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Evaluation of the genotoxicity of the pyrethroid insecticide phenothrin

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ABSTRACT

Phenothrin, a synthetic pyrethroid compound, is widely used to control agricultural and household insects, as well as to eliminate human louse infestation. Toxicity studies on the direct DNA-damaging effect of phenothrin are lacking. We therefore investigated whether phenothrin exposure can lead to increased DNA damage *in vitro* in human peripheral blood lymphocytes and in human hepatocytes. Genotoxicity was evaluated by means of the comet assay modified with formamidopyrimidine DNA-glycosylase post-treatment for the detection of oxidative base-damage in DNA. We also assessed the cytotoxic potential of this compound by use of combined fluorescence viability staining. Our results show that phenothrin induces statistically significant, dose-dependent DNA damage in the absence of marked cytotoxicity at concentrations higher than 20 μM and 50 μM in human blood peripheral lymphocytes and hepatocytes, respectively. Oxidative DNA damage could also be detected in the two cell types, although this did not reach statistical significance. These findings provide evidence of the DNA-damaging potential of phenothrin and call for additional studies to reveal the genotoxic properties of this pyrethroid. The observations also point at the importance of using caution when considering the use of phenothrin.

1. Introduction

Synthetic pyrethroids are among the most common pesticides currently in use worldwide for pest control and community anti-mosquito programmes, a significant part of the population may be exposed to these compounds. Pyrethroids are strongly lipophilic agents that paralyze the peripheral and central nervous system of insects by interacting with sodium channels on nerve membranes [1]. Since they play an effective, therefore extensive, role in outbreak control and in protecting agricultural crops, it is essential to carefully study and analyze the hazards of pyrethroids to human health including their cytotoxic and genotoxic properties, in order to take adequate measures that prevent humans from potential mutagenic and carcinogenic effects.

Phenothrin ($C_{23}H_{26}O_3$) is a synthetic type-I non-cyano pyrethroid insecticide (Fig. 1). Phenothrin was first registered by the US-EPA in 1976 [2]. Recent quantitative data on the production and use of phenothrin are not publicly available; its worldwide production level was estimated 70–80 tonnes per year in 1989 by the World Health Organization [3]. It is widely used in pesticide products as a spray against agricultural and household insects, as well as in the area of public health against insect vectors that can spread communicable diseases among human populations. In addition, phenothrin has therapeutic applications, particularly in the elimination of human louse infestation, in which case it is formulated as a powder, shampoo, or lotion [2,4].

The general population may be exposed to phenothrin through multiple routes such as inhalation of household aerosol sprays, ingestion of food containing residual material, or dermal contact with pediculicides, i.e. medications used to treat lice and scabies infestations. According to deterministic exposure assessments, several residential scenarios could result in exposures of concern, especially the incidental ingestion of residues by toddlers. Application of pediculicides is considered to be a significant source of

Abbreviations: 7-AAD, 7-Aminoactinomycin D; ATSDR, Agency for Toxic Substances and Disease Registry; Calcein AM, acetomethoxy derivate of calcein; Fpg, formamidopyrimidine DNA-glycosylase; HepG2, human hepatoblastoma-derived cell line; IPCS, International Programme on Chemical Safety; LMA, low melting-point agarose; NMA, normal melting-point agarose; *trans*-CDCA, *trans*-chrysanthemumdicarboxylic acid; US EPA, United States Environmental Protection Agency; WHO, World Health Organization.

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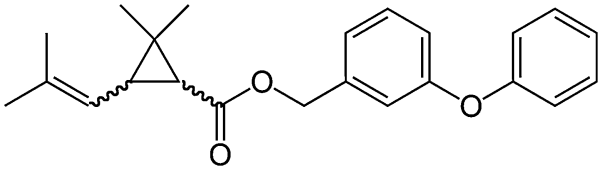

Fig. 1. Chemical structure of phenothrin.

residential phenothrin exposure, too, due to direct dermal contact. Furthermore, occupational scenarios may also pose increased risks of exposure to phenothrin if no protective equipment is supplied to or used adequately by the workers who mix, load and apply the pesticide product. Since the environmental persistence of phenothrin ranges from 1 to 2 days, the exposure from residues in food or drinking water is expected to be very low [2,4]. At present, reliable quantitative data on exposure are not available to permit characterization of a dose-response relationship.

