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SHORT COMMUNICATION

Glutathione in Chlorpyrifos-and Chlorpyrifos-Oxon-Induced Toxicity: a Comparative Study Focused on Non-cholinergic Toxicity in HT22 Cells

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Abstract

Chlorpyrifos (CPF) is a neurotoxic organophosphorus (OP) insecticide widely used for agricultural purposes. CPF-mediated neurotoxicity is mainly associated with its anticholinesterase activity, which may lead to a cholinergic syndrome. CPF metabolism generates chlorpyrifos-oxon (CPF-O), which possesses higher anticholinesterase activity and, consequently, plays a major role in the cholinergic syndrome observed after CPF poisoning. Recent lines of evidence have also reported non-cholinergic endpoints of CPF- and CPF-O-induced neurotoxicities, but comparisons on the non-cholinergic toxic properties of CPF and CPF-O are lacking. In this study, we compared the non-cholinergic toxicities displayed by CPF and CPF-O in cultured neuronal cells, with a particular emphasis on their pro-oxidant properties. Using immortalized cells derived from mouse hippocampus (HT22 line, which does present detectable acetylcholinesterase activity), we observed that CPF-O was 5-fold more potent in decreasing cell viability compared with CPF. Atropine, a muscarinic acetylcholine receptor antagonist, protected against acetylcholine (ACh)-induced toxicity but failed to prevent the CPF- and CPF-O-induced cytotoxicities in HT22 cells. CPF or CPF-O exposures significantly decreased the levels of the antioxidant glutathione (GSH); this event preceded the significant decrease in cell viability. Pretreatment with N-acetylcysteine (NAC, a GSH precursor) protected against the cytotoxicity induced by both CPF and CPF-O. The present study indicates that GSH depletion is a non-cholinergic event involved in CPF and CPF-O toxicities. The study also shows that in addition of being a more potent AChE inhibitor, CPF-O is also a more potent pro-oxidant molecule when compared with CPF, highlighting the role of CPF metabolism (bioactivation to CPF-O) in the ensuing non-cholinergic toxicity.

Highlights

- *Chlorpyrifos and chlorpyrifos-oxon caused toxicity in HT22 cells.*
- *Chlorpyrifos-oxon is more toxic to HT22 cells when compared with chlorpyrifos.*
- *Atropine does not prevent such toxicity.*
- *Chlorpyrifos and chlorpyrifos-oxon caused glutathione depletion.*
- *N-acetylcysteine prevented chlorpyrifos- and chlorpyrifos-oxon-induced toxicity.*

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Introduction

Chlorpyrifos (CPF) (O, O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is an organophosphorus (OP) insecticide widely used in agriculture. It is a lipophilic compound that readily crosses cell membranes (Ki et al. 2013; King and Aaron 2015). Analogous to other OP insecticides, CPF-induced neurotoxicity has been mainly associated with its ability to inhibit acetylcholinesterase (AChE) in the central and peripheral nervous system (CNS and CNP, respectively), thus leading to a cholinergic syndrome. AChE inhibition is particularly relevant when CPF is metabolized to chlorpyrifos-oxon (CPF-O), which possesses higher inhibitory effects on AChE, causing increased toxicity (Eaton et al. 2008; Vale and Lotti 2015).

Several lines of evidence have indicated alternative (non-cholinergic) mechanisms that mediate OP-induced neurotoxicity (Kaur et al. 2007; Sunkaria et al. 2014; Wani et al. 2014; dos Santos et al. 2016). In fact, CPF has been reported to cause oxidative damage, leading to increased reactive oxygen species (ROS) generation, culminating in glutathione (GSH) depletion, lipid peroxidation, and oxidative DNA damage (Giordano et al. 2007; Ki et al. 2013; Lee et al. 2012; Lee et al. 2014; Saulsbury et al. 2009). In addition, CPF has been reported to inhibit mitochondrial complex I activity (Lee et al. 2012), which may also contribute to the production of oxidants.

GSH is a tripeptide found not only in the cytosol but also in the nucleus and mitochondria, where it plays a central role as antioxidant. This molecule is the most abundant and efficient non-protein thiol antioxidant present in cells, where its concentration varies between 1 and 10 mM. GSH can act as a scavenger of reactive species, by direct reaction with radicals, as well as a substrate for the reactions catalyzed by glutathione peroxidase (GPx), glutathione transferase (GST), and glutaredoxins, among others. Depletion of GSH and regulation of some GSH-related enzymes may be associated to critical events in physiological, as well as in pathological conditions that are able to compromise neural cell homeostasis (Ballatori et al. 2009; Mazzetti et al. 2015; Gorelenkova Miller and Mieyal 2019). Of note, CPF-induced toxicity is increased by intracellular GSH depletion (Giordano et al. 2007; Saulsbury et al. 2009).

