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Genotoxic and cytotoxic effects of carbofuran and furadan® on Chinese hamster ovary (CHO_{K1}) cells

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ABSTRACT

The *in vitro* geno- and cytotoxicity exerted by the *N*-methylcarbamate pesticide carbofuran (CF) and its commercial formulation furadan® (F®) were studied in Chinese hamster ovary (CHO_{K1}) cells by several bioassays for both genotoxicity (e.g., the sister chromatid exchange (SCE) and micronuclei (MNi) frequencies), and cytotoxicity (e.g., cell-cycle progression, mitotic index (MI), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and neutral red (NR)). Both CF and F® activities were tested within the range of 5–100 μg/ml. CF within a 10–100 μg/ml concentration-range induced a significant dependent increase of SCE frequency and MNi over control values. At the same concentration-range, F® increased significantly the SCE frequencies over control values although in a non-dependent manner while only an enhanced frequency of MNi was found in those 50 μg/ml-treated cultures. No binucleated cytokinesis-block cells were found in 100 μg/ml F®-treated cultures. The NDI index revealed a delay in the onset of cell-division with 50 and 100 μg/ml of CF and F®, respectively. The delayed rate of nuclear division induced by 100 μg/ml of F® was higher than that induced by an equal concentration of CF. CF and F® induced both a significant concentration-dependent delay in cell-cycle progression and a decrease in the proliferative replication index within 5–100 μg/ml and 50–100 μg/ml concentration-range, respectively. Decreased cell viability was found in up to 26% and 47% in 100 μg/ml CF- and F®-treated cultures, respectively. The NR and MTT assays revealed a clear cell growth inhibition when concentrations of 50 and 100 μg/ml of either CF or F® were employed. Accordingly, the results highlight that CF by itself and F®, even in a greater extend exerts both genotoxicity and cytotoxicity in mammalian cells in culture, at least in CHO_{K1} cells.

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1. Introduction

Carbofuran (CF) is one of the most widely granular employed *N*-methylcarbamate esters. CF is basically a systemic pesticide used in agriculture to control soil-dwelling and foliar-feeding insects, mites and nematodes in grain, forage, vegetable, seed and fiber crops (www.epa.gov). It is an anticholinesterase compound able to inhibit both acethylcholinesterase and butyrylcholinesterase *in vivo* and *in vitro* [1]. The main toxic effect of CF is regarded to result from carbamylation of the catalytic centre of the acethylcholinesterase in the nervous system. This results in accumulation of acetylcholine at nerve synapses and myoneural junctions leading to cholinergic signs and causing toxic effects [2]. The inhibition of the carbomoylated enzyme is labile, of short duration, and reversible compared to that induced by organophosphorus compounds, undergoing spontaneous and rapid reactivation [1,3,4].

In general, *N*-methylcarbamates have low acute toxicity to mammals, and are recognized as non-genotoxic to bacteria, yeast and fungi [5–7] as well as to mammalian cells [8–11]. However, CF has been reported to be mutagenic in the Ames test after metabolic activation with S9-mix as well as in the base substitution mutation JK947 *Salmonella* lactam assay [12]. Furthermore, it has been claimed as a relatively weak mutagen in the *Escherichia coli* repair test [13]. Gentile and coworkers [6] reported the induction of unscheduled DNA synthesis in human lung fibroblasts. No single-strand breaks detected by alkaline sucrose gradient were induced in *in vivo* normal human skin fibroblasts [8] or mice peripheral lymphocytes [14]. However, Naravanemi and Jamil [15] reported its induction in *in vitro* human lymphocytes by the comet assay. The induction of cytotoxicity in human lymphocytes *in vitro* determined by the trypan blue dye exclusion method [15] and mitotic inhibition in mice bone marrow cells *in vivo* have been observed [16]. Furthermore, CF has been reported to induce sister chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) cells [17], and in mice [6] and rats [18] exposed *in vivo*, chromosomal aberrations in human lymphocytes *in vitro* [15] and mice *in vivo* [16], as well as mice sperm abnormalities *in vivo* [16]. Although the induction

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of micronuclei (MNi) has been observed for mice exposed *in vivo* [16], Zhou and collaborators [14] were unable to register such effect not only after CF treatment but also with carbofuranphenol, one of its metabolites. While CF induced *in vitro* DNA fragmentation in rat cortical neurons [19], negative results were observed in Chinese hamster lung fibroblast *in vitro* [20]. Furthermore, CF has been reported to be teratogenic and embryotoxic [1].

