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Evaluation of the genotoxic potential of cypermethrin, chlorpyrifos and their subsequent mixture, on cultured bovine lymphocytes

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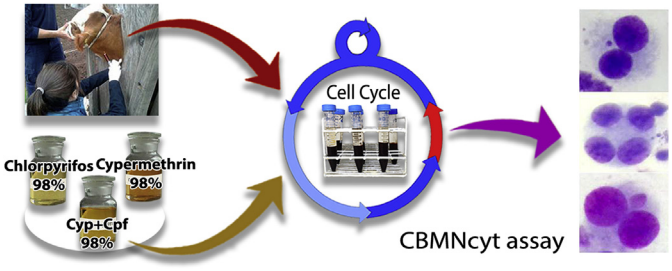
HIGHLIGHTS

- CYP, CPF and their mixture produced genotoxic effects on cultured bovine lymphocytes.
- CYP and the mixture concentrations were correlated with micronucleated cells frequency.
- CPF concentrations show correlation with the frequency of micronuclei and nuclear buds.
- Increasing CPF concentrations induced a decrease in cell proliferation.
- The genotoxicity of veterinary medicines can be evaluated in bovine cell cultures.

GRAPHICAL ABSTRACT



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ABSTRACT

Cypermethrin (CYP) and chlorpyrifos (CPF) are insecticides/parasiticides used in the production of fruits, vegetables and beef cattle. These substances or their metabolites are frequently reported as residues in food, whose consumption in a diet implies a genotoxic risk. The potential for chronic toxicity of CYP and CPF is unclear, and only a few genotoxicological evaluations based on their mixture have been performed. The aim of this study was to evaluate the genotoxic potential of CYP, CPF and CYP + CPF in five concentrations, from 5.9 to 175 μg/mL, on bovine lymphocytes. By means of the cytokinesis-block micronucleus cytome assay, a decrease in the cell proliferation index was observed ($r = -0.89$ $p = 0.04$); and also an increase in the frequencies of binucleated cells (BN) with micronuclei (BNMn) ($r = 0.93$, $p = 0.02$) and BN with nuclear buds (BNBud) ($r = 0.778$ $p = 0.04$), depending on the concentrations of CPF. An increase in BNMn frequencies was observed as a function of CYP concentrations ($r = 0.89$, $p = 0.04$) and also of the CYP + CPF mix ($r = 0.99$, $p = 0.008$). CYP caused greater genotoxic damage (BNMn) than CPF and the mixture on bovine lymphocytes. Cells with simultaneous presentation of micronuclei and nuclear buds were detected, as well as cells with irregular nuclei, something never previously reported, whose origin and significance should be investigated. The genotoxic effect of chlorpyrifos, cypermethrin

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and their mixture on bovine lymphocytes was observed. We recognized the value of the use of primary bovine cultures, animal species adjacent to man in the food chain, for genotoxicity studies.

1. Introduction

Agriculture and livestock have dramatically increased their yields due, in part, to the application of chemical treatments to control pests in crops; and also the use of medicines to help prevent and treat diseases of domestic animals that incur economic losses in production (Pórfido et al., 2014). In crop production, the amount of pesticides being used in the world is increasing (Atwood and Paisley-Jones, 2017). The use of veterinary drugs and some organophosphorus pesticides (OP) in livestock is now a common practice (Pórfido et al., 2014). In the production of beef cattle, pesticides generally enter the live animal, through the consumption of plants contaminated with pesticides, by the application of antiparasitic agents in the animal or in the environment where the beef cattle are being produced (LeDoux, 2011; Katole et al., 2013). The parasiticides are the group of drugs most commonly used in livestock production (Kay et al., 2017) and their use has generated undesirable consequences such as the presence of stable substances in ecosystems, bio-magnification and chemical resistance (Anadón et al., 2009).

There is a coincidence between the groups of pesticide residues in food with respect to the same active ingredients used as insecticides in crops and the medicines in veterinary practices, mainly parasiticides. The actual exposure of the population to certain active ingredients may be higher than expected due to different food preferences, and the potential risks may arise from the consumption of different pesticide residues consumed on a daily basis (Boobis et al., 2008; Crépet et al., 2013). In this context, “aggregate exposure” refers to exposure to a single active ingredient through different routes and “cumulative exposure” refers to the exposure to several active ingredients also through several routes (Meek et al., 2011).

The types of pesticides and their combinations that are used in a region depend to a large extent on the type of crops and the local practices used (Jess et al., 2014). In previous studies, we have shown that, in addition to the individual active principles, the insecticide formulations with chlorpyrifos (CPF) and cypermethrin (CYP) are used in each of the following three food production systems: the production of fruits, vegetables and cattle from the semi-arid region of western Argentina (Ferré et al., 2018).

Chlorpyrifos (O, O-diethyl-O- [3, 5, 6-trichloro-2-pyridinyl] phosphorothioate), CAS No. 2921-88-2, is an OP widely used in food production (United States Environmental Protection Agency, 2016; European Food Safety Authority et al., 2017); and its presence as residues in food has been reported worldwide (Australian Pesticides and Veterinary Medicines Authority, 2009; European Food Safety Authority et al., 2017). In our country CPF is the main insecticide currently applied in agricultural production (Villaamil Lepori et al., 2013). CYP ((1RS) -cis, *trans*-3- (2,2-dichlorovinyl) −2,2-dimethylcyclopropanecarboxylate (RS) -cyano-3-phenoxybenzyl), CAS No. 52 315-07-8 is a synthetic pyrethroid and is also widely used in food production (United States Environmental Protection Agency, 2006). The presence of CYP residues in bovine milk has been reported worldwide (Food and Agriculture Organization of the United Nations- World Health Organization, 2008).