As already mentioned, CPF-O presents higher anticholinesterase activity and is considered more toxic than CPF (Eaton et al. 2008; Vale and Lotti 2015). However, comparisons between their non-cholinergic toxic properties have yet to be addressed. The main objective of this study was to compare the non-cholinergic toxicities displayed by CPF and CPF-O in cultured neuronal cells (HT22 line), with a particular emphasis on their pro-oxidative properties. We hypothesized the potential involvement of GSH depletion in CPF- and CPF-O-induced cytotoxicity.

Material and Methods

Chemicals

Chlorpyrifos (CPF), acetylcholine (ACh), atropine, 5,5-dithiobis-(2- nitrobenzoic-acid) (DTNB), and N-acetylcysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chlorpyrifos-oxon (CPF-O) was purchased from Chem Service (West Chester, PA, USA). Dulbecco's modified Eagle's medium (DMEM) and FBS were obtained from Gibco Life Technologies.

Cell Culture

The HT22 neuronal cell line was kindly donated by Dr. David Schubert (Salk Institute, La Jolla, CA, USA). HT22 cells are a sub-line derived from parent HT4 cells, which were originally immortalized from a culture of hippocampal neurons of mice (Frederiksen et al. 1988). Liu et al. (2009) demonstrated that HT22 cells express cholinergic markers such as choline acetyltransferase (ChAT), the enzyme responsible for synthesis of acetylcholine, an important marker of cholinergic neurons, in addition to the high affinity choline transporter (TAC), acetylcholine vesicular transporter (VAChT), and muscarinic receptors (M1 and M2) (Liu et al. 2009). Through a conventional method (Ellman et al. 1961), which was already standardized in our laboratory (dos Santos et al. 2016), we were unable to detect AChE activity in this cell line, indicating that it represents an optimal model to investigate non-anticholinesterasic toxicity induced by OP pesticides. In agreement, to the best of our knowledge, data showing AChE activity in HT22 cells are lacking. Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (2 mM) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subcultured every 2 days by trypsinization (Trypsin-EDTA at 0.05%) and used between the passages 5–15. All experiments were carried out 24 h after cell seeding. The sequence of each condition's treatment (different compounds or concentrations), as well as their position in the well plates, was randomly performed in an attempt to minimize potential biases. For treatments, CPF and CPF-O were dissolved in dimethyl sulfoxide (DMSO), whose final concentration in the well was 0.1%. Because of the high affinity of CPF and CPF-O by albumin, resulting from their high

lipophilicity, the medium supplemented with 10% FBS was changed by a low-serum (1%)-containing medium before treatments. In parallel experiments, cells were pretreated with 5 mM N-acetylcysteine (NAC) to investigate the protective effect of this GSH precursor against the cytotoxicity induced by CFP and CFP-O. To investigate the possible protective effect of atropine (murcarinic receptor antagonist) against CFP- or CPF-O-induced toxicity, cells were co-treated with atropine (1 mM) and acetylcholine (1 mM) or CFP/CPF-O.

Cell Viability

Cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay as described by Mosmann (1983). This technique is based on the colorimetric conversion of yellow MTT tetrazolium to purple formazan by viable cells. Cells were plated at a density of 3.5×10^3 cells/well (96-well plates) and incubated at 37 °*C* in a humidified 5% CO_2 atmosphere, 24 h before treatment. After each treatment, the culture medium was removed (50 μL was separated for the LDH assay), and 100 μL of a 0.5 mg/mL MTT solution (dissolved in HBSS) at 37 °C was added to each well, followed by 1 h of incubation at 37 °C. After this period, the MTT solution was removed and 100 μL of DMSO was added for solubilization of the formazan crystals. After complete solubilization of the crystals, absorbance was read in a microplate reader (Tecan, Mannedorf, Switzerland) at a wavelength of 540 nm. Cell viability was expressed by the percentage of the control (untreated cells, 100%).

