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Chlorpyrifos induces genotoxic effects in human leukocytes *in vitro* at low concentrations

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ABSTRACT. Chlorpyrifos (CPF) is an organophosphate widely marketed as a pesticide. However, only few studies have investigated its genotoxic potential, especially in human tissues or cells, or the minimal concentration capable of inducing damage. This study analyzed the *in vitro* genotoxic potential of CPF in cultured human leukocytes at concentrations of 3, 35, and 350 μg mL^{-1}. Results demonstrated that the two highest concentrations exhibited an increase (~2-fold) in the micronucleus frequency ($p < 0.01$), the number of numerical chromosomal instabilities (~10–12 times, $p < 0.05$), and the number of apoptotic cells (~10- to 20-fold, $p < 0.001$). These results demonstrate the genotoxic potential of CPF and could open new discussions regarding its safety and toxicology.

Keywords: pesticide; genotoxicity; clastogenic effect; chlorpyrifos.

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Introduction

Pesticides are products use to combat pests and invasive herbs in crops, improving quality and food production, but may lead to relevant poisoning in humans and animals. Chlorpyrifos (CPF) is one of the most commonly used pesticides that belongs to the class of organophosphates whose primary toxic mechanism has been inferred to involve acetylcholinesterase inhibition. However, there is limited information regarding the CPF-induced genotoxic effect in human cells, particularly in normal cells (Darwiche et al., 2018). Nonetheless, Želježić et al. (2017) have described that CPF is safe at doses of up to 3 μg mL^{-1} without causing significant DNA damage or carcinogenicity in human lymphocytes. However, it is plausible to expect that doses close to this concentration (3 μg mL^{-1}) have a much lower plasma peak than own doses.

On the other hand, it has been demonstrated that CPF exposure induces genotoxic effects and different pathologies, such as non-Hodgkin lymphoma, in different biological matrices (Costa, Mello, & Friedrich, 2017). Some of those effects include decreasing the number of bovine spermatozoa *in vitro* (Pallotta et al., 2019), structural changes in the DNA of peripheral rat leukocytes (Ojha & Gupta, 2017), and changes in green algae (Martínez, Marzio, & Sáenz, 2015) and freshwater fishes, such as *Channa punctatus* (Bloch) (Ali et al., 2009), *Labeo rohita* (Ismail et al., 2018), and *Oreochromis niloticus* (Ibrahim, El-Houseiny, Behairy, Mansour, & Abd-Elhakim, 2019).

However, the literature is imprecise regarding the concentration of CPF that is capable of inducing genotoxicity (Zhu et al., 2015). In fact, few studies have demonstrated the genotoxic effect associated with CPF in human tissues or cells as well as the concentration capable of inducing this effect (Okonko, Ikpeme, & Udensi, 2016; Darwiche et al., 2018). Therefore, the range of concentration of CPF that is capable of inducing genotoxic effects remains yet unsolved (Ali et al., 2009).

Hence, the primary objective of this study was to demonstrate the range of concentration of CPF associated with potential genotoxic effects in cultured human leukocytes.

Material and methods

Ethical aspects

The biological matrix and all protocols used in this study were previously submitted and approved by the Ethics Committee of the Federal University of Pampa (n°27045614.0.0000.5323).

Reagents

For this study, all reagents were purchased from Sigma-Aldrish.

Cell culture

The leukocyte culture was prepared according to Güez et al. (2015). Briefly, 8 mL of peripheral venous blood was collected from adult individuals who self-declared that they were healthy, did not use any medicines for the past 3 months, and had no exposure to radiation during the past 6 months. The blood sample (0.5 mL) was added to a culture flask containing 4.5 mL of RPMI 1340 culture medium (pH 7.4), supplemented with fetal bovine serum (10%), phytohemagglutinin (1%), and penicillin/streptomycin (1%). Cells were kept at 37°C for 72 hours at 5% CO_2 atmosphere. The negative control was prepared using 5 mL of RPMI 1340 culture medium, whereas RPMI 1340 culture medium plus 3 μg mL^{-1} of bleomycin was used as the positive control. The following CPF concentrations were tested in this study: 350, 35, and 3 μg mL^{-1}, which were based on a previous study conducted by our research group (data not shown). All cultures and protocols were performed in triplicate.