In the National Plan for the Control of Residues in Foods (CREHA-SENASA), the published reports from 2018 indicated that from 971 samples of slaughtered bovine analyzed for external antiparasitic agents (carbamates, pyrethroids and OP), 5.56% were positive not exceeded (PNE) for pyrethroid residues and in one sample the Maximum Residual Limit (MRL) allowed was exceeded. One sample was PNE for OP residues and another positive was exceeded for the same residue. In a European monitoring program, 80% of the fruit and vegetable samples imported from Argentina showed residues below the MRL, with 5% above. CPF was the main pesticide detected (Hjorth et al., 2011). Residues of CYP have been detected in samples of ground beef meat in Italy (Stefanelli et al., 2009); and OP residues have been detected in meats in China studies (Zhan et al., 2013). CPF and CYP are moderately dangerous (class II) according to their acute toxicity. The LD_{50} administered orally to rats is 135 mg/kg for CPF and 250 mg/kg for CYP (World Health Organization, 2010). However, the main concern underlying this present study is the possible chronic toxicity to consumers. Both chemicals are not included in the IARC monographs (International Agency for Research on Cancer- World Health Organization, 2016). CPF is listed as “evidence of non-carcinogenicity for humans” and CYP as “possible human carcinogen” (United States Environmental Protection Agency, 2015) and both were included as suspects in the endocrine disruptor screening program (United States Environmental Protection Agency, 2015; Kegley et al., 2016).

The genotoxic potential of CPF has been evaluated by cytokinesis block micronucleus (CBMN) assays in human lymphocytes and HepG2 cells (Želježić et al., 2016), in addition a comet assay in human lymphocytes (Sandhu et al., 2013), a micronucleus assay in mice (Yaduvanshi et al., 2012) as well as in rats (Sandhu et al., 2013), all with inconclusive results. Other studies have concluded that CPF is a strongly genotoxic agent that induces simple and double damage to DNA and cellular apoptosis in humans HeLa and HEK293 cells (Li et al., 2015).

Evaluations of CYP genotoxic potential have been reported by the CBMN assay on human lymphocytes (Surrallés et al., 1995); in lymphocytes from rabbits exposed to oral doses of CYP (Vardavas et al., 2016); comet assay in brain cells from rats (Hussien et al., 2013) and in blood and liver cells in fetuses obtained from pregnant female mice exposed to CYP (Murkunde et al., 2012). Both CYP and CPF were evaluated individually in the comet assay in human lymphocytes (Sandal and Yilmaz, 2011), and in the comet assay and UDS assay in mouse hepatocytes (Cui et al., 2011).

It has been reported that CPF and CYP induce oxidative stress in different biological systems, interfere with the synthesis of certain hormones, alter reproductive processes and also affect physical development (Dahamna et al., 2010; Viswanath et al., 2010; Afzal Gill et al., 2011; Kopjar et al., 2018).

In a mixture, pesticides can interact with each other in various ways, depending on the compound itself, the chemical group to which they belong, specific dose and target organs, all of which can lead to various effects. Long-term exposure to low doses of pesticide mixtures is currently considered a more realistic scenario that may involve multiple potential interactions between different pesticides (Hernández et al., 2017). This exposure can have adverse effects on health and could contribute to an increased risk of long-term diseases, including cancer (Parrón et al., 2011),

neurodegenerative diseases (González-Alzaga et al., 2014) and immunological deregulation (Mokarizadeh et al., 2015). Studies on co-expositions of biological systems to CPF + CYP are scarce, especially those that evaluate genotoxic effects.

The objective of this study was to evaluate the genotoxic potential of different concentrations of cypermethrin, chlorpyrifos and their mixture, applied "in vitro" on bovine blood lymphocytes.

2. Materials and methods

2.1. Selection of the non-cytotoxic concentrations of cypermethrin and chlorpyrifos to be applied in the CBMN-cyt assay

Before the implementation of the cytokinesis block micronucleus cytome (CBMN-cyt assay), a supravital test with modified trypan blue was performed (Sigma-Aldrich, 2010) to determine that concentrations of CYP and CPF did not exceed 60% cytotoxicity (OECD, 2016). Blood was extracted with heparin from the jugular vein of a 345 kg, 18 month old Aberdeen Angus castrated male, observed to be in good health and that was being reared in an intensive fattening system with balanced feed supplemented with corn, and with access to water ad libitum. Lymphocytes were obtained by separating with Histopaque 1077 density gradient (Sigma®) and a 4 h cell cultures were achieved in RPMI culture medium (Gibco®) at 38.5 °C. The cultures were exposed to 1.25, 2.5, 5, 10, 20, 40, 80 and160 μg/mL concentrations of CPF and CYP (Chemotecnica®) individually. Both standards were dissolved in DMSO Sigma®. The negative control was performed with DMSO. All cultures were performed in duplicate. The cells were stained with 0.4% trypan blue diluted in sterile physiological solution and observed in a Neubauer chamber with an optical microscope (Nikon®). The observation and cell counts were produced in duplicate for each culture. To obtain the cell viability %, the following formula was applied: (Number of total living cells) ÷ (Total number of cells [alive + dead]) × 100.

