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Cytotoxic and genotoxic profiles of the pyrethroid insecticide lambda-cyhalothrin and its microformulation Karate® in CHO-K1 cells

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ABSTRACT

Lambda-cyhalothrin (LCT) and its microformulation Karate® (25 % a.i.) were analysed for its genotoxicity and cytotoxicity on Chinese hamster ovary (CHO-K1) cells. Cytokinesis-block micronucleus cytome (CBMN-cyt) and alkaline single-cell gel electrophoresis (SCGE) bioassays were selected to test genotoxicity. Neutral red uptake (NRU), succinic dehydrogenase activity (MTT) and apoptogenic induction were employed for estimating cytotoxicity. Both compounds were analysed within a concentration range of 0.1–100 μg/mL. Only LCT produced a significant augment in the frequency of micronuclei (MNs) when the cultures were exposed to highest concentrations of 10 and 100 μg LCT/mL. A noticeable decrease in NDI was observed for cultures treated with LCT at 10 and 100 μg/mL. Karate® induced the inhibition of both the proportion of viable cells and succinic dehydrogenase activity and triggered apoptosis 24 h of exposition. Whilst an increased GDI in CHO-K1 cells was observed in the treatments with 1–100 μg Karate®/mL, the GDI was not modified in the treatments employing LCT at equivalent doses. SCGE showed that Karate® was more prone to induce genotoxic effects than LCT. Only 50 μg/mL of Karate® was able to increase apoptosis. Our results demonstrate the genomic instability and cytotoxic effects induced by this pyrethroid insecticide, confirming that LCT exposure can result in a severe drawback for the ecological equilibrium of the environment.

1. Introduction

The extensive use of pesticides in agricultural production and the control of disease vectors are remarkable parameters of the cultivation strategies in different countries around the world. In 2018, the Food and Agriculture Organization of the United Nations (FAO) informed that the total pesticide trade was estimated at 5.9 million tons, whereas the total pesticide usage reached up to 3.5 million T in 2020 [1,2]. The pesticide industry produces more than 800 active ingredients to manufacture 40, 000 commercial formulations employed in the agricultural sector [2]. Environmental risk assessment associated with the production, storage as well as use and misuse of pesticides plays a decisive role in regulatory decisions since both existing and new compounds should be continuously evaluated to guarantee their appropriate specific safety standards [3]. Far to be true, however, several epidemiological and molecular studies pinpoint that pesticides are closely linked with acute and chronic diseases [4]. In terms of environmental risk and human health, numerous pesticides have been considered as chemical mutagens able to inflict damage to the DNA not only in humans but also in other living organisms. Epidemiological studies have shown that active ingredients possess genotoxic capabilities, increasing the risk of mutations, chromosomal aberrations and/or DNA single- or double-stranded breaks, linked with carcinogenic mechanisms, based upon data reported for mammals, including human beings, and other living species [5,6].

Pyrethroid insecticides are synthetic analogues of natural pyrethrins and encompass a group of six esters of chrysanthemic acid, which have been obtained from the plant pyrethrum *Tanacetum cinerariifolium* since ancient China because of their insecticidal properties. Since the 1970s, synthetic pyrethroids have been developed by complex structural chemical modifications [4]. Lambda-cyhalothrin (LCT) is a member of a class of pyrethroid insecticides. It encompasses one enantiomeric pair of isomers and represents the most biologically active form of cyhalothrin [3]. This compound possesses both insecticide and acaricide properties employed to control an extensive variety of insect pests and diseases. The insecticidal activity of LCT targets the voltage-gated sodium channels in electrically excitable cells, overexciting the nervous system; eventually, the target insect is paralysed and dies quickly [3]. Based on its acute toxicity, LCT has been defined as a ‘‘moderately hazardous’’

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(Class II) substance by the World Health Organization [4]. The U.S. EPA classifies LCT as a chemical ranked in "Group D" since its ability to cause cancer has not been yet determined [7]. Reported data showed that LCT was found in food, usually, less than 0.2 mg/kg and an acceptable daily intake for humans has been set at 0.02 mg/kg body weight [7]. U.S. EPA has established the reference dose at 0.001 mg/kg/day in chronic studies taking into account neurotoxicity symptoms [7]. In rats, LCT is moderately toxic and its acute oral LD50 ranges from 56 to 79 mg/kg body weight [7]. In mice, LCT is extremely toxic and the acute oral LD50 is 19.9 mg/kg body weight [7]. Concentrations of LCT were found in bovine blood, milk and meat [3], and LCT induces a number of toxic side effects, including hepatic and renal toxicity in rats [7]. Other investigations indicated that in rats, LCT affects renal enzyme activity as altered malondialdehyde and protein carbonyl levels, reduced glutathione (GSH) levels and significant changes in renal histopathology were reported [8]. In addition, LCT produces considerable increases in brain, kidney and liver weight and changes in functional alterations in the thyroid glands of rats [7]. However, in rabbits and guinea pigs, LCT did not induce skin irritation and sensitisation, respectively, and is considered a low-toxic compound for skin effects in these mammals [7]. When dogs were chronically fed with LCT, toxicity was observed at 3.5 mg/kg/day and the NOAEL was 0.5 mg/kg [7]. One of the most extensively used *in vitro* methodologies for detecting and quantifying DNA lesions at the chromosomal level is the cytokinesis-block micronucleus cytome (CBMN-cyt) technique, where micronuclei (MN) are identified in binucleated cells at the individual cell level [9–12]. Other biomarkers, as the quantification of nucleoplasmic bridges (NPBs), nuclear buds (NBUDs) as well as necrosis and apoptosis ratios, can also be determined at the same platform of analysis [9,12]. Several investigations have validated that there is a positive relationship between augmented MNs, NPBs and NBUDs and carcinogenicity [13,14].