Numerical chromosomal instability

Numerical chromosomal instability was assessed as described by Yunis (1976). The slides were stained with 5% Giemsa for 3 min. and washed with distilled water. Then, they were dried at room temperature overnight. The assessment was performed using an optical microscope at a magnification of 100× counting 50 metaphases, considering cells with 44–48 chromosomes as normal.

Micronucleus assay

Micronucleus analysis was performed as described by Fenech (2007). The slides were prepared using 100 μL of cell pellets from the cultures (previously centrifuged at 1500 rpm for 10 min.) and stained by the Panoptic method. Then, the slides were evaluated under an optical microscope at a magnification of 100×. The presence of apoptotic cells was also determined, considering the presence of nucleosomes, membrane integrity, and intracellular damage. For each slide, a total of 500 cells were counted.

Statistical analysis

Statistical analysis was performed using one-way ANOVA, followed by Tukey's test for multiple comparisons. Data were expressed as mean and standard deviation, and $p < 0.05$ was considered for statistical significance.

Results

Our results demonstrated the mutagenic potential of CPF in human leukocytes, at least under the tested experimental model conditions and concentrations.

CPF-induced 5- and 4-fold increases in micronuclei at the two highest concentrations (350 μg mL^{-1}, $p < 0.01$, and 35 μg mL^{-1}, $p < 0.001$), respectively, compared with the negative control (Figure 1A).

Similarly, these two concentrations (350 μg mL^{-1}, $p < 0.01$, and 35 μg mL^{-1}, $p < 0.05$) induced ~12- and 9-fold increases, respectively, in the numerical chromosomal instability compared with the negative control (Figure 1B).

CPF treatment also increased the percentage of apoptotic cells at the higher ($p < 0.001$) and intermediate ($p < 0.01$) concentrations by ~12- and 4-folds, respectively, compared with the negative control (Figure 1C).

Figure 2 shows a normal leukocyte and a leukocyte with cellular alterations with or without exposure to CPF. First, from right to left, a normal lymphocyte is shown (Figure 2a). Then, a lymphocyte after exposure to CPF is shown presenting a micronucleus as indicated by the arrow (Figure 2b). An apoptotic leukocyte (after exposure to CPF) is shown with the following characteristics: nucleus undergoing pyknosis, collapsed and with deep wrinkles with dense chromatin in a hooded form, presence of apoptotic corpuscles, cytoplasm bubbles, and membrane integrity (Figure 2c). Finally, a leukocyte with a normal chromosome number in the mitotic process (Figure 2d) and two leukocytes exposed to CPF having a chromosome count well below normal are shown (Figures 2e-f).