2.2. Cytokinesis-block micronucleus cytome assay

Blood was extracted with heparin from the jugular vein under aseptic conditions from a 14 month old Aberdeen Angus steer weighing 298 kg, that had been reared in similar conditions to the previously mentioned castrated male. The extracted blood was stored at 4–8 °C for 2 h until it was processed in the laboratory. The lymphocyte cultures were held in suspension for 72 h at 38.5 °C according to Moorhead et al. (1960) with slight modifications, and the CBMN-cyt was implemented according to Fenech (2000) and OECD (2016). In falcon conical tubes, 0.5 ml of heparinized blood, 4 mL of RPMI 1640 culture medium, 1 mL of fetal bovine serum (Natocor®), 150 μl of phytohemagglutinin M (Gibco®) and 57 μl of streptomycin (10 mg/mL) - penicillin (10 000 IU/mL) (Sigma®), were incorporated. Five cultures were exposed to 175, 87.5, 43.7, 21.8 and 10.9 μg/mL of CYP 98% (Chemotecnica®) concentrations equivalent to 70; 35; 17.5; 8.75 and 4.37% of the LD_{50} (WHO, 2010). Another 5 cultures at 94, 47, 23, 11 and 5.9 μg/mL of 98% CPF (Chemotecnica®) concentrations equivalent to the same percentage of their LD_{50}. The last 5 cultures were exposed to the mixture of the same individual concentrations. The positive control was performed with mytomycin C (MMC) 0.25 μg/mL; the negative control with DMSO 0.49 μl/ml. All cultures were performed in duplicate. The active ingredients to be evaluated were incorporated at the start of the incubation. After 44 h, 5 μg/mL of cytochalasin B (Sigma®) was added and the incubation was then continued; after an additional 28 h, the tubes were centrifuged for 10 min at 3000 rpm; the supernatant was discarded and replaced with a hypotonic solution of cold 0.075 M KCl, the contents were homogenized and allowed to incubate for 2–3 min at 4 °C. They were centrifuged again and the supernatant was replaced with a Carnoy methanol and acetic acid (3: 1) fixative solution. The samples were kept at between 4 and 8 °C for 24 h, then the fixative was renewed 3 times. They were then spread out on clean slides and stained with 10% Giemsa in distilled water for 10 min. We then performed observations by optical microscope (Nikon Eclipse®). The variables analyzed were mononuclear and multinucleated cells (bi, tri, tetra and pentanucleated). The cell proliferation index (CBPI) was obtained by using the formula (M1 + 2 (M2) + 3 (M3 + M4 + M5)/N, where M1 - M5 represents the number of cells with 1–5 nuclei and N the total number of counted cells (Fenech, 2000; OECD, 2016). They were analyzed as: binucleated cell with micronuclei (BNMn), binucleated cell with bud (BNBud), binucleated cell with micronuclei and bud (BNMn + Bud), binucleated cell with nucleoplasmic bridge (BNbridge) (Fenech, 2006, 2007). The binucleated cell variable with irregular nuclei (BNIrr) was determined as a cell with two nuclei, each one with a different size and shape. At each tested concentration, 4000 cells were analyzed (2000 in each duplicate culture). The results are expressed as the frequency of each variable analyzed/1000 cells.

2.3. Quality criteria

The bovine blood used in the CBMN-cyt assay was previously analyzed by gas chromatography in order to verify the absence of CPF and CYP. In 2 mL of plasma, microextraction of analyte in solid phase (SPME) (Tri Plus RSH®) was carried out using fiber of 65 μm PDMS/DVB (Supelco®), stirred at 45 °C, incubated for 15 min, extracted on 30 min, desorption of 30 min and finally being shaken whilst off for 15 s, and on for 2 s. The equipment used was a ThermoScientific ™ TRACE ™ Ultra GC with mass detector (Waltham, Massachusetts, USA) and TRI PLUS RSH autosampler (SPME), which had a 5% phenylmethylpolysiloxane capillary column, which was 30 mm long x 0.25 mm thick and 0.25 mm in diameter. The conditions of the chromatograph were as follows: A MS Xline temperature of 180 °C, an ion source temperature of 150 °C, an ionization mode EI and voltage of 71 eV. The ranges were SIR CYP m/c: 51, 77, 91, 115.127, 152, 163, 165, 181, 208, 209, 379, 415.07; CPF m/c: 97, 197, 199, 314, 316, 125, 201, 65, 349. The conditions of the TRACE chromatograph were: ramp temperature: 40 °C (3 min); 150 °C (4 °C/min, 1 m) 200 °C (6 °C/m, 1 min); 280 °C (10 °C/m, 10 m). S/L Front: Splitless mode T = 250 °C, split flow = 75 mL/m, Splitless = 3 min, Flow = 1.2 mL/m. In the standards of both active principles (Pestanal®), the retention times of each one in sodium chloride solution at 8.5 g/L was determined.

2.4. Statistical analysis

The frequencies of the genotoxicity variables obtained in the culture with MMC were compared with those of the negative control procured by Student's t-test. The type of distribution presented by the analyzed variables (CBPI, BNMn, BNBud, BNMn + Bud, BNbridge, BNIrr) was evaluated by Kolmogorov-Smirnov. After standardization of the data, the ANOVA test was applied to each variable from the 3 treatments of the study, followed by Tukey posttest. In order to analyze if there was any correlation between the tested doses of CYP, CPF, and CYP + CPF with the CBPI index, the frequencies of BNMn, BNBud, BNMn + BNBud, BNIrr, were applied to the Pearson correlation test. To try to identify any correlation between the different variables previously studied in each tested concentration, the same correlation test as previously mentioned was applied. The GraphPad Prism 6.0 was used to obtain the analysis.

3. Results

The chromatographic search for CYP and CPF in bovine blood prior to "in vitro" exposure tests showed that it was free of pesticide residues. The retention time for the 10.7 μg/L CYP standard was 27.4 min and for 10.0 μg/L CPF standard was 39.7 min, according to the mass spectrum obtained from the NIST library (Standard Reference Database, https://www.nist.gov/srd/nist-standard-reference-database-1a-v17). The limit of detection was 3 μg/L for CYP and 1 μg/L for CPF. The limit of quantification was 6 μg/L for CYP and 2 μg/L for CPF. A representative gas chromatogram of CPF and CYP according to the mass spectrum obtained from the NIST library is provided as supplementary material, also the parameters listed by the chromatograph.