Phenothrin is metabolized rapidly by hydrolytic cleavage of the ester bond, followed by oxidation and glucuronidation producing the common urinary metabolite *trans*-chrysanthemumdicarboxylic acid (*trans*-CDCA), the half-life of which for urinary excretion varies from 4 to 12 h after exposure. *Trans*-CDCA is used as a biomarker for internal dose assessment of certain pyrethroid insecticides [5].

Phenothrin is an effective nerve stimulant that affects the conduction of nerve impulses by forcing the sodium channels of insects to remain open; the ensuing excessive sodium discharge eventually leads to paralysis [6]. The sensitivity of human nerves to phenothrin is low, nevertheless studies have reported toxic effects of phenothrin generally observed in humans including symptoms such as dizziness, salivation, headache, fatigue, diarrhoea, and irritability to sound and touch [7].

Although phenothrin was found to be non-mutagenic in *Escherichia coli* strains [8], data provided by mutagenicity and genotoxicity studies with higher organisms are lacking. Its carcinogenic potential was investigated by *in vivo* animal studies, in two of which phenothrin increased the incidence of liver cancer; however, this effect did not reach statistical significance [9,10]. On the basis of these limited findings, phenothrin has been classified by the US-EPA as "not likely to be carcinogenic to humans" [2].

The purpose of the present acute-exposure study was to update the knowledge about the genotoxic properties of phenothrin by use of highly sensitive *in vitro* genotoxicity tests with human peripheral blood lymphocytes and human hepatocytes. The information obtained in this study was then used to make a preliminary evaluation on the appropriateness of the present regulations for phenothrin use.

2. Materials and methods

2.1. Chemicals

Analytical grade d-*trans*-phenothrin (purity 94.5%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Histopaque-1077 and chemicals used for the alkaline comet assay were purchased from Sigma–Aldrich Chemie GmbH (Heidelberg, Germany). The cell-culture medium and the supplements were provided by Gibco (Paisley, UK). The endonuclease formamidopyrimidine DNA-glycosylase (Fpg, FLARE™ Module) was obtained from Trevigen (Gaithersburg, MD, USA). The acetomethoxy derivative of calcein (Calcein AM) and 7-Aminoactinomycin D (7-AAD) fluorescent dyes were purchased from Biotium (Hayward, CA, USA).

2.2. Cell cultures

The human hepatoblastoma-derived cell line HepG2 was purchased from ATCC (Manassas, VA, USA). It provides a frequently used *in vitro* cell system in human toxicological studies on liver cells. The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, and grown as a monolayer in T25 and T75 flasks (TPP, Trasadingen, Switzerland) at 37 °C in a humidified incubator with a 5% CO_2 atmosphere.

Human peripheral blood samples were obtained by venipuncture and collected into heparin-containing vacutainer tubes (BD Vacutainer Systems, Plymouth, UK) from five non-smoking, healthy volunteers (males, aged 25–30 years) with no known previous contact with high concentrations of pesticides. Mononuclear white blood cells were separated from the erythrocytes by density-gradient centrifugation on Histopaque-1077. The buffy-coat was aspirated and re-suspended in RPMI 1640 medium containing 10% foetal calf serum.

2.3. Cytotoxicity assay

Before and after phenothrin treatment, aliquots of cells were immediately subjected to a cytotoxicity assay. Calcein AM and 7-AAD fluorescent dyes were used to co-label the cells. Calcein AM is a non-polar compound that passively crosses the plasma membrane of living cells, where it is cleaved by intracellular esterases to release a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm. 7-AAD is a DNA-intercalating dye that is able to permeate membranes of dead and dying cells, but cannot penetrate plasma membranes of live healthy cells.