Measurement of Cytotoxicity

Using a previously described protocol (Rosa et al. 1997; Petegnief et al. 2003), we measured cytotoxicity with the lactate dehydrogenase (LDH) release assay, which evaluates the integrity of the plasma membrane. This enzyme can be detected in the extracellular environment in the occurrence of damage to the plasma membrane. Cells were plated at a density of 3.5×10^3 cells/well (96-well plates) and incubated at 37 °C in a humidified 5% CO_2 atmosphere, 24 h before treatment. After each treatment, 10 μL of 2% Triton X-100 (0.2% final well concentration) was added in wells as positive controls for cell death (100%). After 15 min of incubation at 37 °C, 50 μL of culture medium was withdrawn and passed to a new 96-well plate. To this plate containing only the culture medium, 200 μL of reaction mix (0.15 M potassium phosphate buffer pH 7.4, containing 4.7 mM $NaHCO_3$, 0.4 mM NADH, and 2.1 mM sodium pyruvate) was added. NADH oxidation was monitored at a wavelength of 340 nm for 150 s using a microplate reader (Tecan, Mannedorf, Switzerland). Release of LDH was expressed as the percentage of positive control (Triton X-100 treated cells, 100%).

Measurement of Reduced Glutathione

Glutathione content was measured as non-protein thiols (NPSH) according to the method previously described by Ellman (1959). The HT22 cells were seeded in 6-well plates at a density of 1.0×10^5 cells/well 24 h before treatment. After each treatment, cells were washed with PBS, collected in 200 μL of PBS/Triton 0.5%. About 20 μL was used to measure the proteins according to Lowry et al. (1951). The remained 180 μL was mixed with the same volume of a 10% trichloroacetic acid solution. After centrifugation (5000×*g* at 4 °C for 10 min), the protein pellet was discarded and free thiol groups were determined in the clear supernatant after reaction with DTNB. Absorbance was read in 412 nm (Tecan, Mannedorf, Switzerland), using GSH as standard. The values are expressed as percent of control in the absence of treatment.

Statistical Analysis

Statistical analysis and graphs were made with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). The analysis was performed using Student's *t* test, one-way or two-way analysis of variance (ANOVA) followed by Tukey's test when appropriated. The results are expressed as mean ± SEM. The difference was considered significant when $P \leq 0.05$.

Results

Cytotoxicity of CPF and CPF-O in HT22 Cells

Initially, the cytotoxicity induced by either CPF or its oxon metabolite (CPF-O) was evaluated. HT22 cells were exposed to increasing concentrations of CPF or CPF-O (0, 0.3, 1, 3, 10, 30, 100, and 300 μM) for 24 h. The chosen concentrations were based on the acute CPF's oral LD_{50} for mice (62.5 mg/kg; ~ 1.8×10^{-4} mol/kg) and rats (223 mg/kg; ~ 6.4×10^{-4} mol/kg), considering that humans are more sensitive to CPF compared with rats (Smegal 2000). Exposure to CPF for 24 h at concentrations of 30, 100, and 300 μM significantly reduced cell viability (13, 17, and 48%, respectively) (Fig. 1A), but no significant effects of CPF exposure were observed on LDH release (Fig. 1C). Exposures to CPF-O at concentrations of 10, 30, 100, and 300 μM led to significant reductions in cell viability 24 h after treatments (22, 34, 50, and 70%, respectively), as shown in Fig. 1B. Exposure to 300 μM CPF-O for 24 h led to a significant increase in LDH release (7.3%) (Fig. 1D). IC_{30} values, which indicate the toxicant's concentration necessary to decrease cell viability by 30% (MTT assay), were approximately 30 μM for CPF-O and 150 μM for CPF, indicating that the oxon metabolite

