by the two FLC-based formulations assayed. Hence, a damaging mechanism mediated by oxidation may be hypothesized for Twin Pack Gold®, since vitE prevented cell death. A plausible explanation for the observed non-vitE protection against genotoxic damage could be related to the inconsistent effects of vitE on oxidative damage in *in vitro* systems [48]. The chemical composition of culture medium and the concentration of antioxidants that can be applied to the culture system are factors confounding the interpretation of *in vitro* results [49]. Preincubation with vitE, 2 h, 50 μg/ml, is not sufficient to protect cells against damage induced by the xenobiotics. The possibility that FLC does not exert genotoxicity through mechanism(s) involving lipid peroxidation cannot be ruled out. Additional studies are needed to investigate the protective effect of vitE against DNA damage induced by FLC.

The sensitivity and specificity of the comet assay can be increased by incubating the cells with lesion-specific endonucleases that recognize particular damaged bases [37]. The utility of this modified method has been well demonstrated [50–53]. In the present study, our data indicate that oxidized pyrimidines and purines are not involved in the induction of primary DNA damage after FLC-, Twin Pack Gold®-, or Rainbow®-exposed CHO-K1 cells.

Our current data are consistent with previous studies and strongly suggest that the mechanisms by which Twin Pack Gold® induces both cytotoxicity and genotoxicity in CHO-K1 cells may be attributable to the presence of xenobiotic(s) included in the excipients of the FLC-based commercial formulation rather than to the active ingredient. The commercial formulation Twin Pack Gold® increased MN frequency in HepG2 and CHO-K1 cultures, whereas neither FLC nor the FLC-based herbicide Rainbow® was able to exert such a genotoxic effect [11,12]. Furthermore, cellular growth inhibition was studied in both cell lines, and the results demonstrated that Twin Pack Gold® was more cytotoxic than both the active ingredient and the formulated herbicide Rainbow® [10,12]. Unfortunately, the origin, identity, and purity of the additive compounds present within the excipient of the commercial formulation Twin Pack Gold® have not been made available to us by the Argentinean manufacturer. According the our administration, there is no requirement for the producer to inform the user of the actual composition of the excipients by listing them on the agrochemical data sheet, which can, thus, be kept as “trade secret”. Several investigations have revealed that the additive xenobiotics present in pesticide commercial formulations can exert cellular damage [54–64]. In agreement, the U.S. Environmental Protection Agency [65,66] has observed that the acute toxicity of a technical active ingredient can differ significantly from that of the end-use formulation containing that active ingredient. Our results are in agreement with this concept.

The molecular mechanisms of FLC-induced genotoxicity and cytotoxicity are not fully known. FLC could damage several cellular targets via ROS generation [26]. Similar to our findings, positive induction of oxidative stress by a FLC-based commercial formulation was reported, although using different endpoints and matrices, by Kaya and Yigit [26,67]. The authors reported that FLC was

able to induce biochemical changes through the generation of ROS in leaves of *Vicia sativa*, increasing the glutathione content and the glutathione transferase and glutathione reductase activities [26]. Furthermore, FLC was also reported to affect both the level of the antioxidant enzymatic defense system and content of malondialdehyde and endogenous salicylic acid in cultured sunflower plants *Helianthus annuus* [67]. Furthermore, recently, Xu et al. [68] suggested that FLC could damage the testes of adult rats by inducting oxidative stress. The authors reported that FLC can induce seminiferous epithelial degeneration, Sertoli cell vacuolization, spermatogenic cell loss, and nuclear damage in rats exposed to 750 mg/kg FLC [68].

Finally, rather than corroborate previous studies related to the induction of DNA strand breaks [11,12] the current results indicate that oxidative stress is mediated in the development of DNA damage caused by the commercial formulation Twin Pack Gold®, but not mediated by purine/pyrimidine oxidation, which could be, most probably, induced by the excipient(s) present in this product, but not in the damage induced by FLC by itself or when present in other technical formulations (at least, Rainbow®). We suggest that the mediation of free radicals in DNA damage induced by the active ingredient FLC is not significant or, if present, is below the threshold limit of the methods we employed for analyses. Further studies are needed to understand the mechanism by which FLC induces DNA damage, at least in CHO-K1 cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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