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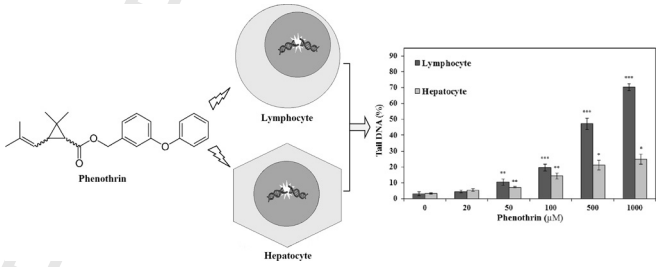
Károly Nagy, Gábor Rácz, Takashi Matsumoto, Róza Ádány, Balázs Ádám*



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Highlights

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- Genotoxicity of the pyrethroid pesticide phenothrin is tested in *in vitro* models.
- Phenothrin induces genotoxic damage in human peripheral lymphocytes and hepatocytes.
- Oxidative DNA damage exerted by phenothrin can be detected.
- Use of phenothrin containing products is recommended with caution.

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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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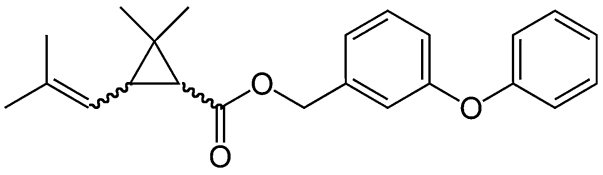

Fig. 1. Chemical structure of phenothrin.

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 (~2×10^5 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.

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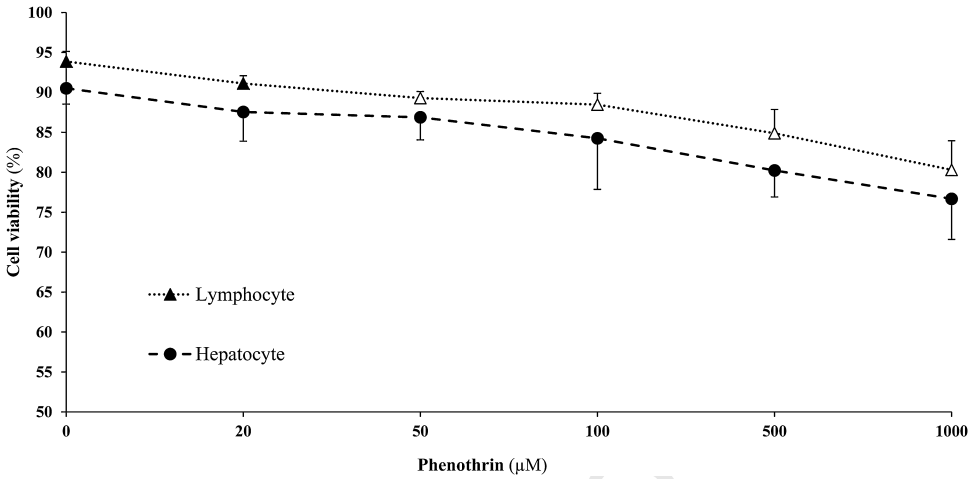

Fig. 2. Effect of phenothrin on cell viability. The data points represent the means ± SEM of repeated experiments. Statistically significant difference ($p < 0.05$) with the corresponding untreated control was determined by Student's *t*-test and is indicated by the open symbol.

To determine cytotoxicity, an FITC filter for Calcein AM and a TRITC filter for 7-AAD were used to excite the co-labelled cells. The survival rate was determined by visual examination of 10 randomly selected non-overlapping fields per slide. Each field contained 10–30 images. Cell viability was expressed as the mean of the percentages of living cells from repeated experiments. The proportions of living cells observed in technical replicates were subjected to statistical analysis.

For the assessment of genotoxicity, the Comet Imager v.2.2.1. Software (Meta-Systems GmbH, Germany) was used to analyze 2 × 50 randomly captured comets from duplicate slides, and to compute the DNA-damage parameters. The percentage of DNA in the tail (%, tail DNA), tail length (μm) and tail moment (a combined descriptor considering both tail length and the fraction of DNA migrated in the tail) were measured to quantify the DNA damage. The results are presented as mean of the median values of DNA-damage parameters from repeated experiments. The medians of technical replicates were subjected to statistical analysis.

