Oxidative stress and inflammatory response of skin fibroblasts exposed to chlorpyrifos

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† Presented at the Biosystems in Toxicology and Pharmacology – Current challenges (BTP-2022), 8-9 September 2022

Abstract: Chlorpyrifos (CPF) is a widely used insecticide. The aim of this work was to study the effect of CPF in skin fibroblasts exposed to concentrations detected in human skin and unleash underlying cellular mechanisms. Fibroblasts were exposed to different concentrations (0.36-250 µM) of CPF pure alone or in a commercial CPF mixture (Lethal-20), for 6 days. In conclusion, prolonged exposure to 250 µM of CPF pure and 125 µM of Lethal-20 caused significant loss of fibroblast’s viability. Moreover, the toxicity of this pesticide in fibroblasts is evidenced by the induction of oxidative stress and stimulation of the production of interleukin (IL-6).

Keywords: Chlorpyrifos; Immunotoxicology; Inflammation; Oxidative stress; skin fibroblasts; IL-6

1. Introduction

Chlorpyrifos (CPF) is an organophosphorus pesticide used to control various insects and protect corn, grain, rice, cotton, fruit and vegetables. CPF can cross skin barrier and reach many body cells’ [1–4] and in animal models, revealed toxicities due to acute and chronic exposures, mainly against neurological, endocrine, and cardiovascular systems. It can also induce dermal and immunotoxicity [5]. CPF was shown to affect the vitamin D3 metabolism in skin cells, and the proliferation, and Reactive Oxygen Species (ROS) production in cancer cells [2,5,6]. In neonatal rats, CPF increased the expression of pro-inflammatory cytokines, such as IL-6, TNF-α and the inflammation mediator HMGB1, and the activation of NF-kB in the amygdala tissues [7]. CPF-induced inflammation through microglia, in neonatal rats, accounts for neurotoxicity [8]. Yet little is known about the toxic and immunomodulatory effects of environmental CPF dosage in human skin cells. In this work we have assessed the effect of CPF in the viability of skin fibroblasts using concentrations up to 250 µM that represent environmental and acute exposure of humans [3,4]. The effects on cell viability, oxidative stress response and inflammatory response were addressed.

2. Materials and Methods

Human skin fibroblast cell line GM03349 was obtained from the Cell Bank at Coriell Institute for Medical Research (USA) and cultured in DMEM low glucose medium	
supplemented with 1% (v:v) penicillin/streptomycin (10.000 U/mL; 10 mg/mL), and 1% (v:v) L-glutamine (200 mM) and 10% (v:v) fetal bovine serum (FBS) (all purchased from Gibco (Thermofisher, USA)). For the incubation with the toxicants, DMEM was supplemented as above except for the 2% FBS. The toxicants’ concentrations tested refer to the active compound CPF- either pure or in the commercial mixture (Lethal 20). 6-carboxy-2,7’-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA) were from Invitrogen (Thermofisher, USA). CPF pure, Luperox (tert-Butyl hydroperoxide, tBH), dimethyl-sulfoxide (DMSO) were from Sigma Aldrich (USA). The commercial mixture (Lethal 20) was purchased from Insecticides India Limited (India). IL-6 ELISA Kit was purchased from Immunotools (Germany). For viability assay, cells were seeded in 96-well plates with a concentration of 1x10^4 cells/mL and exposed for 6 days to different concentrations (0.36 to 250 µM) of the toxicants diluted in medium with DMSO, using the resazurin-based assay as described in [9]. As negative control, cells were cultured in parallel only with the medium with DMSO. For ROS production assay, cells were incubated in medium with the toxicants for 3h. As negative control, cells were cultured only with the medium with DMSO and, as positive control, Luperox (tert-Butyl hydroperoxide, tBH) 100 µM was used as oxidative stress inductor. After exposure, the intracellular ROS production was measured by the conversion of Carboxy-H2DCFDA to fluorescent DCF in a microplate assay. For Inflammatory cytokine production (IL-6) assay, cells were incubated in medium and exposed to 125 and 250 µM chlorpyrifos pure or Lethal 20, for 6 days. The production of IL-6 was measured in the culture supernatants by ELISA as described in [10]. Statistical analysis was performed using ratio paired t test.