In the cell viability assay with trypan blue it was observed that, of the CPF and CYP exposure concentrations tested, none of them resulted in the death of >60% of the cultured lymphocytes. The highest concentrations tested (160 and 80 μg/mL), resulted in between 9 and 12% of cell mortality; whilst the negative controls caused between 6 and 13%. A table with cell viability data of bovine lymphocytes exposed to 5 concentrations of CPF and CYP in cell cultures is provided as supplementary material.

The following non-cytotoxic concentrations of CPF/CYP were selected to evaluate their genotoxic potential: 94/175.0 μg/mL (70.0% of the equivalent LD_{50}); 47.0/87.5 μg/mL (35% of the LD_{50}); 23.0/43.7 μg/mL (17.50% of the LD_{50}); 11.0/21.8 μg/mL (8.75% of the LD_{50}) and 5.9/10.9 μg/mL (4.37% of the LD_{50}).

Fig. 1 shows cell types that were found in the implementation of the CBMN-cyt assay in bovine lymphocytes. We identified and quantified a cell type that had not been reported in the previously validated assay on human lymphocytes (Fenech, 2006). They are binucleated cells but their nuclei were found to have different shapes and/or sizes within them and we have named them as Binucleated cells with irregular nuclei (BNIrr).

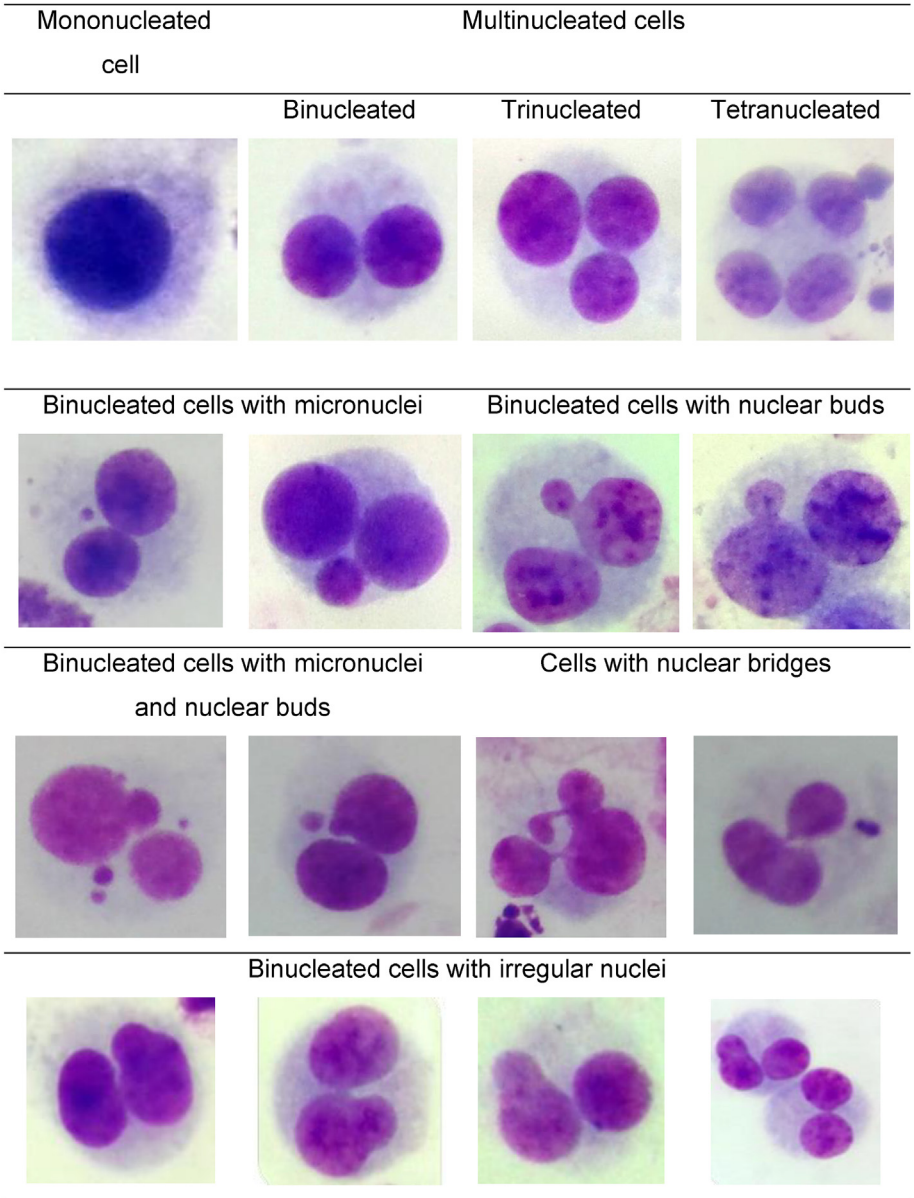

Fig. 1. Bovine lymphocytes exposed to cypermethrin, chlorpyrifos and their mixture, and types of nuclear abnormalities observed in the Cytokinesis Block Micronucleus Cytome assay.

Table 1
Biomarkers of cyto- and genotoxic effect in bovine lymphocytes exposed to cypermethrin, chlorpyrifos and their mixture, obtained by the Cytokinesis-block micronucleus cytome (CBMN-cyt) assay.