The alkaline SCGE assay, also recognised as the comet assay, was first developed and optimised by Singh and co-workers [15]. It is a sensitive, reliable and fast approach for estimating DNA lesions/repair in eukaryotic isolated cells and can be employed in a variety of applications [15,16]. It reveals single and/or double DNA-strand breaks, alkali-labile sites (apurinic/apyrimidinic sites), DNA-DNA/DNA-protein cross-linking as well as incomplete excision repair sites [15]. The SCGE, therefore, represents a promising methodology for predicting responses to xenobiotics that are affected by DNA structure in areas such as genotoxic biomonitoring, ecological testing, human diseases and cancer studies [16,17].

Various cytotoxicity methods are routinely used to identify chemicals with toxic properties [18]. Among them, the neutral red uptake (NRU) and the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) methodologies are single colorimetric tests used for determining cell proliferation and lethal cytotoxicity in monolayer cultures [19–22]. Overall, the toxicity of xenobiotics leading to cell death can include a necrotic or apoptotic critical process that depends on both the cell type and the nature and dose of the death trigger [23]. In general terms, uncontrolled rather than programmed cell death implies the main pathway involved in cell death [23]. Unlike necrosis, apoptosis encompass a coordinated and energetic-dependent pathway that involves the activation of a series of cysteine-aspartic proteases recognised as caspases [23,24]. Several environmental toxins inflict their toxic processes by the regulation of apoptosis signalling, such as different chemicals, pesticides, pharmaceutical residues and heavy metals [20,22,24–26].

In genotoxic studies, the appliance of a battery of bioassays is widely recommended for the detection of xenobiotics that potentially induce carcinogenic and other genetic diseases to predict their threshold of chemical safety [27]. Together with genotoxic properties for regulatory purposes, such results are also mandatory to examine the general toxicity, carcinogenicity, reproductive toxicity, and cyto- and genotoxic biomarkers for determining the risk assessment before the chemicals are introduced into the market, particularly chemical compounds for direct human use [27]. Therefore, an emergent concern over the incidence of clastogenic and genotoxic compounds, such as pesticides, in the environment is mandatory to proceed for its detection as well as understand their genotoxicity profiles in multiple ways for the protection of the biota including human beings.

The present investigation aimed to determine the genotoxic and cytotoxic properties of the synthetic insecticide LCT and its commercially used micro-formulated product Karate® (25 % a.i.) through several cytogenetic bioassays. The *in vitro* genotoxicity was conducted by CBMN-cyt and SCGE assays, and basal cytotoxicity was assessed through NRU and MTT estimation. In addition, the cellular response in terms of exploring the apoptosis pathway induced by the pyrethroid insecticide in CHO-K1 cells was analysed.

2. Materials and methods

2.1. Chemicals

Lambda-cyhalothrin [(RS)-alpha-cyano-3-phenoxybenzyl 3-(2-chloro-3,3,3-trifluoropropenyl) 2,2,-dimethylcyclopropanecarboxylate, Pestanal®, CAS 91465-08-6] was acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Karate® (25 % LCT, excipients q.s.) was obtained from Syngenta Agro S.A., Argentina. Ethanol (EtOH, CAS 64-17-5), dimethyl sulfoxide (DMSO, CAS 67-68-5), neutral red dye (CAS 553-24-2), MTT (CAS 57360-69-7), and propidium iodide (PI) (CAS 25535-16-4) were supplied by Merck KGaA (Darmstadt, Germany). Cytochalasin B from *Dreschslera dematioidea* (CAS 14930-96-2) was purchased from Cayman Chemical (Michigan, USA). Annexin V-FITC was acquired from Invitrogen Molecular Probes® (Carlsbad, CA, USA), and bleomycin (Blocamycin®) was purchased from Gador S.A. (Buenos Aires, Argentina). All other chemical compounds and solvents of analytical grade were obtained from Sigma-Aldrich Co.

2.2. Cell cultures and chemical treatment for the CBMN-cyt assay

The CHO-K1 (CCL-61; American Type Culture Collection, Rockville, MD, USA) cells were preserved at 37 °C in a 5 % CO_2 atmosphere in Ham's F-10 culture medium supplemented with 10 % foetal calf serum, 100 units penicillin/mL, 10 μg streptomycin/mL (both from Gibco, Grand Island, NY, USA) and 8 μg tylosin/mL (TiloZur® from Weizur, Buenos Aires, Argentina). The experimental design was set up with cultures at the log phase of growth. Cells were placed onto sterilised 22 × 22 mm coverslips in 35 mm Petri dishes (1.2×10^4 cells) in a final medium culture volume of 3 mL/dish. Treatments with the a.i. and the commercial insecticide were performed 24 h after plating, as recommended elsewhere [22,28,29]. The LCT was prepared in DMSO and then diluted in serum-free medium, whereas Karate® was prepared directly in culture medium. Both compounds were diluted so that addition of 100 μL into 2.9 mL of culture would allow pesticides to reach the required concentration ranges. Cells were treated with equimolar concentrations of 0.1, 1, 10 and 100 μg LCT/mL or 0.1, 1, 10 and 100 μg LCT/mL contained in Karate® formulation product during for 24 h. The concentrations tested refer to the nominal concentrations of the LCT in the commercial formulation Karate® (25 % a.i., Syngenta S.A., Argentina). To estimate the concentrations selected in the CBMN-cyt assay, preliminary experiments were implemented following the OECD recommendations [11], choosing the range of concentrations based upon the cytotoxicity detected in the NRU assay after 24 h of exposition. Additionally, the tested concentrations were selected on the basis of data reported previously by Naravaneni and Jalil [30] and Saleem et al. [31]. For the treatments, the final DMSO concentration was below 1 % (v/v). Parallel to the treatments, 3 μg of cytochalasin B/mL was added, and the cells were cultured (37 °C in a 5 % CO_2 atmosphere) for an additional 24 h until collecting. A negative control group (cells without treatment), solvent (cells exposed to > 1 % DMSO) and a positive control (cells exposed to 1.0 μg/mL BLM) were run in parallel with pyrethroid-treated

cultures. The pH of the medium was not altered by the treatments (range 7.2–7.4). Each experiment was repeated three times, and cultures were performed in duplicate for each experimental and time point. The same batches of culture medium, serum and reagents were used throughout the experiment. Once the culture period ended, the cells were prefixed with 1000 μL of methanol/glacial acetic acid (6:1) added directly to each dish (−20 °C, 15 min). Subsequently, the supernatant was removed, and the cells were fixed with methanol/glacial acetic acid (6:1) for 15 min and finally stained with 3 % aqueous Giemsa solution for 10 min. Then, the coverslips were air-dried and placed down onto precleaned slides using Permount mounting medium.