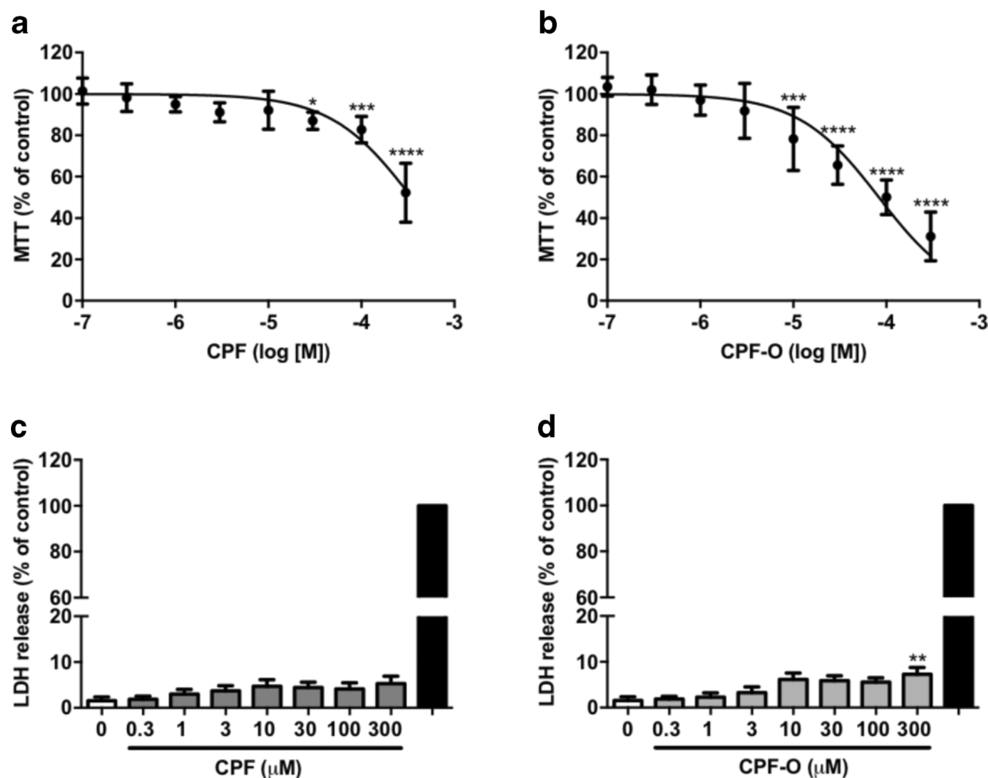

Fig. 1 Concentration-dependent cytotoxicity of CPF and CPF-O in HT22 cells. Cells were exposed to increasing concentrations of CPF (**A** and **C**) or CPF-O (**B** and **D**) (0, 0.3, 1, 3, 10, 30, 100 and 300 μM) for 24 h. At the end of the treatments, the cell viability was evaluated by the MTT reduction assay (**A** and **B**). Results are expressed as percentage of control (untreated cells, 100%). Lactate dehydrogenase (LDH) release (**C** and **D**) is expressed as percentage of the positive control (cells treated with 2% Triton X-100 for 5 min, 100%, black bar). Each bar represents the mean ± S.E.M of 7 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ indicate the difference when compared with the control group based on one-way ANOVA followed by Tukey's post hoc test

(CPF-O) is approximately 5-fold more toxic to HT22 cells compared with CPF.

Based on the IC_{30} values (Fig. 1 A and B), additional time-response experiments were performed with fixed concentrations of CPF and CPF-O. HT22 cells were exposed to the correspondent IC_{30} values (150 μM CPF or 30 μM CPF-O) for 1, 3, 6, 24, and 48 h. At the end of the treatments, the MTT reduction and the LDH release assays were performed. Even though significant decreases in cell viability were observed at 24 and 48 h after treatments with both CPF (Fig. 2A) and CPF-O (Fig. 2B), no significant decreases in cell viability were observed up to 6 h after treatments. Significant increases in LDH release were observed at 24 and 48 h after treatment with CPF-O (Fig. 2D), but not CPF (Fig. 2C).

CPF and CPF-O Reduce Glutathione Content, and NAC Protects against CPF- and CPF-O-Induced Cytotoxicity in HT22 Cells

Based on the absence of significant decrease in cell viability at 6 h after treatments with either CPF (150 μM) or CPF-O (30 μM) (IC_{30} values) (Fig. 2 A and B), GSH levels were determined at this time-point to specifically investigate events preceding (mediating) cytotoxicity. Exposures to either 150 μM CPF or 30 μM CPF-O for 6 h led to a significant (~60%) decrease in NPSH levels (Fig. 3 A and B, respectively). Based on these results, we investigated the role of GSH in CPF- or CPF-O-cytotoxicity by pretreating cells with N-acetylcysteine (NAC), a GSH precursor. NAC pretreatment (1 h) significantly protected HT22 cells against CPF- and CPF-O-induced decrease in cell viability (Fig. 3 C and D). We also observed that 5 mM NAC pretreatment (1 h before the pesticides challenge) increased NPSH levels at 6 h after pesticide treatments even in cells challenged with 150 μM CPF (~730% increase in NPSH) and 30 μM CPF-O (~540% increase in NPSH) (not shown), clearly indicating that the performed NAC-based protocol was effective in increasing intracellular NPSH levels.