CF is recognized either as a highly toxic pesticide for human health by inhalation and ingestion, or moderately toxic by dermal absorption and when affecting nervous system functions. However, this carbamate has been classified as moderately toxic (class II) by the EPA in spite of the great variety of occupational activities that involve the use of CF around the world (www.epa.org). Due to the lack of both human cancer epidemiology and positive rodent cancer bioassays data, the IARC has not listed this *N*-methylcarbamate pesticide as carcinogen so far (www.iarc.fr).

Due to its widespread employment in agriculture and household, contamination of food, water and air has become serious and adverse health problem for humans, animals and wildlife. Epidemiological studies suggested that long-term exposure to CF may be associated with increased risk of gastrointestinal, neurological, cardiac dysfunction and retinal degeneration [21,22]. Therefore, the contamination of environment with CF can easily occur, especially in those countries where it is still in use and the probability of long-term low dose exposure becomes increased. The importance of further studies on this type of pesticide to achieve a complete knowledge on its genetic toxicology seems to be, then, more than evident.

In the present study we employed the SCE, cytokinesis-block MNi frequencies, 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 endpoints to characterize the genotoxicity and cytotoxicity exerted on Chinese hamster ovary (CHO_{K1}) cells by CF and furadan® (47% CF), one of its commercial formulation currently employed in Argentina.

2. Materials and methods

2.1. Chemicals

Carbofuran (CF, 2,3-dihydro-2,2-dimethyl-7-benzo-furanil methylcarbamate, CAS 1563-66-2), 5-bromo-2′-deoxyuridine (BrdUrd, CAS 59-14-3), cytochalasin B from *Dreschslera dematioidea* (Cyt-B, CAS 14930-96-2), dimethyl sulfoxide (DMSO, CAS 67-68-5), ethidium bromide (CAS 1239-45-8), acridine orange (CAS 10127-02-3), neutral red dye (NRD, CAS 553-24-2), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, CAS 57360-69-7), were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Furadan® (F®, 47% carbofuran; excipients c.s.) was kindly provided by FMC Argentina S.A. (Buenos Aires, Argentina). Bleomycin (BLM) (Blocamycin®) was kindly provided by Gador S.A. (Buenos Aires, Argentina).

2.2. SCE and cell-cycle progression and cell viability assays

2.2.1. Cell cultures and pesticide treatment

Chinese hamster ovary (CHO_{K1}) cells were grown in Ham's F10 medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum (Gibco), 100 units/ml penicillin (Gibco) and 10 μg/ml streptomycin (Gibco) at 37 °C in a 5% CO_2 atmosphere. Experiments were set up with cultures in the log phase of growth. Cells were seeded in T25 flasks at a density of 3.5×10^5 cells/flask. Treatments with the test active ingredient and formulated product were performed 24 h after plating. Prior to use, CF was dissolved in DMSO and then diluted in culture medium while furadan® was directly diluted in culture medium. Both CF and F® were appropriate diluted to reach the required final concentration by adding 100 μl into culture medium (0–100 μg/ml). So that, the DMSO 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, 1 h-pulse treatment) were run simultaneously with pesticide-treated cultures. None of the treatments produced significant pH changes in the culture medium. Afterwards, 10 μg/ml bromodeoxyuridine (BrdUrd) (Sigma Chemical Co.) were incorporated into cultures and then the cells incubated under 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.

2.2.2. Chromosome preparations

During the last 3 h of culture, the cells were treated with 0.2 μg/ml colchicine (Sigma Chemical Co.). Cells were detached with a rubber-policeman, collected by centrifugation, hypotonically shocked (0.075 M KCl, 37 °C, 17 min) and fixed in methanol/acetic acid (3:1). Chromosome spreads were obtained using the air-drying technique.

2.2.3. Fluorescence-plus-Giemsa (FPG) method for sister chromatid differentiation

Chromosome spreads were stained using the FPG technique for sister chromatid differentiation as previously described elsewhere [23]. Slides were coded and scored blind by one researcher.