2.3. CBMN-cyt assay

The CBMN-cyt methodology was performed following the recommendations of Fenech [9], with minor adjustments as described elsewhere [20]. At least 1000 binucleated (BN) cells were blind-scored at 1000× magnification, and the number of BN cells with one, two or three MNs was quantified in BN cells [9]; NPBs and NBUDs were blind-scored from 1000 BN cells per experimental point at 1000× magnification, as described previously [9,10,20]. A minimum of 500 cells per experimental point were registered, and the nuclear division index (NDI) was estimated [32].

2.4. Cell cultures and chemical treatment for the SCGE assay

The CHO-K1 cells were placed (3.5×10^4 cells/mL) and maintained for 90 min in the presence of the chemicals (37 °C, 5 % CO_2 atmosphere). Both LCT and Karate® were used at final equal concentrations of 0.1, 1, 10 and 100 μg/mL as stated in *Subsection 2.2*. To estimate the concentrations employed in the SCGE assay, a preliminary viability test after a short pulse treatment of 90 min was implemented, and this exposure time was adequate to yield observable genotoxicity. The concentration-range that allows the survival of a sufficient number of cells to perform the SCGE analysis was selected. The negative control group, solvent and positive control groups (1.0 μg BLM/mL) were run in parallel with pyrethroid-treated cultures. The SCGE and cell viability assays were run immediately after a 90-min pulse treatment period. Each experiment was performed in triplicate.

2.5. SCGE assay

The alkaline SCGE assay [15] was performed with minor adjustments, as stated elsewhere [20–22]. A solution containing 1 % normal-melting agarose in distilled water was prepared. Normal-melting agarose (200 μL) was placed onto slides previously cleaned with 96 % ethanol, spread evenly and heated at 37 °C for 20–30 min. In addition, a 1 % low-melting agarose solution in distilled water was prepared, and 150 μL of this solution was added to 7.0×10^3 cells (50 μL cell suspension plus 150 μL low-melting agarose), covered with a coverslip and allocated at 4 °C for 10 min. Next, the slides were immersed in ice-cold freshly prepared lysis solution (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 the dark for a period of 1 h (4 °C). Subsequently, the slides were placed in 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 neutralised with a solution containing 0.4 M Tris-HCl, pH 7.5 and stained with 4'−6'-diamino-2-phenylindole (DAPI) (Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA). The slides were analysed 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 non-overlapping cells. The DNA damage was categorized into four groups (I: undamaged; II: minimum damage; III: medium damage; IV: maximum damage, as suggested previously by [16]. The results were expressed as the mean number of damaged nucleoids (sum of classes II, III and IV) and the mean comet score for each treatment group. Additionally, a genetic damage index (GDI) was estimated for each test compound using the formula $GDI = [(I) + 2(II) + 3(III) + 4(IV)]/N_{(I\text{-}IV)}$, where I–IV represent the nucleoid type, and N_I–N_{IV} represent the total number of nucleoids scored [33].

2.6. Cell viability assay

The proportion of viable cells was examined using the ethidium bromide/acridine orange methodology [34]. Experiments were performed in triplicate. Cell viability was monitored 90 min after insecticide treatment; at least 500 cells were counted per experimental point, and the results were expressed as the percentage of viable cells of all cells.

2.7. NRU and MTT assays

Briefly, 1×10^5 CHO-K1 cells/mL were cultured in Ham's F10 culture medium on 96-well microplates for 24 h. Afterwards, the culture medium was removed, and the cells were treated with LCT and Karate® within the 0.1–100-μg/mL concentration range for 24 h. Subsequently, 5 % EtOH-treated and 0.1 % DMSO-treated cells were used as positive and solvent vehicle controls, respectively. The NRU assay was performed as described elsewhere [35]. After treatment, the cells were incubated for an additional 3-h lapse with 100 μg/mL neutral red dye dissolved in PBS and subsequently washed with PBS to remove the dye. The MTT assay was performed as described previously [25]. After treatment, 20 μL of MTT was added for an additional 3-h period. After the incubation period, the formazan crystals were dissolved in 100 μL of DMSO, and the plates were read spectrophotometrically at 550 nm (Sunrise AbsReader, Tecan Austria GmbH, Salzburg, Austria). The results were expressed as the mean percentages of lysosomal and mitochondrial activities from three independent experiments performed in parallel.

2.8. Cell cultures and insecticide treatment for flow cytometry analysis

The CHO-K1 cells were seeded in 35 mm Petri dishes at a density of 2.5×10^5 cells/mL and treated for 24 h after plating (37 °C, 5 % CO_2 atmosphere). Both LCT and Karate® were diluted so that the addition of up to 20 μL into cultures resulted in final concentrations of 5 and 50 μg/mL. Controls were run simultaneously with LCT-treated cultures. After an incubation period of 24 h (37 °C, 5 % CO_2 atmosphere), cells were processed for flow cytometry using Annexin V-FITC/PI double staining. Controls and treated cells were washed with PBS and resuspended in 25 μL of binding buffer, supplemented with 2.5 μL of Annexin V-FITC, incubated for 15 min at room temperature in the dark, washed and resuspended in 300 μL of binding buffer. Immediately before performing the analysis, 3 μL (10 μg/mL) of the PI was added. The cell populations were differentiated as viable (Annexin V negative/PI negative), early-apoptotic (Annexin V positive/PI negative), late-apoptotic (Annexin V positive/PI positive), and necrotic (Annexin V negative/PI positive), using a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). The results were expressed as the mean percentages of alive, early-apoptotic, late-apoptotic and necrotic cells of all cells from three independent experiments performed in parallel.