Active principle	μg/mL	Number of cell types/1000 cells					
		CBPI	BNMn	BNBud	BNMn + BNBud	BNBrid	BNIrr
CPF	5.9	1.327 ± 0.095	13.000 ± 1.414	29.000 ± 9.899	0.000 ± 0.000	0.000 ± 0.000	13.500 ± 2.121
	11.0	1.303 ± 0.106	16.000 ± 5.657	30.000 ± 11.314	0.500 ± 0.707	1.000 ± 0.000^{cfg}	18.000 ± 1.414
	23.0	1.296 ± 0.059	16.000 ± 5.657	37.000 ± 9.899	0.500 ± 0.707	0.000 ± 0.000	21.50 ± 2.121
	47.0	1.289 ± 0.055	19.000 ± 1.414	37.000 ± 7.071	0.000 ± 0.000	0.000 ± 0.000	21.000 ± 2.828
	94.0	1.262 ± 0.003	21.000 ± 4.243	41.000 ± 9.899	1.000 ± 1.414	0.000 ± 0.000	27.000 ± 7.071
CYP	10.9	1.308 ± 0.034	20.000 ± 2.828	28.000 ± 0.000	0.500 ± 0.707	0.000 ± 0.000	18.500 ± 0.707
	21.8	1.280 ± 0.011	25.000 ± 1.414	34.000 ± 2.828	0.500 ± 0.707	0.500 ± 0.707	24.500 ± 3.536
	43.7	1.227 ± 0.004^{a}	24.000 ± 11.314	31.000 ± 7.071	2.000 ± 0.000	0.000 ± 0.000	24.000 ± 2.828
	87.5	1.218 ± 0.020^{b}	36.000 ± 5.657^{cde}	32.000 ± 8.485	0.000 ± 0.000	1.500 ± 0.707^{chi}	25.000 ± 5.657
	175.0	1.213 ± 0.004^{b}	37.000 ± 1.414^{cde}	36.000 ± 11.314	1.000 ± 1.414	0.000 ± 0.000	26.500 ± 2.121
CPF + CYP	10.9 + 5.9	1.262 ± 0.045	15.000 ± 4.243	36.000 ± 2.828	0.500 ± 0.707	0.000 ± 0.000	20.000 ± 1.414
	21.8 + 11.0	1.261 ± 0.007	16.000 ± 2.828	36.000 ± 2.828	0.000 ± 0.000	0.000 ± 0.000	20.000 ± 1.414
	43.7 + 23.0	1.232 ± 0.011^{a}	17.000 ± 4.243	37.000 ± 7.071	2.000 ± 2828	0.000 ± 0.000	23.000 ± 1.414
	87.5 + 47.0	1.233 ± 0.021^{a}	20.000 ± 5.657	39.000 ± 1.414	0.000 ± 0.000	0.000 ± 0.000	23.000 ± 4.243
	175.0 + 94.0	1.217 ± 0.018^{b}	29.000 ± 7.071	39.000 ± 4.243	0.000 ± 0.000	0.000 ± 0.000	24.500 ± 3.536
MMC	0.25	1.175 ± 0.018	42.000 ± 5.657^{a}	37.000 ± 7.071	0.500 ± 0.707	1.500 ± 0.707	36.500 ± 7.778
DMSO	0.50	1.362 ± 0.006	13.000 ± 1.414	30.000 ± 2.828	0.000 ± 0.000	0.000 ± 0.000	18.5000 ± 4.243

All data are presented as means ± standard deviation of cultures performed in duplicate. CYP: cypermethrin, CPF: chlorpyrifos, MMC: mitomycin C, DMSO: dimethyl sulfoxide, CBPI: cytokinesis-block proliferation index, Binuclear cell with micronuclei (BNMN), binuclear cell with bud (BNBud), Binuclear cell with micronuclei and Bud (BNMNBud), Binuclear cell with nucleoplasmic bridge (BNbrid), BNIrr: binuclear cell with irregular nuclei. a = * b = ** c = ***against DMSO; d = ** against CPF; e = ** against CPF + CYP; h = ****against CPF; i = **** against CPF + CYP; f = *against CYP; g = *** against CPF + CYP.

In Table 1 the frequencies of all the cell types analyzed in the cultures that were exposed to the different concentrations of CYP, CPF and their mixture are presented.

The dose-response correlation between the concentrations used and the frequency of the biomarkers are shown in Figs. 2–4.

The increase in CPF concentrations in culture showed a correlation with the decrease in the CBPI of the assay: r = −0.89, p = 0.04 (Fig. 2).

The correlation of the active principle concentrations tested versus binucleated cells with nuclear alterations was positive for CPF with BNMn r = 0.93, p = 0.02; CYP with BNMn r = 0.89, p = 0.04; CPF + CYP with BNMn r = 0.99, p = 0.008 (Fig. 3); CPF with BNBud r = 0.778, p = 0.04 Fig. 4).

The possibility of any correlations between the different nuclear alterations was analyzed in order to see if the mechanisms of formation of each nuclear alteration could be related. In the cultures exposed to CPF positive correlations were found between BNMn and BNBud (r = 0.87, p = 0.05); BNMn and BNIrr (r = 0.92, p = 0.02); and between BNIrr and BNBud (r = 0.94, p = 0.01). In cultures exposed to CYP, a positive correlation was found between the frequencies of BNIrr and BNBud (r = 0.89, p = 0.03). While in cultures exposed to CPF + CYP, a positive correlation between BNIrr and BNBud (p = 0.04) was detected.