3. Results and Discussion

3.1. Effect of Chlorpyrifos in cell viability of fibroblasts

The effects of exposure of skin fibroblasts to CPF on cells’ viability were assessed using the resazurin test. For pure CPF and a commercial mixture (Lethal 20 solvent extract) at concentrations below 125 µM there was no loss of fibroblast viability when exposed to any of the formulations. Curiously, the commercial mixture showed a more pronounced effect on viability compared to the pure compound. As shown in Fig. 1, at 125 µM the cell viability was 15.8% with Lethal 20 while there was no loss of viability with CPF pure. At 250 µM of Lethal 20, the cell viability was completely lost, while with pure CPF the viability was 19.0%.

**Figure 1- Effect of CPF in viability of fibroblasts.** a) The fibroblasts were incubated with culture medium with different concentrations of CPF either pure or in the commercial mixture-Lethal 20. After 6 days, the cell viability was evaluated by resazurin assay. Graph shows the percentage of viable cells relative to non-treated cells at day 0 (100% viability). Values are mean ±SEM (n=3). b) Images from microscopy (magnification 10x) of fibroblasts exposed for 4 days to the CPF pure (CPF) or to Lethal20 at 250 µM.

3.2. ROS generation in fibroblasts by chlorpyrifos

ROS formation was assayed after fibroblasts exposure for 3h. As shown in Fig 2, ROS production increased 1.4 folds and 1.3 folds when the cells were exposed, respectively, to 250 µM of pure CPF or Lethal-20. These results are in accordance with what has been demonstrated *in vitro* in human neuroblastoma SH-SY5Y cells for which it was proposed that CPF-mediated induction of oxidative stress was followed by cell apoptosis [11].
Figure 2- Effect of CPF in fibroblasts’ ROS production. The fibroblasts were incubated with culture medium with 250 µM of CPF or Lethal 20. Luperox (ter-Butyl hydroperoxide, tBH) solution 100 µM was used as positive control. After 3h, the production of ROS was measured by Carboxy-H2DCFDA microplate assay. Graph shows the fold increase in fluorescence relative to control assays with no compound added (n≥3).

3.3. Immunomodulatory effect of chlorpyrifos in fibroblasts

To evaluate the immunomodulatory effect of CPF in fibroblasts we have assessed the production of the pro-inflammatory cytokine IL-6, after cells were exposed for 6 days. As shown in Fig. 3, IL-6 production is dose dependent for both formulations. IL-6 secretion was more pronounced in cells exposed to Lethal 20 (2.4-fold increase, at 250 µM) as compared to CPF pure (1.8-fold increase, at 250 µM). IL-6 is a multifunctional cytokine that is implicated in various inflammatory conditions. Nasal fibroblasts exposed to diesel exhaust particles or by synovial fibroblasts exposed to particulate matter produce IL-6, suggesting the possible implications of IL-6 in the pathophysiology of diseases like allergic rhinitis and chronic rhinosinusitis or osteoarthritis [12,13]. Fibroblasts are important sources of IL-6, whose expression has been reported as induced by ROS [14]. In this work we show for the first time that exposure to CPF stimulates the production of IL-6 by skin fibroblasts probably due to the increased ROS generation. It is probable that other pro-inflammatory cytokines are upregulated thus pointing CPF mechanism of action has an important inflammation inducer.

Figure 3- Effect of CPF on the production of IL-6. The fibroblasts were incubated with DMEM with 2% FBS with CPF pure or lethal 20 (125 and 250 µM). After 6 days, the production of IL-6 was measured in the culture supernatants by ELISA. Values are mean ±SEM (n=2).

4. Conclusion

CPF is still considered a health issue that assumes a great relevance in countries where high concentrations were identified in the skin of agricultural workers. Here, we show how CPF affects skin fibroblasts’ physiology, resulting in huge loss of cell viability at 250 µM, and increasing ROS and cytokine IL-6 production. The effect of CPF on cytokine production shows its important implication in inflammatory responses, ultimately leading to disease, and pin-points potential therapeutic targets to treat chronic or acute exposure to CPF.

Author Contributions: Investigation, Z.S. and D.A.; writing—original draft preparation, Z.S and P.V.; writing—review and editing, D.M.S. and R.L.; supervision, P.V. All authors have read and
agreed to the published version of the manuscript.

**Funding:** This research was funded by Fundação para a Ciência e Tecnologia (FCT – Portugal) through the research project PTDC/BIA-MB/31864/2017. It was also funded by FCT- Portugal in the scope of the unit projects UIDP/04378/2020 and UIDB/04378/2020 (UCIBIO), LA/P/0140/2020 (4iHB), LA/P/0045/2020 (ALiCE), UIDB/50020/2020 and UIDP/50020/2020 (LSRE-LCM).

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