2.2.4. Cell-cycle kinetics and mitotic index

A minimum of 100 metaphasic cells per experimental point from each experiment 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 previously described [24]. The MI was determined by scoring 2000 cells from each experimental point and expressed as 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) ($f = MI_t/MI_c$) [25].

2.2.5. Sister chromatid exchange analysis

For the SCE assay, a total of 25 well-spread diploid M_2 cells metaphases were scored per experimental point from each experiment. Data were expressed as the mean number of SCEs/cell ± S.E. from 75 cells.

2.3. Flurochrome-mediated viability test

At the end of the culture period, cell viability was determined using the ethidium bromide/acridine orange assay as described elsewhere [26]. Briefly, one aliquot of 5 μl of a 1:1 freshly prepared mixture of ethidium bromide (100 μg/ml) and acridine orange (100 μg/ml) was mixtured with 50 μl of the cell suspension under study. Afterwards, the cells were analyzed using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. At least 500 cells were counted per experimental point from each experiment, and the results expressed as percentage of viable cells among all cells.

2.4. Neutral red dye assay

The assay is based on the initial protocol described by Borenfreund and Puerner [27]. Briefly, 2×10^4 CHO_{K1} cells/ml were cultured in Ham's F10 medium on 96-well microplates during 24 h. Afterwards, the culture medium was removed and the cells were treated with CF and F® within the range of 0–100 μg/ml for 24 h. A 0.5% ethanol-treated and 1% DMSO-treated cells were used as positive and negative controls, respectively. Following exposure to tested chemicals, cells were incubated for an additional 2 h period in the presence of 100 μg/ml NRD dissolved in serum-free culture medium. The absorbance at 540 nm was recorded using the microplate spectrophotometer system (7530 Microplate reader, Cambridge Technology Inc., USA). Results were expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel.

2.5. MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

The methodology was performed following the technique described in detail by Kosmider and collaborators [28]. Briefly, 2×10^4 CHO $_{K1}$ cells/ml were cultured in Ham's F10 medium for 24 h on 96-well microplates. Afterwards, the culture medium was removed and the cells were treated with CF and furadan® within a 0–100 μg/ml range for 24 h. A 0.5% ethanol-treated and 1% DMSO-treated cells were used as positive and negative controls, respectively. Following exposure to tested chemicals, 40 μl of MTT (2 mg/ml) were added for an additional 2 h period. Then, the formazan crystals were dissolved in 100 μl of DMSO. Absorbance at 550 nm was measured with a spectrophotometer (7530 Microplate reader, Cambridge Technology Inc., USA). Results were expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel.

2.6. Micronuclei assay

2.6.1. Cell cultures and pesticide treatment

Chinese hamster ovary (CHO_{K1}) cells were grown in Ham's F10 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco), 100 units/ml penicillin (Gibco) and 10 μg/ml streptomycin (Gibco) at 37 °C in a 5% CO_2 atmosphere. Experiments were set up with cultures in the log phase of growth. The cells were seeded onto pre-cleaned cover 22 mm × 22 mm cover slips in 35-mm Petri dishes at a density of 1.2×10^4 cells/dish. Treatments were performed as indicated for SCE assay (see Section 2.2.1). Afterwards, 3 μg/ml Cyt-B (Sigma Chemical Co.) was incorporated into cultures and then the cells 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.

2.6.2. Slide preparations

At the end of the culture period, the cover slips were rinsed with Hank's balanced salt solution (3 times, 2–5 min each) and exposed to a mild hypotonic shock *in situ* using 0.075 M KCl (37 °C, 5 min). Afterwards, the cells were fixed with 100% (v/v) cold methanol and cover slips were stained with May-Grünwald-Giemsa. After staining, the cover slips were air-dried and then placed down onto pre-cleaned slides using Depex mounting medium.

2.6.3. Examination of slides and assessment of nuclear division index (NDI)

Slides were coded and scored blind by one researcher at 600× magnification. Criteria of examination of the slides was performed following the recommendations suggested by Fenech [29]. A minimum of 500 viable cells per experimental point was scored to determine the percentage of cells with one, two, and three or more nuclei and the NDI was calculated for each experimental point according to the method of Eastmond and Tucker [30].