2.9. Statistical analysis

Data obtained from NRU, MTT and flow cytometry were analysed applying one-way ANOVA. Variables were tested for normality with the Shapiro-Wilk test, and homogeneity of variances among groups was verified by Bartlett's test. Pair-wise comparisons among the different groups were made using the post-hoc Tukey honest significant difference test. The SCGE and CBMN-cyt bioassays were evaluated by the $\chi 2$

test. To verify a concentration-dependent response of the treatments, Spearman's rank order linear correlation analysis was performed. The selected significance level was 0.05 unless indicated otherwise. All data analyses were performed in the RStudio software (2020).

3. Results and discussion

Total MN frequencies and nuclear abnormalities in binucleated CHO-K1 cells are show in Table 1. The DMSO treatment did not modify MN and nuclear abnormalities frequencies in regard to negative control values ($p > 0.05$). An increased level of MNs was registered in those BLM-treated cultures (positive control) compared to the values observed in the control solvent group ($p < 0.001$). A significant induction of MNs was observed after treatment only with the highest doses of 10 and 100 μg LCT/mL ($p < 0.05$), whereas no MN induction was achieved after treatment with lower concentrations compared to solvent control values (Table 1). When cells were treated with 10 and 100 μg LCT/mL, the total MN frequency enhancement was due to an increase in the frequency of binucleated cells carrying two MNs ($p < 0.05$ and $p < 0.01$, respectively) as well as by an increased frequency of binucleated cells carrying three MNs ($p < 0.001$). No significant increase in MN frequency was observed in Karate®-treated cultures ($p > 0.05$). A correlation test showed that the total frequency of MNs increased as a positive function of the LCT concentration ($r = 0.62$, $p < 0.001$) titrated into cultures. When other nuclear abnormalities were analysed on the same platform, positive control exerted a significant increase in NBUD ($p < 0.01$) and NPB ($p < 0.001$) frequencies. The frequency of NBUDs was significantly increased when cells were exposed to 1–10 μg LCT/mL ($p < 0.01$; $p < 0.001$), and a significant increase in the frequency of NPBs was reached only after treatment with 100 μg/mL of LCT compared to solvent control values ($p < 0.01$). Contrarily, no variations in the frequencies of other nuclear abnormalities were observed in Karate®-treated cultures ($p > 0.05$). Finally, significant alterations in the NDI were found in cultures treated with LCT for the 10- and 100 μg/mL treatments ($p < 0.05$). A correlation analysis demonstrated that the NDI decreased in a concentration-dependent manner when LCT was added to the cultures ($r = -0.47$, $p < 0.001$).

Genotoxic studies have been extensively employed as biomarkers for toxicological regulatory matters and to determine the toxic profiles of pesticides [6]. Although LCT is an extensively employed manufactured insecticide, with several adverse health effects for humans and other organisms [7], studies at the cellular level to reveal its primary toxicity mechanism(s) are scarce [7]. The discussion on the possible genotoxic properties of LCT remains controversial. According to the U.S. EPA [7], LCT is considered either weakly mutagenic or non-mutagenic in bacterial systems. At the chromosomal level, LCT did not increase MNs in *in vitro* and *in vivo* [36,37]. However, LCT was able to enhance the MN occurrence in several non-traditional target species such as the mosquito fish *Gambusia affinis* [38], the fish *Cheirodon interruptus interruptus* [39], the nibble fish *Garra rufa* [40] and the American bullfrog *Rana catesbeiana* [41]. In laboratory models, LCT induced an enhancement in the proportion of MNs in bone marrow, gut epithelial and the peripheral blood of treated rats [42–45]. When other nuclear abnormalities were evaluated, the pyrethroid insecticide was linked to a significant enhancement in the occurrence of cells carrying notched, lobed and blebbed nuclei in erythrocytes of *G. affinis* [38] and *Prochilodus lineatus* [37]. The induction of structural chromosomal aberrations was observed in Wistar rats [43,44]. Aziz and Rahem [46] observed an enhancement in the frequency of structural and numerical chromosomal aberrations after oral repeated administration in Swiss albino mice when analysing bone marrow cells and primary spermatocytes. Similarly, LCT increased the proportion of structural chromosomal aberrations in *in vitro* human lymphocytes [30]. Besides, in an *in vitro* study using the commercial formulated product Karate®, a dose-dependent enhance in the incidence of chromosomal aberrations was described when rabbit peripheral blood lymphocytes were treated [47]. Our current results show the capability

Table 1
Micronucleus (MNs), nuclear abnormalities and nuclear division index (NDI) values observed in CHO-K1 cells after 24 h treatment with lambda-cyhalothrin (LCT) and Karate®[a].

Compound	Concentration (μg/mL)	MN frequencies	Micronucleated cell distribution			NBUDs	NPBs	NDI
			1 MN	2 MN	3 MN			
Control		15.30 ± 0.67	13.97 ± 0.67	0.66 ± 0.19	0.00 ± 0.00	65.09	1.33	2.13 ± 0.05
DMSO (solvent control)		13.94 ± 0.06	10.33 ± 0.04	1.49 ± 0.33	0.50 ± 0.24	78.32	3.00	1.93 ± 0.02
LCT	0.1	15.65 ± 1.71	14.31 ± 0.57	0.66 ± 0.57	0.00 ± 0.00	60.18	2.33	1.90 ± 0.00
	1	18.55 ± 0.52	16.57 ± 0.68	0.99 ± 0.68	0.00 ± 0.00	105.76***	1.66	1.90 ± 0.01
	10	23.14 ± 2.11*	16.57 ± 1.08	2.44 ± 1.08*	0.99 ± 0.33***	87.64**	1.63	1.80 ± 0.02*
	100	23.65 ± 0.94*	15.49 ± 0.23	3.00 ± 0.23**	0.50 ± 0.24***	58.63	5.00**	1.82 ± 0.03*
Karate®	0.1	21.90 ± 0.84	15.30 ± 0.96	1.66 ± 0.77	1.00 ± 0.33***	60.82	3.33	1.92 ± 0.02
	1	21.63 ± 0.88	17.25 ± 0.69	1.33 ± 0.88	0.67 ± 0.19***	59.10	2.32	1.90 ± 0.02
	10	ND ± ND	ND ± ND	ND ± ND	ND ± ND	ND	ND	ND ± ND
	100	ND ± ND	ND ± ND	ND ± ND	ND ± ND	ND	ND	ND ± ND
BLM (positive control)	1	74.53 ± 3.05***	58.70 ± 2.30***	5.92 ± 0.60***	1.33 ± 0.51***	87.64**	8.16***	1.97 ± 0.02