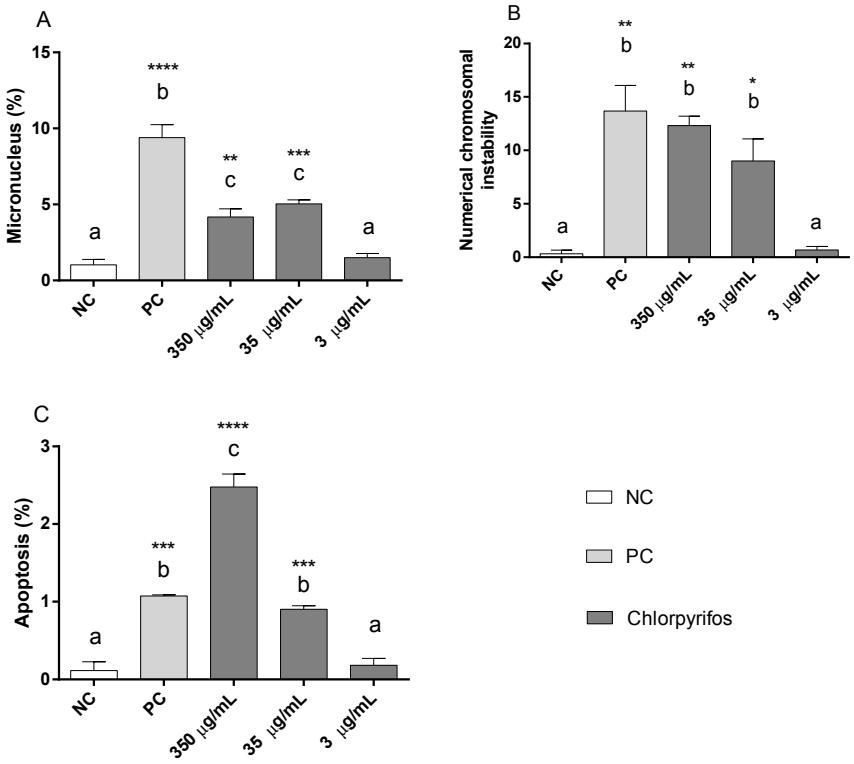

Figure 1. A) Percentage of micronucleus, B) numerical chromosomal instabilities, and C) apoptotic cells from cultured human leukocytes exposed to different concentrations of CPF. Data expressed as mean and standard deviation, n = 3, $p < 0.05$; one-way ANOVA, post-hoc Tukey's test. The superscripted letters in the bars represent significant statistical differences between the groups. NC = Negative control; PC = positive control; CPF = Chlorpyrifos.

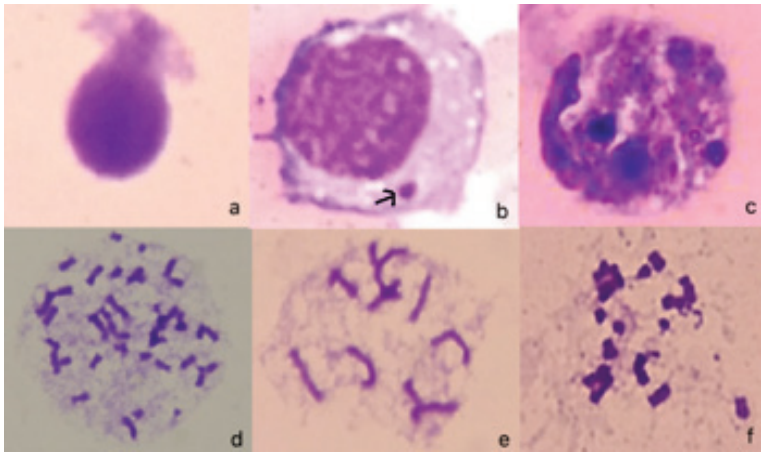

Figure 2. a) Normal lymphocyte; b) Lymphocyte with micronucleus (the arrow indicates the micronucleus in its cytoplasm); c) Leukocyte in apoptosis where is evidenced nucleus in picnose, collapsed and wrinkles deeply with density chromatin under hooded form, presence of apoptotic corpuscles, cytoplasm bubbles, and the membrane integrity; d) Mitosis with normal numeric chromosome count from a leukocyte no exposure to CPF; e-f) Numerical chromosomal alteration (with lower chromosome counts) from leukocyte in mitosis exposure to CPF.

Discussion

The presence of few cells with micronucleus is considered as normal in humans. These micronuclei are related to ruptures and rearrangements in the chromosome structure. However, a significant increase in the number of micronuclei is linked to the presence of a mutagenic process that, in the majority of cases, was induced by xenobiotics (Fenech, 2007).

Mutagenesis may cause damages in the offspring and, at other times, be a part of the process that results in carcinogenicity (Zhang & Vijg, 2018). Li et al. (2015) demonstrated that CPF could induce breaks in the DNA structure in cultured human lymphocytes in a concentration-dependent manner (35, 70, 105, and 175 µg mL^{-1}).

Breaks in the DNA structure, in turn, can induce changes in the sequencing of the genetic code when they are inappropriately repaired. In other words, they may corroborate with the onset of mutations. In our study, we observed that the two highest concentrations of CPF could induce a clastogenic process in human leukocytes, demonstrating their mutagenic potential for this type of human cells.

Chromosomal instability is a phenomenon that may alter specific genes associated with cell growth regulation, differentiation, and defense, such as expression of antioxidant molecules and mechanisms of repair of DNA damage. All these factors, combined or isolated, may contribute to the occurrence of neoplastic events (Pereira, Grácio, Teixeira, & Magro, 2015).

Recently, Mužinić, Ramić, and Želježić (2019) demonstrated that CPF treatment induced missegregation and aneuploidy in 9, 18, X, and Y chromosomes from human peripheral blood lymphocytes at low concentrations (0.623, 16.66, and 26.2 ng mL^{-1}) but did not cause micronuclei incidence. This phenomenon was attributed to defective sister chromatid cohesion, which generates chromosomal instability but does not necessarily induce micronucleus formation. However, in our study, we have clearly demonstrated the presence of both numerical chromosomal instability and micronucleus frequency, although at a concentration more than that tested by the above-cited authors.

