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Proceeding Paper

Oxidative Stress and Inflammatory Response of Skin Fibroblasts Exposed to Chlorpyrifos †

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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 a significant loss of the 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

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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 the skin barrier and reach many body cells [1–4], and in animal models, it was revealed it induced toxicities due to acute and chronic exposures, mainly against the neurological, endocrine, and cardiovascular systems. It can also induce dermal and immunotoxicity [5]. CPF was shown to affect vitamin D3 metabolism in skin cells, the proliferation of cancer cells, 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 on the viability of skin fibroblasts using concentrations up to 250 μM, which represents environmental and acute exposure of humans [3,4]. The effects on cell viability, the oxidative stress response and the 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 (Camden, NJ, 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, Waltham, MA, USA)). For the incubation with the toxicants, DMEM was supplemented as above, except for the 2% FBS. The toxicants´ concentrations tested refers to the active compound—CPF—either pure or in the commercial mixture (Lethal 20). The acquisition of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Carboxy-H2DCFDA) was from Invitrogen (Thermofisher, Waltham, MA, USA). CPF pure, Luperox (tert-Butyl hydroperoxide, tBH), and dimethylsulfoxide (DMSO) were from Sigma Aldrich (St. Louis, Missouri, USA). The commercial mixture (Lethal 20) was purchased from Insecticides India Limited (Delhi, India). IL-6 ELISA Kit was purchased from Immunotools (Friesoythe, Germany). For viability assay, cells were seeded in 96-well plates with a concentration of 1×10^5 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 with only the medium with DMSO. For ROS production assay, cells were incubated in medium with the toxicants for 3h. As negative control, cells were cultured with only 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-H_2DCFDA 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 on Cell Viability of Fibroblasts

The effects of exposure of skin fibroblasts to CPF on the 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 with the effect of the pure compound. As shown in Figure 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%.

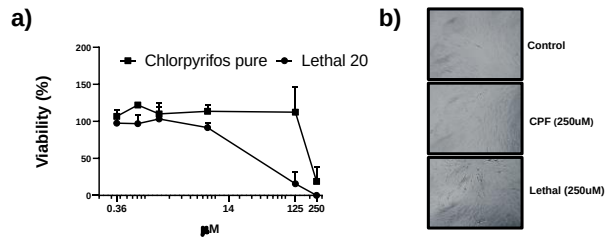

Figure 1. Effect of CPF on viability of fibroblasts. (**a**) The fibroblasts were incubated with culture medium with different concentrations of CPF, either pure or in the commercial mixture Lethal 20. After 6 days, the cell viability was evaluated by resazurin assay. Graph shows the percentage of viable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

3.2. ROS Generation in Fibroblasts by Chlorpyrifos

ROS formation was assayed after fibroblast exposure for 3 h. As shown in Figure 2, ROS production increased 1.4-fold and 1.3-fold 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].

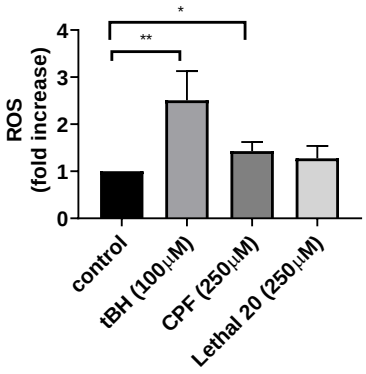

Figure 2. Effect of CPF on fibroblasts' ROS production. The fibroblasts were incubated with culture medium with 250 μM of CPF or Lethal 20. Luperox (ter-Butyl hydroperoxide, tBH) solution 100 μM was used as positive control. After 3 h, the production of ROS was measured by carboxy-H_2DCFDA microplate assay. Graph shows the fold increase in fluorescence relative to control assays with no compound added ($n \geq 3$).

3.3. Immunomodulatory Effect of Chlorpyrifos in Fibroblasts

To evaluate the immunomodulatory effect of CPF in fibroblasts, we assessed the production of the pro-inflammatory cytokine IL-6, after cells were exposed for 6 days. As shown in Figure 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 with 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 synovial fibroblasts exposed to particulate matter produce IL-6, suggesting the possible implications of IL-6 in the pathophysiology of diseases such as 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 to the CPF mechanism of action as an important inflammation inducer.

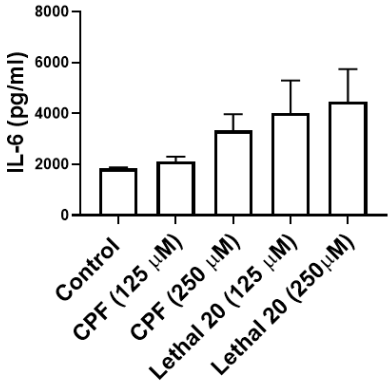

Figure 3. Effect of CPF on the production of IL-6. The fibroblasts were incubated with DMEM with 2% FBS with CPF pure or Lethal 20 (125 and 250 μM). After 6 days, the production of IL-6 was measured in the culture supernatants by ELISA. Values are mean ± SEM (n = 2).

4. Conclusions

CPF is still considered a health issue that assumes 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 a 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.

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Institutional Review Board Statement: The study was conducted in accordance with the Declartion of Helsinki. Human skin fibroblast cell line was obtained from the Cell Bank at Coriell Institute for Medical Research.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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