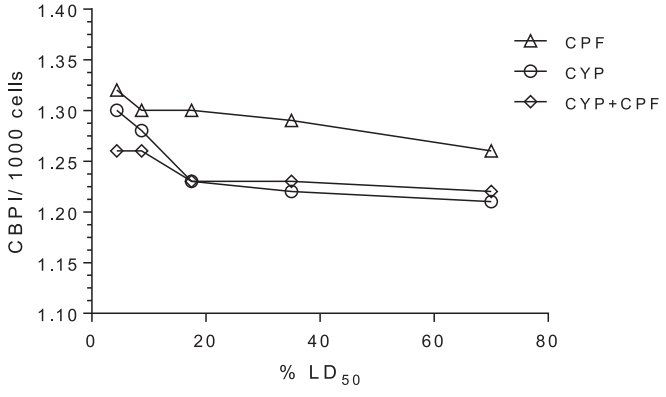

Fig. 2. Effect of cypermethrin, chlorpyrifos and their mixture on the cellular proliferation of bovine lymphocytes exposed in vitro. CPF: chlorpyrifos, CYP: cypermethrin, CBPI: cytokinesis-block proliferation index. % LD_{50}: percentage of the CPF or CYP LD_{50} in rats (WHO, 2010). The indices are means obtained with CBPI from duplicate cultures. % LD_{50} and (the equivalent μg/ml of CPF/CYP in culture): 4.37% DL_{50} (5.90/10.90 μg/ml), 8.75% LD_{50} (11.00/21.80 μg/ml); 17.50% LD_{50} (23.00/43.70 μg/ml); 35% LD_{50} (47.00/87.50 μg/ml); 70% LD_{50} (94.00/175.00 μg/ml). Correlations: CPF with CBPI r = −0.89, p = 0.04.

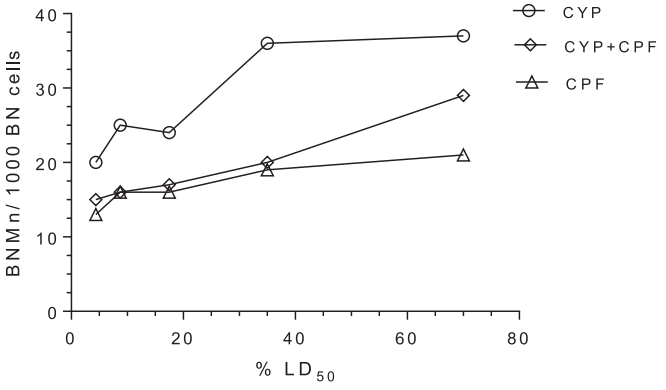

Fig. 3. Effect of cypermethrin, chlorpyrifos and their mixture on the Binuclear cells with micronuclei frequencies of bovine lymphocytes exposed in vitro. CPF: chlorpyrifos, CYP: cypermethrin, BNMn: Binuclear cells with micronuclei. BN: binucler cells. % LD_{50}: percentage of the CPF or CYP LD_{50} in rats (WHO, 2010). % LD_{50} and (the equivalent μg/ml of CPF/CYP in culture): 4.37% LD_{50} (5.90/10.90 μg/ml), 8.75% LD_{50} (11.00/21.80 μg/ml); 17.50% LD_{50} (23.00/43.70 μg/ml); 35% LD_{50} (47.00/87.50 μg/ml); 70% LD_{50} (94.00/175.00 μg/ml). Correlations: CPF with BNMn r = 0.93, p = 0.02; CYP with BNMn r = 0.89, p = 0.04; CPF + CYP with BNMn r = 0.99, p = 0.008.

4. Discussion

The genotoxicity assay was performed following OECD guidance, which is periodically reviewed. The highest chemical concentration must be determined in order to be tested, which does not produce excessive cytotoxicity, precipitation and changes in pH or osmolarity (OECD, 2016). This was determined as detailed in section 2.1. CYP, CPF and the mixture concentrations, starting from 5.9 μg/mL CPF and 10.9 μg/mL CYP, were correlated with micronuclei or bud frequencies. These concentrations are higher than those detected environmentally. In our country the presence of CPF

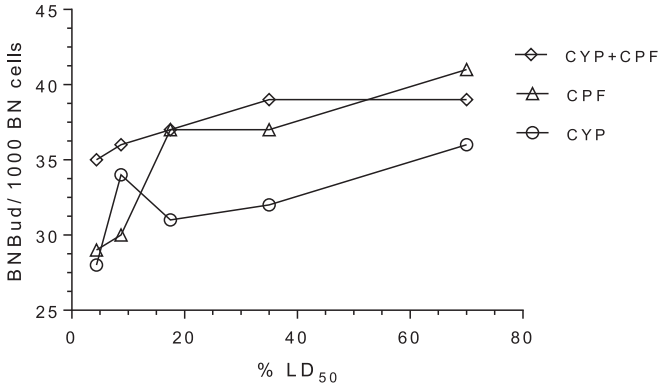

Fig. 4. Effect of cypermethrin, chlorpyrifos and their mixture on the Binuclear cells with buds frequencies of bovine lymphocytes exposed in vitro. CPF: chlorpyrifos, CYP: cypermethrin, BNBud: Binuclear cells with buds. % LD_{50}: percentage of the CPF or CYP LD_{50} in rats (WHO, 2010). % LD_{50} and (the equivalent μg/ml of CPF/CYP in culture): 4.37% LD_{50} (5.90/10.90 μg/ml), 8.75% LD_{50} (11.00/21.80 μg/ml); 17.50% LD_{50} (23.00/43.70 μg/ml); 35% LD_{50} (47.00/87.50 μg/ml); 70% LD_{50} (94.00/175.00 μg/ml). Correlations: CPF with BNBud r = 0.778, p = 0.04.

has been confirmed in soils, surface waters and groundwater in 3 distant areas, with a concentration up to 1.16 μg/L (Etchegoyen et al., 2017). In addition to the intensive agricultural use of the active principles, a variety of therapeutic formulations of CPF and CYP are marketed in Argentina for use in cattle, CYP 20% + CPF 50%; CYP 20%; CYP 10% + Etion 40%, all immersion baths (http://www.motivar.com.ar/2015/12/listado-aprobado-de-garrapaticidas); CYP 20% + CPF 50% for spray use; CYP 6%; CYP 5% + CPF 2.5% + piperonyl butoxide (PBX) 1%; CYP 4% + CPF 4% + PBX 4% all for pour on use (https://www.sani.com.ar/producto.php?id_producto=338), only to exemplify the diversity of concentrations in market.