Furthermore, Thakur, Dhiman, and Mantha (2018) reported that CPF-induced oxidative stress promoted lung cancer cell survival and proliferation through alterations in the APE1-dependent BER pathway at a concentration range of 35-175 μg mL^{-1}. This mechanism (oxidative stress) appears to play a relevant role in CPF-induced pathogenesis beyond the association with its genotoxic effects.

The presence of errors in the final product after cell replication and subsequent DNA repair is unexpected. Hence, there was an increase in the number of chromosomal abnormalities after exposure to xenobiotics (such as CPF), demonstrating their ability to cause genetic imbalance in cells (Mužinić et al., 2019). In our study, CPF-induced clastogenic process in human leukocytes at the two highest tested concentrations. Thus, the highest concentration of CPF is the plasma peak for rodents, which implies that it is plausible to suggest that these tested concentrations may be similar to the peak for humans.

Chromosomal abnormalities may lead to inadequate conditions to maintain the life of a cell. Consistent with this, programmed cell death is a physiological process in the life cycle of a cell or in response to injury against cells. Thus, an increase in the frequency of apoptotic cells after exposure to xenobiotics is related to the presence of irreparable cell damage (Kawakami, Liu, & Dmitrovisky, 2019).

Adedara et al. (2015) demonstrated that CPF treatment induced apoptosis and DNA chain fragmentation in *Drosophila melanogaster* fed with CPF at a concentration of 0.15 μg g^{-1} of feed, which was attributed to its ability to generate oxidative stress. Our data corroborate with these findings, because CPF also induced apoptosis in human leukocytes at the concentration of 35 μg mL^{-1}.

The literature describes the mechanisms involved in the genotoxic effect on different human cells, both normal and pathological. One of such studies has demonstrated that CPF treatment increased cell membrane injury in HepG2 cells, increased the intracellular free Ca^{2+} concentration, and decreased the mitochondrial transmembrane potential, in addition to inducing slight apoptosis (Zhou & Li, 2018). CPF-induced apoptotic effect was also observed in three testicular cell lines through the generation of reactive oxygen species at a concentration range of 3.5-17.5 μg mL^{-1} (Chen et al., 2018), as well as in HEK293 human embryonic kidney cells, but in this case, it was intermediated by IL6 (van Emmon, Pan, & van Breukelen, 2018).

In this context, Nolan, Rick, Freshour, and Saunders (1984) demonstrated that oral administration of 0.5 mg^{-1} kg^{-1} $body^{-1}$ weight of CPF in humans reached a plasma peak of ~1 μg mL^{-1}, which was able to decrease acetylcholinesterase activity. Considering that this plasma peak reached after the administration of only one dose, it is reasonable to suggest that repeated exposures may increase the concentration of CPF plasma peak. In addition, besides the alteration of acetylcholinesterase activity, CPF could generate genotoxic effects. Considering these data, the US Food and Drug Administration (FDA), as well as other regulatory agencies from different countries, has banned the use of CPF in the US. Unfortunately, this caution has been ignored by other countries.

For several decades, issues concerning the use of experimental models, particularly related to the ethical aspects, have been discussed. More recently, and in this context, *in vitr*o models have been used to elucidate mechanisms and establish safe concentration limits of exposure to xenobiotics. In this context, it would be appropriate to mention the study of Želježić et al. (2017) on human lymphocytes (as aforementioned), which

reported that doses up to 3 μg mL^{-1} were incapable of causing DNA damage. These authors performed their experiments using lower concentrations than those we used with which the genotoxic effect was observed. In fact, to our knowledge, the literature has no reports of the concentration limit of CPF that is capable of inducing genotoxic effects in normal human cells. Hence, in an unprecedented manner, our results have demonstrated the concentration range that is capable of inducing genotoxic effects in normal human cells.

Conclusion

This study has demonstrated the genotoxic effect of CPF *in vitro* in human leukocytes at the concentration of 35 μg mL^{-1} by causing the following alterations: micronuclei, numerical chromosomal abnormalities, and apoptotic cells. However, further studies are required to elucidate whether the mechanisms of these phenomena are mediated through the same pathways already described in the literature.

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