2.6.4. Micronuclei (MNi) analysis

For MNi assay, at least 500 binucleated (BN) cells were scored blind by one researcher at 600× magnification per experimental point from each experiment. The number of BN cells with none, one, two, three or more MNi was determined in BN cytokinesis-block cells following the examination criteria reported by Fenech [29]. Briefly, the criteria employed in identifying MNi were: diameter smaller than 1/3 of that of the main nuclei, non-refractibility, same staining intensity as or lighter than that of the main nuclei, no connection or link with the main nuclei, no overlapping with the main nuclei, MNi boundary distinguishable from main nuclei boundary, and no more than 4 MNi associated with the nuclei.

2.7. Statistical analysis

The two-tailed Student's *t*-test was used to compare pooled data of three independent experiments as mean values of SCE/cell and micronuclei/cell, MTT and neutral red data between treated and control groups. A χ^2-test was employed for cell-cycle progression and MI data. SCE, frequencies of M_1 and M_2 cells, PRI, MI, and NDI data were evaluated by regression analysis. The level of significance chosen was 0.05 unless otherwise indicated.

3. Results

Since no difference of SCEs, cell-cycle progression, PRI, MI, micronuclei frequencies, cell viability, and mitotic proliferative factor values were observed between untreated and negative controls (untreated and DMSO-treated cells), pooled data are presented for control values.

The results of SCE analysis in CHO_{K1} cells treated with different concentrations of CF and F® and the positive control (BLM) are presented in Table 1. As expected, results showed statistically significant differences between negative and positive controls ($P < 0.01$). The SCE frequencies observed either in CF- or F®-treated cultures were significantly higher than those of negative control cultures with concentrations higher than 10 μg/ml ($P < 0.05$). No SCE induction was achieved when cells were treated with 5 μg/ml of both CF and F® ($P > 0.05$). While only SCE frequencies induced by CF increased in a concentration-dependent manner ($r = 0.98$, $P < 0.01$) not such an effect was observed in those F®-treated cultures ($r = 0.72$, $P > 0.05$). Equal concentrations of CF and F® induced equivalent enhancement in the frequency of SCE within the 10–100 μg/ml concentration-range ($P > 0.05$) (Table 1).

A significant delay in cell-cycle progression were observed in both CF- and F®-treated cultures within 5–100 μg/ml concentration-range (Table 1). BLM treatment produced a significant inhibition of the cell-cycle progression compared with the corresponding negative control values ($P < 0.01$). A regression test showed that the number of M_1 was enhanced as a function of the concentration of both CF ($r = 0.98$, $P < 0.01$) or F® ($r = 0.99$, $P < 0.01$) titrated into cultures. Concomitantly, a concentration-dependent decrease in the number of M_2 cells was observed either in CF-treated ($r = -0.98$, $P < 0.01$) or in F®-treated cultures ($r = -0.99$, $P < 0.01$). A significant decrease in the PRI was observed in cultures treated with 50–100 μg/ml of CF ($P < 0.05$ and $P < 0.001$, for 50 and 100 μg/ml, respectively) and F® ($P < 0.01$ and $P < 0.001$, for 50 and 100 μg/ml, respectively). A regression test showed that the PRI decreased as a function of either the concentration of CF ($r = -0.98$, $P < 0.01$) or F® ($r = -0.99$, $P < 0.01$) titrated into cultures (Table 1).

The MI data for CF- and F®-treated cultures are presented in Table 1. BLM (positive control) treatment produced a clear depression of the MI compared with the corresponding control values ($P < 0.01$). For both chemicals, a progressive concentration-related inhibition of MI was observed when 10–100 μg/ml were employed ($P < 0.001$ and $0.05 > P < 0.01$, for CF and F®, respectively). A regression test showed that the MI decreased as a function of either the concentration of CF ($r = -0.88$, $P < 0.05$) or F® ($r = -0.95$, $P < 0.01$) titrated into cultures. When either 100 μg/ml of CF or F® were used, the MI of cultures decreased over control values ($f = 1.00$) by a mean f of 0.35 ± 0.03 and a mean f of 0.32 ± 0.02, respectively (Table 1).

A significant decrease of cell viability was observed after 100 μg/ml of CF-treatment ($P < 0.05$) and when 50 μg/ml ($P < 0.05$) or 100 μg/ml of F® ($P < 0.001$) where titrated into cultures (Fig. 1). Treatments with 5–50 μg/ml of CF or 5–10 μg/ml of F® did not altered cell viability ($P > 0.05$). The decrease in cell viability induced by 100 μg/ml of F® was higher than that induced by an equal concentration of CF ($P < 0.001$) (Fig. 1).