The implementation of the CBMN-cyt assay allows the evaluation of the cytostatic, cytotoxic and genotoxic effect of the active principles under study (Fenech, 2007). The cytostatic effects were evaluated with the CBPI index. The CBPI indicates the average number of cell cycles experienced by a cell. A CBPI equal to 1 means that all the cells are mononuclear, which means that 100% cytostasis has occurred (OECD, 2016). The CBPI is biologically more relevant than the nuclear division index (NDI) (Surrallés et al., 1995), and that is why it was used in this study. All concentrations of CYP, CPF and CYP + CPF evaluated induced a CBPI> 1 (Table 1). The CBPI results define if the cultures are developing and multiplying normally, in this assay the highest concentrations of CYP and the mixture tested induced a decrease in the CBPI (Fig. 2).

Another cytotoxicity indicator cells could not be evaluated in these assays because in the final stages of cell cultures a hypotonic solution of KCl was used that produced the destruction of necrotic and apoptotic cells (Fenech, 2000). The test was validated in bovine lymphocytes exposed to MMC, which tripled the frequency of binucleated cells with micronuclei as detected in the negative control and doubled the frequency of irregular cells. In addition, binucleated cells with nucleoplasmic bridges and with micronuclei + nuclear buds were only induced with MMC, and were absent in the negative control (Table 1). Other chemical compounds evaluated in CBMN-cyt bovine lymphocytes were doramectin (Anchordoquy et al., 2019), thiacloprid (Galdíková et al., 2015; Schwarzbacherová et al., 2018) bisphenol A (Šutiaková et al., 2014). These reports have also been able to evaluate clastogenic and aneugenic effect, through cultures of bovine lymphocytes.

The CPF + CYP mixture induced BNMn frequencies slightly higher than those observed for CPF individually and lower than those found in CYP exposures. These results are in agree with the findings to be found in related literature where it is established that co-exposure to pyrethroids and OP can cause toxicokinetic interactions. The highly toxic CPF-oxon metabolite is an irreversible inhibitor of CYP hydrolysis mediated by esterase enzymes, thus decreasing the activity of these enzymes responsible for the cleavage of the ester bond in the pyrethroid molecule whilst increasing the toxicity of the mixture (Idris et al., 2012; Hernández et al., 2017, 2013; Hernández and Tsatsakis, 2017). It should be clarified that the trials of the present study were carried out on lymphocytes, cells that possess toxic metabolic capacity, because they present enzymes of cytochrome P450 complex (Coutiño Rodríguez et al., 2010).

The concentrations tested for both compounds are comparable because they were calculated in relation to a range of 4–70% of the LD_{50} reported for both individually. At the tested concentrations of CYP, CPF and CYP + CPF, the BNMn frequencies were mostly higher than those of the negative control (Table 1), with statistical differences in two concentrations of CYP (87.5 and 175.0 μg/ml). BNMn frequencies were statistically higher in CYP than in CPF, and lower in the mixture than in any of the individual insecticides. However, we believe that the most important finding of this study is the relationship between the genotoxic response and the concentration of the active principles, demonstrated with the three treatments for BNMn and with CPF for BNBud (Figs. 3 and 4). Damage to DNA was also dose-dependent for the CPF concentrations tested on Hela and HEK293 cells (Li et al., 2015).

At the molecular level, MN can be originated by several different processes, such as, unrepaired double-stranded DNA breaks, scission DNA repair as well as the fragmentation of chromosomal material, when buds have formed, additionally by hypomethylation of the cytosine located in centromeric and pericentromeric sequences; for defects in the assembly of the mitotic spindle and the control point of mitosis, and finally by abnormal centrosome amplification (Fenech et al., 2011). On the other hand, nuclear buds are the product of amplified DNA, DNA repair complexes and possibly chromosomes in excess of aneuploid cells that are selectively localized to specific sites in the periphery of the nucleus and are eliminated as nuclear buds during the S phase (Fenech et al., 2011).

Micronuclei and nuclear buds have their formation mechanisms through an interdependent relationship that could support the higher frequencies of BNBud compared to those of BNMn. It is important to mention that what the observer recognizes as a micronucleus is actually a detained instance of a rapid dynamic process (in min), which may also imply that MNs are re-incorporated into the main nucleus, re-expelled or merged (Yasui et al., 2010). Whereas the buds, according to the moment in which they were formed, have a band of either narrow or wide nucleoplasmic material (Fig. 1). Because of this, microscopic observation is complex; so we adhere to the idea that the classification of cells requires a subjective decision by the researcher that can sometimes be difficult to make (Heddle et al., 2011). In the present study we are able to report the simultaneous presentation of binucleated cells with MN and nuclear buds (BNMn + BNBud) (Fig. 1) at low frequency, but not observed in humans in other published studies (Fenech et al., 2011); nor in CBMN studies conducted in cattle (Kadmiri et al., 2006; Lee et al., 2007; Šutiaková et al., 2014). The concentration of 17.5% LD_{50} of CYP and the CYP + CPF mixture induced the highest frequency of BNMn + Bud; even higher than the reported frequency for the positive control. In the negative control culture, this nuclear anomaly was not detected. Nucleoplasmic bridges were induced by MMC, and with only a concentration of CPF or CYP. In cultures with mixtures, the situation

was alleviated. This was also observed in the frequency of micronuclei as indicated above.