[a] Results are presented as mean values of MNs or nuclear abnormalities/1000 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ±standard error of the mean.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significant differences with respect to control values.
NBUDs, nuclear buds; NPBs, nucleoplasmic bridges.
ND, not determined.

of the pyrethroid insecticide to increase the proportion of MNs at concentrations higher than those tested *in vitro* in mammalian cells. Additionally, the formulated product Karate® exhibited no clastogenicity on CHO-K1-cultured cells after a 24-h treatment. The current results agree with the genotoxic profile of LCT previously reported in other studies conducted at the chromosomal level *in vitro* and *in vivo* systems [40,43,44,48]. When other nuclear chromatin instabilities were analysed in the current study, NBUDs and NPBs were significantly enhanced in LCT-treated cells. The origin of NBUDs is associated with an aneuploid mechanism possibly originating from failure in tubulin polymerization, hence eliminating from the main nucleus as amplified DNA, unresolved DNA repair complexes, and DNA excess from cells with nuclear anomalies [12]. In the case of NPBs, they originate during the anaphase from dicentric chromosomes, which may occur due to telomere end-fusions, misrepair of DNA disruptions or failure of complete chromatid separation of sister chromatids [12]. Nuclear anomalies such as MNs, NPBs and NBUDs can lead to genetic imbalance and are in the background of carcinogenesis; their occurrence is an important parameter for estimating the risks of pesticides [10,12]. The results of the present investigation confirm the assumption that LCT can induce nuclear anomalies and could be categorised as a damaging agent with genotoxic potential at the chromosomal level.

Table 2 shows the results of the SCGE assay performed in CHO-K1 cells after a 90-min pulse-treatment with LCT or Karate® within the 0.1–100 μg/mL concentration range. The proportion of damaged cells of in the solvent-treated cultures were consistent with the observed frequencies for the control cultures ($p > 0.05$). After treatment of BLM (positive control) an increase in the proportion of damaged cells as well as an increment in the GDI values compared to solvent control values ($p < 0.001$). Worth mentioning, this enhancement did not significantly alter the proportion of viable cells. After treatment with 10 and 100 μg LCT/mL, an enhancement in the proportion of damaged nucleoids was observed due an increase in type III ($p < 0.001$) and IV nucleoids ($p < 0.01$) compared to solvent control values. None of the LCT treatments induced an enhancement of GDI values or an alteration in the proportion of cell viability. When Karate® was assayed, an enhancement in the proportion of damaged cells was found after all concentration treatments ($p < 0.05$ and $p < 0.001$). The proportion of damaged cells was achieved by decreasing the frequencies of type I ($p < 0.05$ and $p < 0.001$) and increasing the frequencies o type II, III and IV ($p < 0.01$ and $p < 0.001$) comets, respectively (Table 2). The GDI values induced by Karate® treatments are presented in Table 2. Karate® treatments induced an enhancement of the GDI values when concentrations of 1 ($p < 0.05$), 10 ($p < 0.001$) and 100 μg/mL ($p < 0.001$) were added to the cultures. No significant decrease in cell viability was observed for cultures treated with Karate®. Regression analysis demonstrated that the total number of damaged cells was directly correlated with both LCT ($r = 0.64$; $p < 0.05$) as well as Karate® ($r = 0.94$; $p < 0.001$) concentrations added to CHO-K1 cultures. Overall, the SCGE assay demonstrated that Karate® was more prone to induce genotoxic effects than the active ingredient.

Scarce information is available regarding LCT-induced DNA single-strand breaks estimated by the SCGE methodology. Saleh et al. [49] reported a significant increase in DNA damage when exposing the Sf9 insect cell line to LCT. Similarly, Abdallah et al. [50], Naravaneni and Jamil [30] and Zhang et al. [51] demonstrated a genotoxic potential of the active ingredient LCT in *in vitro* systems. Vieira and dos Reis Martinez [52] reported a significant enhancement in the proportion of DNA lesions after treating specimens of *P. lineatus* with the commercial formulation Karate Zeon© CS (5 % LCT). Our present results are in agreement with the findings of these previous investigations, reinforcing the potential of LCT to damage DNA, at least at the concentrations assayed in the present study. Here, LCT generated DNA single-strand breaks in a dose-dependent way for both treatments in CHO-K1 cultures. Similarly, Zhang et al. [51] described the same pattern when employing the mouse macrophage cell line RAW 264.7. The authors demonstrated that LCT exerts DNA lesions through oxidative damage, reflected by an enlargement in comet tail length and a diminution in the integrity of the nucleoids. Zhang et al. [51] stated that LCT contains chloride atoms and proposed that LCT may perform oxidative DNA damage *via* a similar pathway to that of organochlorine pesticides. Finally, we also observed that the capability of LCT and Karate® to induce DNA lesions was not similar for all assayed concentrations. Karate® was more prone to exert genotoxicity than the LCT for a given concentration. Additional studies are required to understand the formation of DNA damage in LCT-treated mammalian cells and corroborate whether oxidative damage is involved as suggested elsewhere Zhang et al. [51].