A loss of lysosomal activity, indicated by a decrease in uptake of NR, was observed when CHO_{K1} cells were exposed to 50 and 100 μg/ml of CF ($P < 0.01$; $P < 0.001$, respectively) and with all concentrations of F® assayed ($P < 0.001$) (Fig. 2). A regression test showed that lysosomal activity decreased as a function of the pesticide concentrations only in those CF-treated cultures ($r = -0.98$, $P < 0.01$). A similar trend was observed in F®-treated cultures although not reaching statistical value ($r = -0.73$, $P > 0.05$) (Fig. 2). Alteration in the energetic cell metabolism induced by the pesticides measuring the ability of the mitochondrial succinic dehydrogenase was further analyzed by the MTT assay. The results

Table 1
Sister chromatid exchange (SCE) frequencies, proliferative rate index (PRI), mitotic index (MI), and mitotic proliferative factor (*f*) values in control, carbofuran- and carbofuran-containing technical formulation furadan®-treated Chinese hamster ovary (CHO_{K1}) cells[a]

Dose (μg/ml)	Carbofuran				Furadan®			
	SCE frequencies[b]	PRI	MI[b]	*f*	SCE frequencies[b]	PRI	MI[b]	*f*
0	9.86 ± 0.27	1.95	87.29 ± 0.33	1.00 ± 0.00	10.02 ± 0.24	1.94	82.33 ± 0.24	1.00 ± 0.00
5	10.49 ± 0.32	1.89	72.29 ± 0.32	0.82 ± 0.02	10.99 ± 0.28	1.88	72.78 ± 0.19	0.88 ± 0.01
10	10.72 ± 0.31*	1.76	54.43 ± 0.30***	0.62 ± 0.03	11.65 ± 0.27**	1.86	61.56 ± 0.29*	0.74 ± 0.06
50	11.88 ± 0.33***	1.61*	50.29 ± 0.33***	0.57 ± 0.01	11.80 ± 0.36***	1.54**	57.22 ± 0.36**	0.69 ± 0.03
100	13.15 ± 0.73***	1.10***	31.29 ± 0.66***	0.35 ± 0.03	12.03 ± 0.37***	1.20***	26.76 ± 0.76***	0.32 ± 0.02
BLM	25.72 ± 0.25***	1.60*	36.50 ± 0.33**	0.41 ± 0.03	25.72 ± 0.25***	1.60*	36.50 ± 0.33**	0.41 ± 0.03

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; significant differences with respect to control values. BLM, bleomycin (1 μg/ml, positive control).

[a] Cells were treated 24 h after seeding with test compounds and harvested 24 h later.

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

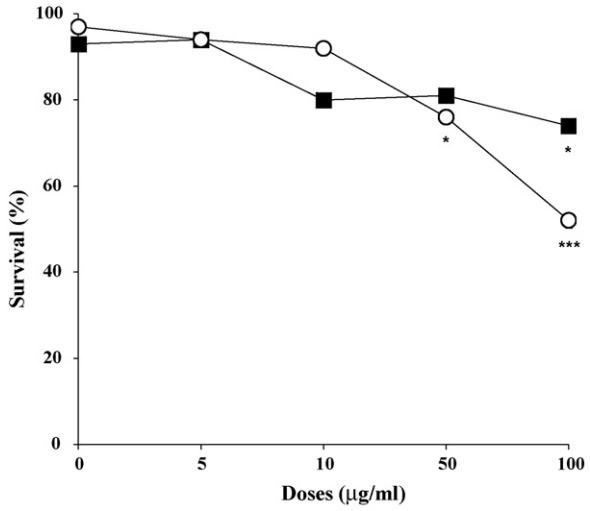

Fig. 1. Effect on *in vitro* treatment with carbofuran (black squares) and furadan® (empty circles) on CHO_{K1} cells. Cell viability was determined using the ethidium bromide/acridine orange assay and expressed as the proportion of living cells at 24 h after pesticide treatment. For each experimental point, pooled data from three independent experiments are reported as mean values ± S.E. of the mean (*y*-axis) and plotted against pesticide concentration (*x*-axis). $^{*}P < 0.05$; $^{***}P < 0.001$.

presented in Fig. 3 show a decrease in the MTT dye reduction to an insoluble violet formazan product when CHO_{K1} cells were exposed to 50 and 100 μg/ml of either CF or F® ($P < 0.01$ and $P < 0.001$, respectively). Fig. 3 also shows that 50 and 100 μg/ml of CF induced a lower alteration in cellular metabolism than the equivalent concentration of F® ($P < 0.05$).