In this study, we have reported binucleated cells with irregular nucleus (BNIrr) (Fig. 1). This category has not been described for CBMN-cyt in humans (Fenech, 2000, 2006; 2007; Fenech et al., 2011), nor in the other CBMN- cyt assays in bovines, previously mentioned. This type of cell was present in all cell cultures. In addition, in the positive control test, the CBIrr frequency was the highest, which makes us believe that it is derived from a genotoxic effect. More studies are needed to clarify the importance of these new cell category obtained in the CBMN-cyt assay in bovines.

Numerous "in vitro" genotoxic evaluation tests have been published with OP and pyrethroids, however, very few of them correspond to the evaluation of CYP and CPF pesticides, and even less so to the combination of CYP + CPF. Others exposed bovine lymphocytes at different concentrations of pyrethroid supermerthrin, similar in structure to CYP, detecting a clastogenic effect (Dianovský and Šiviková, 1997). The effect on the genetic material of low exposures of CPF by CBMN-cyt in human lymphocytes has been reported by Želježić et al. (2016), which agree with our findings in the production of BNMn, BNBud and BNBridge indicating genetic damage; while at the same time there was a decrease in the CBPI. Želježić et al. (2016) also reported that the exposure of HepG2 cells to CPF presented BNMn, BNbud and BNBridge frequencies much higher than those observed in lymphocytes exposed to the same concentrations of CPF, but their use of transformed cells justifies these differences. Finally, these authors reported that when increasing the frequency of BNMn, those of BNBuds also increased, which is also in agreement with the findings of our present study in the CPF genotoxicity test.

Our results show a higher frequency of BNMn in cultures with CYP, like Noaishi et al. (2013), who's assigned to CYP the greatest potential for the induction of chromosomal aberrations in rats exposed to the mixture of CYP 5% + CPF 24%. The authors suggested that CYP, due to its small molecular size and high liposolubility, managed to cross the cell membranes more easily than CPF. Other evaluations of the genotoxic potential of CYP by CBMN in human lymphocytes have been carried out and reported (Surrallés et al., 1995). These authors reported a mild genotoxic effect of CYP on lymphocytes "in vitro"; whereas in our study we demonstrated a significant effect ($p < 0.0005$) in comparison with negative control for the concentrations of 175 and 87.5 μg/mL. The authors only evaluate the presence of BNMn, without considering the cytome approach (Surrallés et al., 1995).

The genotoxic potential of CPF has also been studied through other genotoxicological assays, the comet assay in human lymphocytes (Sandal and Yilmaz, 2011), and mouse hepatocytes (Cui et al., 2011). Also, the genotoxic potential of CPF was evaluated by means of the MN assay in mouse bone marrow with an induction of micronuclei in polychromatic erythrocytes (Yaduvanshi et al., 2012) and in a 14 - day trial on rats, with results showing greater damage in males (Sandhu et al., 2013).

The results obtained suggested the possible existence of an antagonistic effect between CPF + CYP in terms of the genotoxic effect with the effects being lower than expected. Some authors reported increased acute toxic effects from the mixture (Zhou et al., 2011; Zhang et al., 2017), others different types of interaction, potentiation or antagonism, depending on concentrations (Bonansea et al., 2016). In insects, the combination CYP + CPF was antagonistic when used in a 1:1 ratio in *Musca domestica* (Khan et al., 2013) and also in *Bemisia tabaco* (Ahmad, 2007).

There are no reports available on genotoxicity studies of the CYP + CPF mixture in cells of bovine origin, nor on the application of CBMN-cyt with this mixture. Meanwhile, rats have been exposed to a mixture of CYP 5% + CPF 24% with increased chromosomal aberrations only in high doses (1/20 LD_{50}) (Noaishi et al., 2013). More recently, the genotoxic effect of CYP + CPF with MN has been evaluated in the bone marrow of rats exposed for 65 days with increased MN (Okonko et al., 2016). The genotoxic effects of the combination of CYP + CPF, found in our trial with the genetic damage variables, do not enable us to confirm a synergistic interaction between CYP and CPF as reported in the experimental acute conditions of β-CYP and CPF in zebrafish (Zhang et al., 2017) and earthworms (Zhou et al., 2011). In other genotoxicity studies, in mice exposed to the mixture, an increased frequency of chromosomal aberrations and MN was reported with respect to those observed individually, as we do, and interactive effects are assumed to be less toxic (Chauhan et al., 2016). According to the frequencies of the most significant nuclear abnormalities in the analysis of genetic damage, we can observe that the mixture induced frequencies of intermediate values between those observed in the exposures to CPF and CYP individually. So, we can assume a situation of antagonism, which means that the general effect is less than expected from the sum of the separate effects. It is a point that should be considered, since antagonism does not represent a problem for risk assessment (Hernández et al., 2017) and should therefore be reevaluated for confirmation.

We propose that the same experimental design used in this study should be used with human lymphocytes in order to compare the resultant effects with those observed in our study. We postulate the use of bovine lymphocytes for the evaluation of the active principles used in the production of animals, and also of those detected in meat destined for human consumption. In conclusion, CPF, CYP and CPF + CYP induced an increase in BNMn, and CPF an increase in BNBud, dependent on the concentration. An antagonistic effect of the CPF + CYP mixture was evidenced in concentrations equivalent to 35 and 70% of LD_{50}. CYP caused greater genotoxic damage than the mixture, by means of BNMn.

Declarations of competing interest

None.

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Appendix A. Supplementary data

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