In the present study, Figs. 1 and 2 show the values obtained from the NRU and MTT assays, respectively. EtOH-treated cultures (positive control) produced a statistically significant cell inhibition in CHO-K1 cells compared to the values observed in the control solvent group ($p < 0.001$). Lysosomal and mitochondrial activities were not significantly modified by any LCT concentration assayed. On the other hand, there was a significant inhibition in lysosomal activity when CHO-K1 cells were exposed to 5 μg Karate®/mL ($p < 0.01$) and 10–100 μg Karate®/mL ($p < 0.001$). When exposed to Karate®, CHO-K1 cells showed an inhibition of mitochondrial activity for 50- and 100-μg/mL treatments ($p < 0.05$ and $p < 0.001$, respectively). The NRU was able to reveal cytotoxicity in Karate®-treated cultures after exposure to up to 5 μg/mL. The MTT assay showed that 50 μg Karate®/mL was required to significantly reduce mitochondrial metabolism. The difference observed in

Table 2
Analysis of DNA damage measured by comet assay in CHO-K1 cells exposed during 90 min to lambda-cyhalothrin (LCT) and Karate®.

Compound	Treatment (μg/mL)	Proportion of damaged nuclei (%)[a]					DNA damage (%)[b]	GDI[c]	Viablility (%)
		Type 0	Type I	Type II	Type III	Type IV			
Control		0.00	90.26	8.76	0.65	0.33	9.74 ± 1.20	1.11 ± 0.01	99.33 ± 0.67
DMSO (solvent control)		0.00	92.89	5.02	0.79	1.30	7.11 ± 1.13	1.10 ± 0.05	99.33 ± 0.38
LCT	0.1	0.00	86.96	9.06	3.26*	0.72	12.53 ± 3.30	1.17 ± 0.05	97.73 ± 0.29
	1	0.00	85.15	11.88	1.98	0.99	14.87 ± 1.27	1.19 ± 0.03	98.47 ± 0.28
	10	0.00	83.17	8.58	5.94***	2.31**	16.86 ± 5.17*	1.27 ± 0.10	95.87 ± 0.81
	100	0.00	80.54	12.08	6.04***	1.34	19.48 ± 1.02**	1.28 ± 0.01	94.34 ± 1.36
Karate®	0.1	0.00	83.35	12.42	3.59***	0.65	16.65 ± 2.53*	1.21 ± 0.02	99.37 ± 0.63
	1	0.00	77.45*	15.81	3.25**	3.49***	22.37 ± 5.20***	1.33 ± 0.09*	97.40 ± 2.18
	10	0.00	64.26***	28.44	2.96**	4.33***	35.74 ± 7.59***	1.47 ± 0.02***	98.05 ± 0.98
	100	0.00	28.78***	50.23**	12.03***	8.95***	71.21 ± 2.70***	2.01 ± 0.02***	95.68 ± 2.96
BLM (positive control)	1	0.00	31.80***	26.94	7.66***	33.59***	68.20 ± 1.54***	2.43 ± 0.09***	99.33 ± 1.39

[a] I–IV indicate grades of DNA damage as mean values of pooled data from three independent experiments.
[b] Results are expressed as mean values ±standard error of three experiments.
[c] Genetic damage index (GDI).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significant differences with respect to control values.

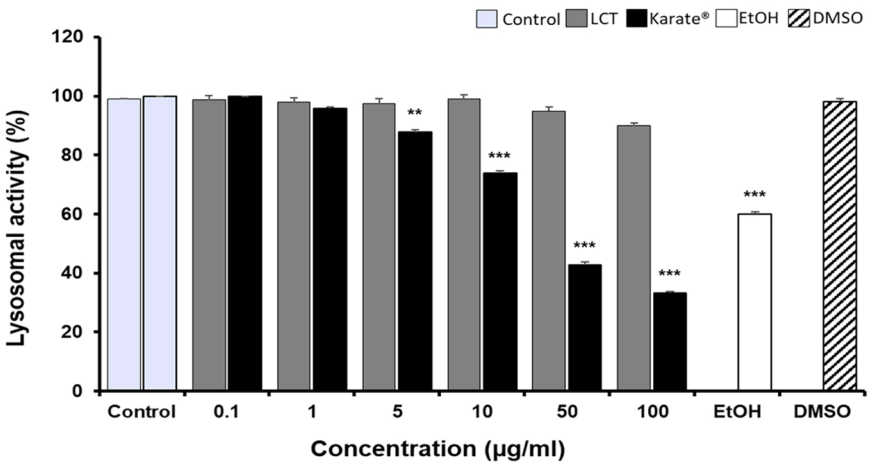

Fig. 1. Lysosomal activity evaluated by the NRU assay in LCT- and Karate®- treated CHO-K1 cells. Results are expressed as the mean percentage of cell viability from three independent experiments performed in parallel (*y*-axis) and plotted against the insecticide concentration (*x*-axis) employing 0.1–100 µg/mL concentration-range of LCT and Karate®. 5 % ethanol-treated cells were employed as positive control. $^{**}p < 0.01$; $^{***}p < 0.001$; significant differences with respect to solvent control values. Values were expressed as mean values ± standard error of the mean, n = 3.