Table 2 summarizes the results of the analysis of pesticide-induced MNi in BN cytokinesis-block CHO_{K1} cells. The frequencies of MNi in the BLM (positive control) treatment cells were significantly increased as compared to the control cultures ($P < 0.001$). A progressive concentration-related induction of MNi was observed after CF-treatments with 10–100 μg/ml ($P < 0.001$). No enhancement of the MNi frequency was observed in those 5 μg/ml CF-treated cells ($P > 0.05$). A regression test showed that the frequency of MNi increased as a function of the concentration of CF ($r = 0.97$, $P < 0.01$) titrated into cultures (Table 2). On the other hand, the frequency of MNi was significantly higher only in 50 μg/ml of F®-treated cultures ($P < 0.001$) than that of control cultures. While 5–10 μg/ml of F® did not increase the frequency of micronucleated cells, no BN cytokinesis-block cells were found in 100 μg/ml-treated cultures. The NDI index was found to be disturbed in both 50–100 μg/ml CF- and F®-treated cultures. As indicated by the NDI values, while treatments with 5–50 μg/ml of CF or 5–10 μg/ml of F® did not alter the rate of nuclear division ($P > 0.05$), 50 μg/ml of CF or 100 μg/ml of F® induced a delay in the onset of cell-division ($P < 0.001$ for CF and F®, respectively). The delayed rate of nuclear division induced by 100 μg/ml of F® was higher than that induced by an equal concentration of CF ($P < 0.001$) (Table 2).

4. Discussion

In the present report, the genotoxicity and cytotoxicity of the systemic pesticide CF and the CF-containing technical formulation F® were evaluated *in vitro* on CHO_{K1} cells. The study was conducted

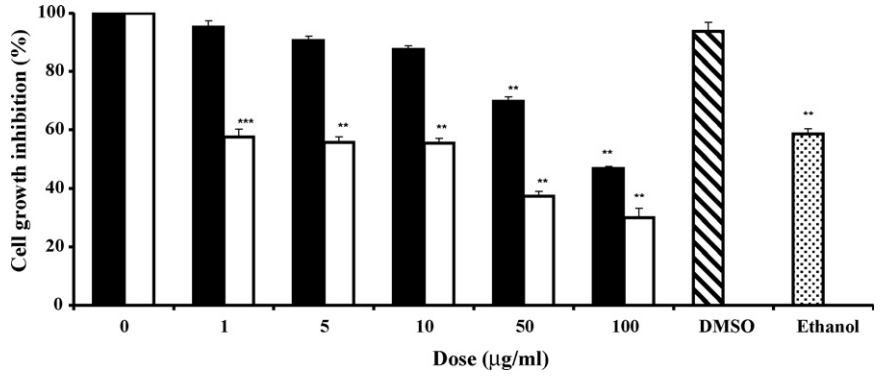

Fig. 2. Lysosomal activity assessed with the neutral red assay (NR) in carbofuran-treated (black bars) and furadan®-treated (white bars) CHO_{K1} cells. Cultures were incubated for 2 h with neutral red dye after 24 h of the pesticide treatment. Results were expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel (*y*-axis) and plotted against the pesticide concentration (0–100 μg/ml concentration-range; *x*-axis). A 0.5% ethanol-treated (dotted bar) and 1% DMSO-treated cells (stripped bar) were used as positive and negative controls, respectively. $^{**}P < 0.01$; $^{***}P < 0.001$.