Atropine Does Not Protect HT22 Cells Against CPF- and CPF-O-Induced Cytotoxicity but Protects Against ACh-Induced Cholinergic Over-stimulation

To investigate if the decrease of GSH levels observed after CPF or CPF-O treatments is not related to anticholinesterase effects, further experiments with acetylcholine and atropine were performed. Atropine is a competitive non-selective muscarinic antagonist (King and Aaron 2015), whose administration can reverse clinical signs of muscarinic toxicity in the cases of cholinergic neurotoxicity induced by OF (King and Aaron 2015). Acetylcholine caused a concentration-dependent decrease in cell viability in HT22 cells (Fig. 4A), and atropine significantly protected against the acetylcholine-induced toxicity (Fig. 4C). Of note, atropine did not protect against CPF- (Fig. 4D) and CPF-O-induced toxicity (Fig. 4E), suggesting that the decrease in cell viability induced by CPF

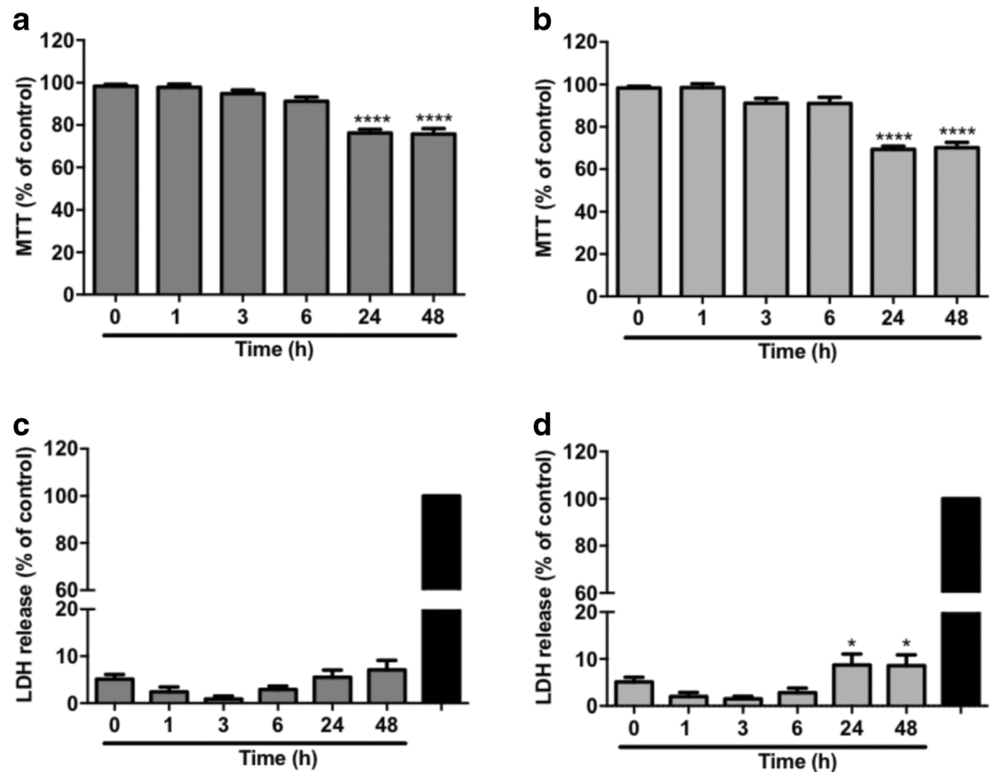

Fig. 2 Time-dependent cytotoxicity of CPF and CPF-O in HT22 cells. Cells were exposed to 150 μM of CPF (**A** and **C**) or 30 μM of CPF-O (**B** and **D**) for 1, 3, 6, 24, and 48 h. At the end of the treatments, cell viability was evaluated with the MTT reduction assay (**A** and **B**) and the results are expressed as percentage of control (untreated cells, 100%). Lactate dehydrogenase (LDH) release (**C** and **D**) is expressed as percentage of the positive control (cells treated with 2% Triton X-100 for 5 min, 100%, black bar). $*p < 0.05$ and $****p < 0.0001$ indicate the difference when compared with the control group by one-way ANOVA followed by Tukey's post hoc test