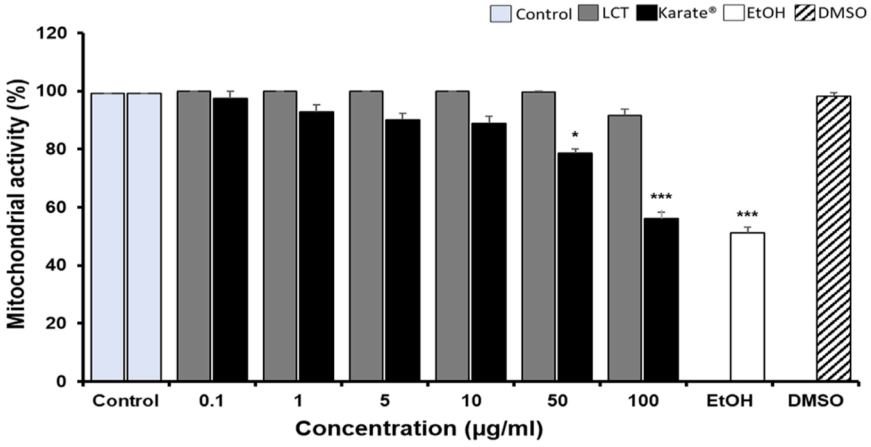

Fig. 2. Cellular metabolism inhibition evaluated by the MTT assay in LCT- and Karate®-treated CHO-K1 cells. Results are expressed as the mean percentage of cell viability from three independent experiments performed in parallel (*y*-axis) and plotted against the insecticide concentration (*x*-axis) employing 0.1–100 µg/mL concentration-range of LCT and Karate®. 5 % ethanol-treated cells were employed as positive control. $^{*}p < 0.05$; $^{***}p < 0.001$; significant differences with respect to solvent control values. Values were expressed as mean values ± standard error of the mean, n = 3.

these two cytotoxic assays may be explained by the fact that the Karate®-induced cytotoxicity was differentially estimated by MTT and NRU bioassays. Whilst at low concentrations, Karate® increases the cytotoxic response *via* lysosomal pathway, a 10-fold increase in the concentration was required for Karate® to reduce cellular metabolism *via* mitochondrial activity. This response may be explained by the fact that the NRU assay has been reported to be more sensitive than MTT methodology to detection cytotoxicity as stated by Repetto et al. [53]. Previous studies showed that LCT induced a reduction in cell viability in several established cell lines when employed in MTT assay [51] and in

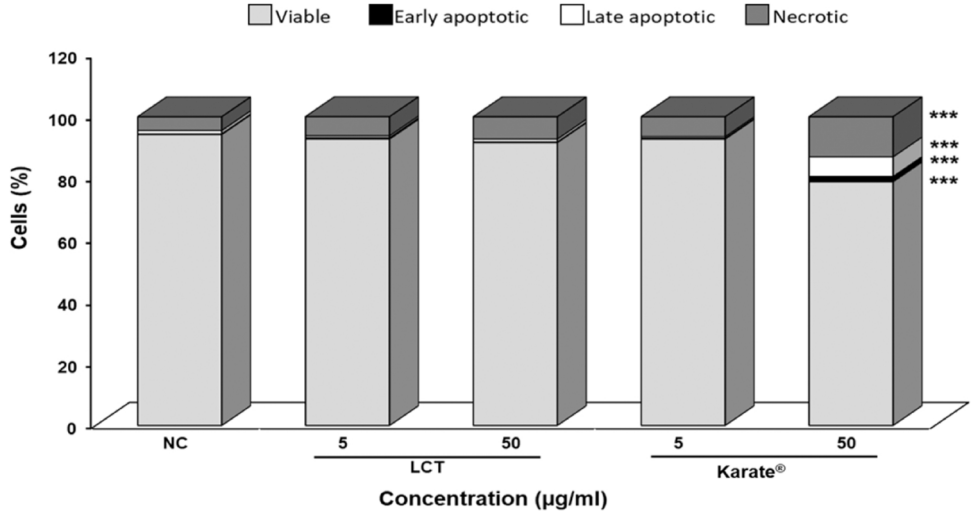

Fig. 3. LCT and Karate® induce apoptosis in CHO-K1 cells. Cultures were treated with 5 µg/mL and 50 µg/mL of LCT and Karate® and processed 24 h later with Annexin V-FITC/PI staining for quantification of the incidence of apoptosis by flow cytometry. The percentages of cells from the three independent experiments after flow cytometry analysis are shown. $^{***}p < 0.001$; significant differences with respect to solvent control values.

human and rat astrocytes when WST-1 assay was applied [54]. Also, LCT induced cytotoxicity in human lymphocyte when the mitotic index and NDI were performed [36,55]. Similarly, cytotoxicity evaluated by NRU and Kenacid Blue protein assays employing fish cell lines revealed that whilst the insecticide produced a significant reduction in RTG-2 viability but not in the RTL-W1 cell line, the Kenacid blue protein assay showed a poor response for both cell lines [56]. Although previous reports demonstrated cytotoxicity induction by LCT in several *in vitro* models [36,51,55], in the present study, the pyrethroid did not exert such effect, at least within the concentration-range and time exposition tested. One possible explanation could be related to the fact that different cell lines were employed as biotic matrices and their different origins might be reflected in divergent responses at both physiological and functional levels after LCT treatment. Differences in the cellular responses after LCT exposure employing various types of cells and growth conditions were also described by Hsu et al. [54] and Babin and Tarazona [56].

Fig. 3 shows the flow cytometry analyses of cells exposed to 5 and 50 μg/mL LCT and Karate®. The proportions of live, early- and late-apoptotic as well as necrotic cells in the DMSO-treated cultures were consistent with the observed frequencies for the control cultures, and thus, pooled data are presented for the control culture values. As mentioned above, after LCT treatment, no significant alterations in the proportions of cells were found compared to the control values ($p > 0.05$) (Figs. 3 and 4). When 50 μg Karate®/mL was employed, a statistically significant enhancement in early- and late-apoptotic as well as necrotic cells was achieved ($p < 0.001$), with a concomitant decrease in the proportion of live cells ($p < 0.001$) compared to control values (Figs. 3 and 4). Cell death through apoptogenic mechanisms is a process tightly controlled [24]. A number of signals, such as changes in protein expression, protein interactions, post-translational protein modifications and lysosomal destabilisation are involved as promotors of a series of consecutive molecular reactions [24]. Our present results are in agreement with the previously reported results, which showed that LCT induced apoptosis in *in vitro* human lymphocytes after treatment with 15 μM of LCT contained in a commercial formulated product [36] and in human astrocytes treated with 10 and 15 μM of LCT [54]. Martínez et al. [57], using liver cells of male Wistar rats, reported that pure LCT increased the mRNA expression of *p53*, *Casp-3* and *Bax*, which play an important role in apoptotic cell death. Our assessment of the apoptotic potential observed only after treatment of CHO-K1 cells with Karate® reflects the competence of the microformulation to exert damage *via* an apoptotic pathway. Overall, our current apoptotic findings are in accordance with the results found after the application of the NRU assay in Karate®-treated CHO-K1 cells for 24 h. Several studies have investigated the role of the lysosomal pathway in apoptosis in a number of pathological situations [58]. Our results support the concept of lysosome participation in the caspase-dependent pathway during apoptosis. Further studies are required to obtain a comprehensive knowledge of the possible mechanisms through Karate® exerts its toxic effects.