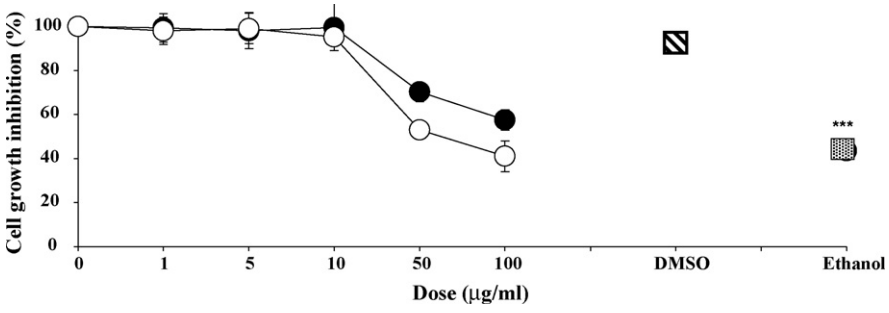

Fig. 3. Cellular metabolism inhibition evaluated by MTT assay in carbofuran-treated (black circles) and furadan®-treated (white circles) CHO_{K1} cultures. Cultures were incubated for 2 h with MTT after 24 h of the pesticide treatment. Results were expressed as mean percentage of cell growth inhibition from three independent experiments performed on parallel (*y*-axis) and plotted against the pesticide concentration (0-100 μg/ml concentration-range; *x*-axis). A 0.5% ethanol (dotted square)-treated and 1% DMSO (stripped square)-treated cells were used as positive and negative controls, respectively. $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2
Micronuclei (MNi) induction and nuclear division index (NDI) values for control, carbofuran and carbofuran-containing technical formulation furadan®-treated in binucleated cytokinesis-blocked Chinese hamster ovary (CHO_{K1}) cells[a]

Dose (μg/ml)	Carbofuran				Furadan®			
	MNi frequencies[b]	Micronucleated cell distribution[c]		NDI[d]	MNi frequencies[b]	Micronucleated cell distribution[c]		NDI[d]
		1 MNi	2 MNis	3 MNis		1 MNi	2 MNis	3 MNis	
0	43.00 ± 0.008	36.33 ± 0.40	3.33 ± 0.40	0.00 ± 0.00	2.07 ± 0.04	43.33 ± 0.008	38.33 ± 2.43	2.00 ± 0.69	0.33 ± 0.40	2.07 ± 0.04
5	44.67 ± 0.008	38.33 ± 2.12	2.67 ± 2.12	0.33 ± 0.40	1.93 ± 0.14	42.67 ± 0.008	38.13 ± 3.82	3.55 ± 0.69	0.33 ± 0.40	1.97 ± 0.04
10	50.67 ± 0.009***	39.33 ± 2.51	5.67 ± 0.80	0.00 ± 0.00	1.97 ± 0.04	44.00 ± 0.008	33.66 ± 2.80	3.33 ± 0.80	0.00 ± 0.00	1.97 ± 0.04
50	65.33 ± 0.010***	50.33 ± 1.60***	7.00 ± 3.18*	0.33 ± 0.40	1.80 ± 0.07	68.33 ± 0.010***	51.00 ± 2.01***	7.67 ± 2.12**	0.67 ± 0.40	1.60 ± 0.00**
100	73.67 ± 0.011***	55.00 ± 1.83***	7.33 ± 2.80*	1.33 ± 1.60	1.57 ± 0.14***	ND		ND		1.13 ± 0.04***
BLM	85.00 ± 0.010***	79.00 ± 0.010***	3.00 ± 0.010***	0.00 ± 0.00	1.40 ± 0.03***	85.00 ± 0.010***	79.00 ± 0.010***	3.00 ± 0.010***	0.00 ± 0.00	1.40 ± 0.03***

ND, not determined. *$P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; significant differences with respect to control values. BLM, bleomycin (1 μg/ml, positive control).

[a] CHO_{K1} cells were treated 24 h after seeding with test compounds and cytochalasin B, and harvested 24 h later.

[b] Results are presented as mean MNi cells/500 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.

[c] Results are presented as number of cells carrying 1, 2 or 3 MNis among 1500 binucleated cytokinesis-blocked cells of pooled data from three independent experiments ± S.E. of the mean.

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

using several bioassays for both genotoxicity (e.g., SCE and MNi frequencies), and cytotoxicity (e.g., cell-cycle progression, MI, MTT, and NR). Overall, both chemicals induced higher SCE and MNi frequencies compared to control values. When not toxic, either CF or F® altered the cell-cycle progression, the mitotic and the nuclear proliferative indices as well as increased lysosomal damage and decreased cellular metabolism. Furthermore, at equal concentrations the F®-induced cytotoxicity was greater than that induced by the pure pesticide. Hence, our observations showed the ability of the active ingredient and the formulated product to induce both genotoxicity and cytotoxicity *in vitro*, at least under the experimental conditions used in the present study.