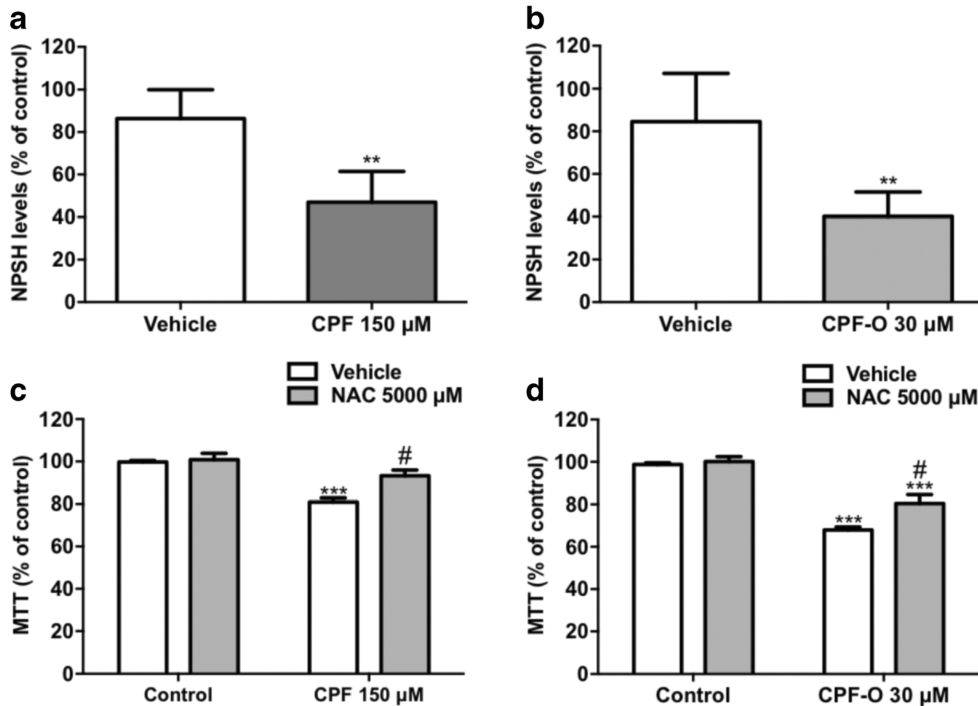

Fig. 3 Effect of CPF and CPF-O on GSH levels and the effect of NAC on CPF and CPF-O induced cytotoxicity. HT22 cells were exposed to 150 μM CPF (**A**) or 30 μM CPF-O (**B**). After 6 h, NPSH levels were determined. The results are expressed as percentage of control (untreated cells, 100%). Each bar represents the mean ± SEM of 5 independent experiments. $**p < 0.01$ indicates the difference when compared with the control group according to Student's *t* test. HT22 cells were pretreated for 1 h with 5 mM NAC followed by exposure to 150 μM CPF (**C**) or 30 μM CPF-O (**D**) for 24 h. Viability assay was performed by the MTT method, and the results are expressed as percentage of control (untreated cells, 100%). Each bar represents the mean ± S.E.M of 8 independent experiments. $**p < 0.01$ and $***p < 0.001$ indicate the difference when compared with the control group and $\#p < 0.05$ when compared with the CPF group 150 μM (**C**) or CPF-O 30 μM (**D**) by two-way ANOVA followed by Tukey's post hoc test