Pesticide products are typically a combination of one or more active ingredients together with other inert adjuvants which are used to improve the pesticide action of the active ingredient(s). However, in some cases, the excipients possess *per se* health risks for humans and other organisms. According to the U.S. EPA [59], approximately 50 % of inert adjuvants authorised for pesticide manufacture possess low or low/moderate risk. Several reports confirmed that excipients present in products formulations can exert toxicity by themselves, separate from the pure compound [20,22,29,60–63]. Our current observations suggest that the introduction of DNA single-strand breaks, cytotoxicity and apoptosis induced by Karate® is most probably due to the existence of xenobiotic(s) involved in the adjuvant(s) of the LCT-based microformulation, enhancing the toxic profile of the pyrethroid commercial product. A deeper understanding of the toxic effects of the excipients is therefore essential to support the latter possibility.

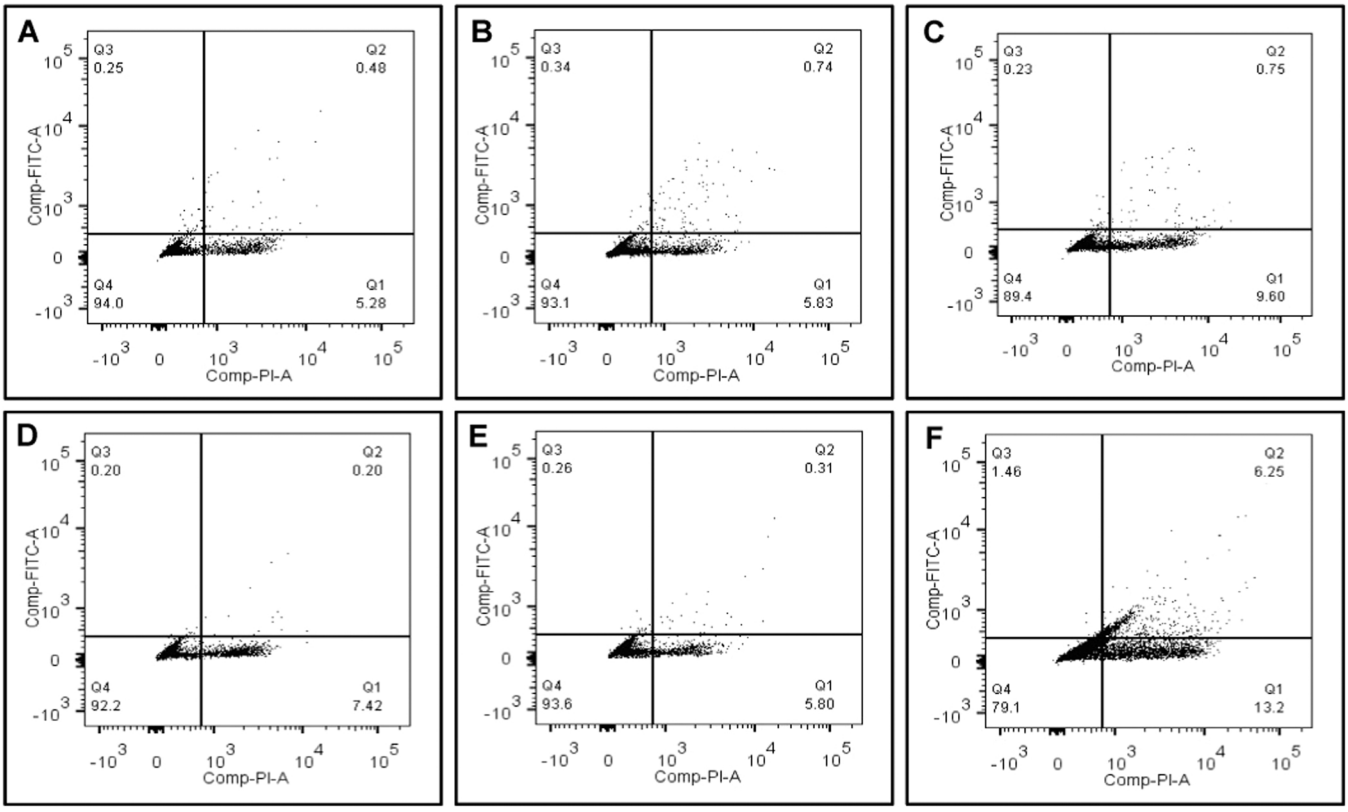

Fig. 4. LCT and Karate® induce apoptosis in CHO-K1 cells. Cultures were treated with the test compounds and processed 24 h later with Annexin V-FITC/PI staining for quantification of the incidence of apoptosis by flow cytometry. Representative dot plot analyses showing (A–D) control, (B) 5 μg/mL LCT-treated, (C) 50 μg/mL LCT-treated, (E) 5 μg/mL Karate®-treated, (F) 50 μg/mL Karate®-treated cultures. ***$p < 0.001$; significant differences with respect to solvent control values.

Finally, we demonstrated that LCT provokes DNA damage in mammalian cells, promoting genomic instability at the chromosomal level, which could be critical against the background of carcinogenesis. Our results also revealed that the microformulation Karate® may cause DNA single-strand breaks, cytotoxicity and apoptosis in CHO-K1 cells. Data here presented might be employed to increase the existing information about LCT toxicity for its safe usage in agronomical and household practices. However, further studies should explore the mechanisms by which LCT negatively impacts mammalian cells at a genetic level.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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