CF represents an acute poison when absorbed into the gastrointestinal tract by inhalation of dust and spray mist and to a lesser extend through the intact skin. *N*-Methylcarbamate esters are converted by nitrosation into *N*-nitroso derivatives under conditions pertaining mammalian stomach and/or in the presence of nitrites or nitrogen oxides [31] or in the colon by intestinal bacteria [32]. Although less toxic than the parental insecticides to mammals, they are, however, agents of quite extraordinary carcinogenic and mutagenic potency [8,11,14,17,20,33]. However, the mechanism/s by which *N*-nitroso carbamates exert genotoxicity is not fully established so far. Accordingly, the non-consistent results reported so far by the different research groups about the deleterious effects of the *N*-methylcarbamate pesticides could be attributed to the ability to convert this type of pesticides into their *N*-nitroso derivatives by the different cellular systems employed in those studies. We report here a delay in the cell-cycle progression and in the nuclear division index as well as a significant but low induction of SCEs in CHO cells after treatment with CF and F®. The possibility that the CHO_{K1} strain cells we employed were able to metabolize CF into its very well known deleterious and genotoxic *N*-nitroso derivatives could not be ruled out.

Investigations have reported that CF does not induce apoptosis in Chinese hamster lung fibroblast cells *in vitro* [20]. Hence, the decrease in the cell viability revealed in our study in CF- and F®-treated CHO_{K1} cultures by different bioassays could be more related to cell necrosis rather than to cell death by pesticide-induced apoptosis. Moreover, years ago it has been observed that this pesticide is able to induce not only apoptosis in rat cortical neurons *in vivo* but also a down-regulation of the surface α7-subunit of acetylcholine receptors [19]. Whether the former or the latter possibility is the correct mechanism to explain our observations remains unknown. However, the lack of apoptotic figures in our MNi analysis could discard the cellular death process as a consequence of the induction of apoptosis, being most probably due to the cytotoxicity exerted directly by the *N*-methylcarbamate pesticide CF.

Our findings are in accordance with the properties of the carbamates pesticides, and especially with previous studies demonstrating the CF-induced MNi in bone marrow cells [16,34] and in human lymphocytes of pesticide-manufacturing workers [35]. It has been also claimed that CF-induced MNi would most probably be the result of lagging chromosomes due to spindle failure and/or induced abnormalities rather than chromosome break induction [35]. Evidences have been reported that disturbances in the spindle apparatus of mammalian cells are observed after treatment with several carbamate pesticides [36,37]. Further experiments are under development to analyze whether CF and/or F® are able to introduce disturbances or abnormalities in the spindle apparatus.

As a commercial insecticide formulation, F® contains a 53% of excipients theoretically not possessing deleterious genotoxic and/or cytotoxic effect/s. Unfortunately, the identity of the components present in the excipient formulation was not made available to us by the manufacturer. Several investigations have proved that

commercial formulations have ability to induce DNA damage, and in most of the cases such injury differs to the pure pesticide effects [38–42]. In agreement, our results demonstrated that the excipient contained in F® affects the genotoxic potential of the insecticide, possessing its component/s the ability of induce cellular damage. Two plausible explanations for this peculiar observation could be hypothesized. Though almost improbable, the possibility that the concentration of CF incorporated in the technical formulation could be higher than 47% as indicated by the manufactures could not be discarded. Finally, the possibility that during the manufacture of the F®, the parental CF could be converted into any NO-CF derivatives cannot be ruled out. In spite of any putative plausible explanation, it seems evident that the component/s of the excipient from the Argentinean CF-containing technical formulation F® were able to enhance the CF-induced both genotoxicity and cytotoxicity, at least in CHO_{K1} cells.

Finally, our study clearly demonstrate that CF by itself and even in a greater extend its Argentinean formulation F® exert both genotoxicity and cytotoxicity in mammalian cells in culture, at least in CHO_{K1} cells. Further studies are required in order to analyze whether these effects are committed to CHO_{K1} cells or can be extensive to other cell types either *in vivo* or *in vitro*.

Conflict of interest statement

None.

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