Both fluorescent dyes were dissolved in PBS to a final concentration of 2 μM each. Of this working solution, 200 μl were added to the cell pellets (1×10^5 cells), and incubated for 30 min at 4 °C, protected from light. The labelled cells were washed and re-suspended in ice-cold PBS buffer. Forty microliters of the cell suspension were placed on a microscope slide for immediate microscopical examination.

2.4. In vitro treatment

Before treatment, HepG2 cells were seeded into six wells of a 12-well plate (2×10^5 cells/well) and allowed to grow to 80–90% confluence. The human peripheral blood lymphocytes were also partitioned at a cell density of 2×10^5 cells/ml medium into six wells of a 12-well-plate on the day of the experiment.

The cells were exposed to increasing doses of phenothrin (20, 50, 100, and 1000 μM) in the corresponding cell-culture medium. The stock solution and the dilution series (100 μM, 10 μM, 1 μM) were made in methanol. Aliquots of different concentrations of phenothrin solution or solvent control were added to the cell cultures and incubated for 1 h at 37 °C. The methanol content in the cell culture fluid was 10% (v/v) for each treatment, the concentration found to be non-genotoxic and non-cytotoxic in our previous experiments (unpublished).

Following incubation, the HepG2 cells were washed and scraped from the wells in order to avoid trypsin-induced DNA damage. All cultures were centrifuged and the cells re-suspended in the corresponding serum-free medium at a density of 2000 cells/μl.

2.5. Genotoxicity test

The alkaline version of the single-cell gel-electrophoresis assay (comet assay) was performed as described by Singh et al. [11] with slight modifications, *i.e.* the use of restriction-endonuclease digestion immediately after induction of the DNA damage [12].

Degreased frosted slides were first covered with 1% normal melting-point agarose (NMA), which was scraped off the slide before use. The slides were then coated with three layers: 1% NMA covered with 0.75% low melting-point agarose (LMA) containing the cells ($\sim 2 \times 10^5$ per slide, 10 μl) and topped with a 0.75% LMA layer (final volume 100 μl each). After solidification, the embedded cells were lysed (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris base pH 10, 1% sodium N-lauroyl sarcosinate and 1% Triton X-100 added fresh) at 4 °C for at least 1 h, protected from light. After lysis, the DNA was allowed to unwind for 20 min in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) and subjected to electrophoresis in the same buffer for 20 min at 0.8 V/cm and 300 mA in a horizontal electrophoresis chamber (Bio-Rad, Richmond, CA, USA) connected to an EPS 600 electrophoresis power supply (Pharmacia Biotech, Uppsala, Sweden). The buffer level was adjusted to maintain a constant current. Finally, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris base–HCl, pH 7.5) to remove excess alkali and detergent. After drying, each slide was stained with ethidium bromide (20 μg/ml) and stored in a humidified container at 4 °C until analysis.

For the detection of oxidative DNA damage, formamidopyrimidine DNA-glycosylase (Fpg), a lesion-specific restriction endonuclease that recognizes oxidized purines and pyrimidines, was used [13]. After lysis, two additional steps were included in the comet assay: slides were washed three times in FLARE buffer (1 mM HEPES-KOH, pH 7.4, 100 mM KCl) over a 30-min period at room temperature, and then incubated for 45 min at 37 °C with Fpg diluted in enzyme-reaction buffer (FLARE buffer plus BSA). Concentrations of the enzyme were adjusted according to the protocol provided by the manufacturer [14], applying 0.2 μl of enzyme in 75 μl enzyme-reaction buffer per slide. Slides treated with buffer alone were used as negative control. The slides were then processed as described above.

2.6. Image and data analysis

The fluorescence signal was detected at 400× magnification with a Zeiss Axioplan epi-fluorescence microscope equipped with a CCD camera connected to an image-analysis system.