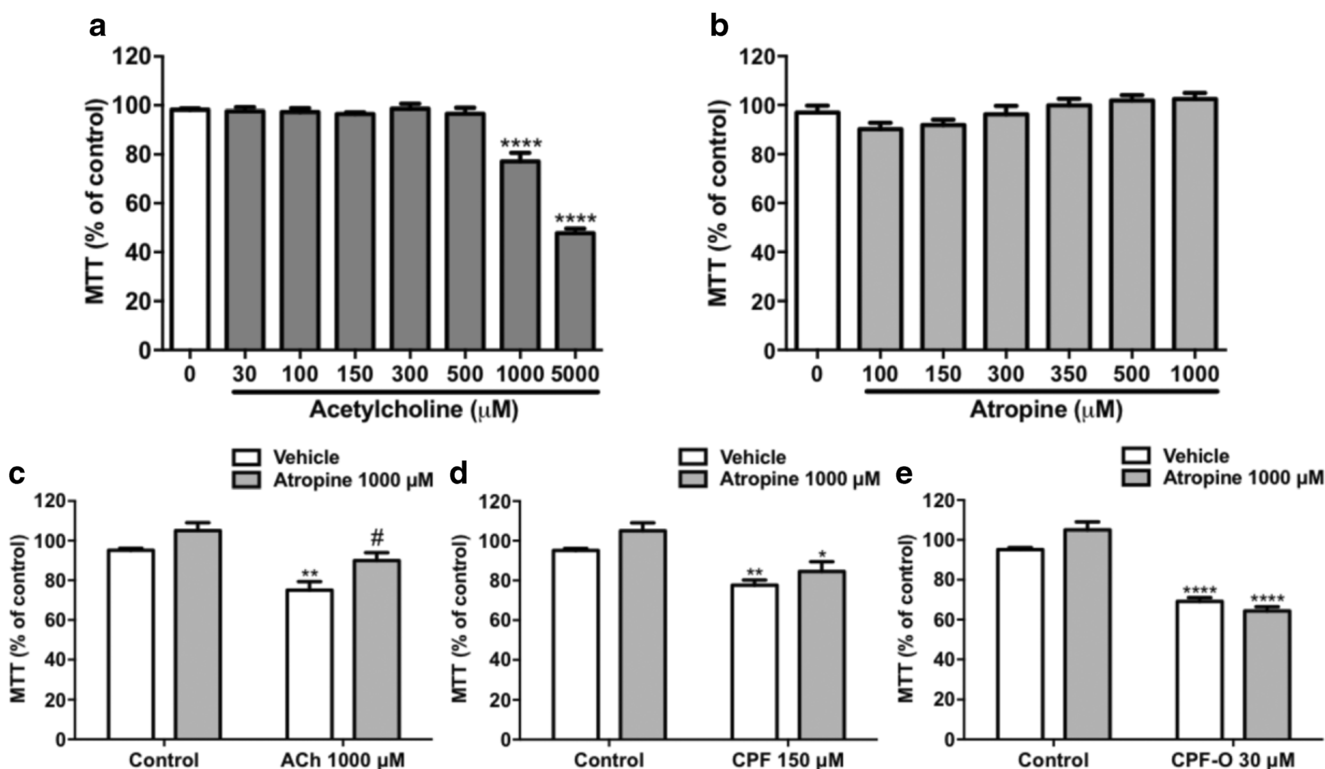

Fig. 4 Effect of atropine on the cytotoxicity induced by CPF, CPF-O, and acetylcholine. HT22 cells were exposed to increasing concentrations of ACh (0, 30, 100, 300, 500 μM, 1 and 5 mM) (**A**) and increasing concentrations of atropine (0, 100, 150, 300, 350, 500 μM, and 1 mM) (**B**) for 24 h. In addition, HT22 cells were co-exposed for 24 h with 1 mM atropine and 1 mM ACh (**C**), 150 μM CPF (**D**), or 30 μM CPF-O (**E**). After 24 h of treatment, the cell viability assay was performed by the MTT method and the results are expressed as percentage of control (untreated cells, 100%). Each bar represents the mean ± S.E.M of 6–9 independent experiments. ***$p < 0.001$ and ****$p < 0.0001$ indicate difference when compared with the control group, according to one-way ANOVA (A) followed by Tukey's post hoc test. ****$p < 0.0001$,**$p < 0.01$, and *$p < 0.05$ indicate difference when compared with the control group, based on two-way ANOVA, and #$p < 0.05$ indicates difference when compared with the 1 mM Ach (**C**, **D**, and **E**)

and CPF-O is not related to stimulation of muscarinic receptors resulting from anticholinesterasic effects of these pesticides.

Discussion

This study showed that (i) CPF-O, the major oxon metabolite generated after CPF biotransformation, displayed higher toxicity when compared with CPF in cultured neuronal cells (HT22 line); (ii) this toxicity, which was not connected with anticholinesterase effects, was related to the higher pro-oxidative effect of CPF-O toward GSH; and (iii) GSH depletion was a key event in the non-cholinergic toxicity induced by CPF and CPF-O, since a GSH precursor prevented this toxic effect. Previous studies indicate that CPF-induced toxicity in both the central and peripheral nervous system (CNS and CNP) is predominantly associated with the inhibition of acetylcholinesterase (AChE), thus leading to a cholinergic syndrome (Eaton et al. 2008). Because oxons have higher inhibitory potency for AChE compared with the correspondent phosphorothioate precursors, AChE inhibition is particularly relevant when CPF is metabolized to CPF-O. In fact, CPF-O has higher inhibitory effects for AChE, thus causing higher toxicity when compared with CPF (Vale and Lotti 2015). Our novel study demonstrated that both CPF and CPF-O significantly decreased cell viability in HT22 line. However, the concentration of CPF-O necessary to decrease cell viability by 30% was 5-fold lower compared with CPF, indicating the former was more toxic than CPF in an experimental condition where the anticholinesterase effects were not central in mediating cytotoxicity. In corroboration, treatment with 30 μM CPF-O oxidized GSH content similar to 150 μM CPF, suggesting that the oxon compound (or further CPF-O metabolite(s)) has higher oxidative properties toward GSH compared with CPF. Although available data indicate significant contribution of CPF activation to CPF-O to the cholinergic toxicity resulting from CPF exposures (Khokhar and Tyndale 2012), our study is the first to report a potential role of CPF metabolism (bioactivation to CPF-O) in the etiology of non-cholinergic toxicity. The idea that non-cholinergic toxicity is taking place in our experimental model is based on the fact that atropine, a muscarinic receptor antagonist that protected against acetylcholine-induced toxicity, did not

protect against CPF- or CPF-O-induced toxicity. These results strongly indicate that the observed decrease of cell viability induced by CPF and CPF-O was not related to a potential stimulation of muscarinic receptors resultant from previous anticholinesterase effect of these pesticides.

The tripeptide GSH (γ-l-glutamyl-l-cysteinylglycine) is the most important low-molecular sulfhydryl antioxidant, which is present intracellularly in millimolar concentrations. Its most important functions, which are particularly dependent on the thiol group of its cysteinyl residue, are related to the detoxification of either endogenous oxidants or xenobiotics (Deponte 2013; Farina and Aschner 2019), including pesticides, such as CPF (Mahmoud et al. 2019). Of note, CPF-induced toxicity is increased upon intracellular GSH depletion (Giordano et al. 2007; Saulsbury et al. 2009). Our results indicate an important role for GSH in mediating CPF- and CPF-O-induced toxicity in HT22 cells. Because both CPF and CPF-O decreased GSH levels in a time-point that preceded the significant decreases in cell viability (6 h), and given that NAC pretreatment prevented CPF and CPF-O-induced cell toxicity, the results highlight the relevance of GSH depletion to the observed cytotoxicity. This idea is reinforced by the fact that both CPF and CPF-O have been reported to interact with GSH (Fujioka and Casida 2007; Mekonnen et al. 2019), although comparative analyses concerning their affinities for GSH are still lacking. Our results are also in accordance with results from a clinical randomized trial showing the beneficial effects of NAC therapy in OP-poisoned patients (El-Ebiary et al. 2016).

As already mentioned, the CPF or CPF-O concentrations used in this study (0, 0.3, 1, 3, 10, 30, 100, and 300 μM) were based on the acute CPF's oral LD_{50} for mice (62.5 mg/kg; $\sim 1.8 \times 10^{-4}$ mol/kg) and rats (223 mg/kg; $\sim 6.4 \times 10^{-4}$ mol/kg). Available data indicate that an acute human exposure to CPF (attempted suicide by pesticide ingestion) led to a serum CPF level of 9.4 mg/L (~ 30 μM) (Martínez et al. 2004), indicating that the concentrations used in our in vitro protocol may be found in human blood after acute and high-level exposures (Martínez et al. 2004). However, the concentrations used in our protocol are unlikely to be found in the blood of subjects chronicaly exposed to CPF in low-dose occupational/environmental scenarios, where blood CPF concentrations seem to be at the nanogram per liter range (Barr et al. 2002; Whyatt et al. 2005). Anyway, it is important to note that the initial half-life of CPF is quite short (Nolan et al. 1984; Timchalk et al. 2002). In fact, at least at relatively high doses, CPF is quickly metabolized to CPF-O and other metabolites, with an apparent half-life of 1 h or less (Eaton et al. 2008).

In summary, the results presented herein indicate that the pro-oxidant properties of both CPF and CPF-O trigger their toxicities in HT22 cells. Because NAC pretreatment protected against CPF- and CPF-O-induced toxicity in HT22 cells, GSH depletion seems to represent a key event in mediating their non-cholinergic toxicities. Moreover, this is the first study to compare the non-cholinergic toxicities of CPF and its oxon metabolite, showing that in addition to being a more potent AChE inhibitor, CPF-O is also a more potent pro-oxidant molecule when compared with CPF, highlighting the role of CPF metabolism (bioactivation to CPF-O) in the etiology of non-cholinergic toxicity.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interest.

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