Experiments were independently performed three times with the HepG2 cells and five times with human peripheral blood lymphocytes from five different donors. Simple linear regression analysis was used to assess the association between DNA-damage levels and doses of phenothrin. The cell viability and the central values of DNA damage induced by various doses of phenothrin in repeated experiments were statistically compared with that of untreated cells by use of Student's *t*-test. A p-value ≤ 0.05 was considered to correspond with statistical significance.

3. Results

3.1. Phenothrin-induced cytotoxicity

Both human peripheral blood lymphocytes and human hepatocytes showed high initial viability (>90%) in all experiments. Exposure to phenothrin during 1 h induced limited cell death in both cell types in a concentration-dependent manner (Fig. 2).

Although the dose–response curves follow a similar pattern for both cell types, a statistically significant decrease in cell viability could only be observed in lymphocytes at concentrations ≥50 μM. All samples showed a relatively high viability (>77%) after the treatment. Therefore, the genotoxic effects of phenothrin observed in the dose range used are unlikely to be caused by apoptotic processes.

3.2. Phenothrin-induced genotoxicity

Exposure to phenothrin induced a dose-dependent increase of DNA damage in both cell types measured as tail DNA and tail length. This finding clearly indicates the genotoxic potential of this pyrethroid pesticide (Fig. 3).

A moderate increase of DNA-damage values was observed in the 0–50-μM concentration range, followed by a considerable increase up to 1000 μM. Human peripheral blood lymphocytes had significantly higher absolute values of both DNA-damage parameters than hepatocytes in the higher concentration range (50–1000 μM). There was a statistically significant positive correlation between DNA damage and phenothrin concentration in the lymphocytes (tail DNA: $r = 0.982$, $p < 0.001$ and tail length: $r = 0.957$, $p < 0.01$) as well as in the hepatocytes (tail DNA: $r = 0.912$, $p < 0.05$ and tail length: $r = 0.848$, $p < 0.05$). The slope of the linear regression line for both indicators was found to be steeper for the lymphocytes (tail DNA: $\beta = 0.066$ and tail length: $\beta = 0.005$) than for the liver cells (tail DNA: $\beta = 0.02$ and tail length: $\beta = 0.001$).

The lowest concentration of phenothrin that produced a statistically significant increase in DNA damage was 50 μM for both cell types when the damage was measured as % tail DNA. When the DNA damage was measured as tail length, the lowest effective concentration of phenothrin was 20 μM for the lymphocytes, and 100 μM for the hepatocytes.

The nature of phenothrin-induced DNA damage was further investigated with the modified comet assay, which included lesion-specific restriction endonuclease Fpg, which allows detection of oxidative DNA damage [13]. Fpg-dependent oxidative DNA damage in the two cell types was expressed as the difference between tail-moment values detected with or without Fpg digestion in the comet assay (Fig. 4). Treatment with phenothrin apparently increased the level of oxidized DNA bases in both cell types, although no statistically significant difference in DNA-damage level was observed between the control and Fpg-incubated cells. The extent of Fpg-detected DNA damage showed a clear dose-dependence. A significant linear correlation was observed between the oxidative DNA damage and phenothrin concentrations in human peripheral blood lymphocytes (tail moment: $r = 0.959$, $p < 0.01$) as well as in liver cells (tail moment: $r = 0.924$, $p < 0.01$). The slope of the linear regression line was found to be steeper in the lymphocytes (tail moment: $\beta = 0.0011$) than in the hepatocytes (tail moment: $\beta = 0.0005$) in the concentration range tested. The results show a lower level of oxidative DNA in the liver cells than in the lymphocytes.

4. Discussion

Phenothrin is a synthetic pyrethroid commonly used for controlling various insects in households and agriculture, among other applications, but its genotoxic/mutagenic activity is poorly investigated. In the present study, we utilized the comet assay to examine the possible DNA-damaging effect of phenothrin and determined the dose-response characteristics of phenothrin-mediated DNA

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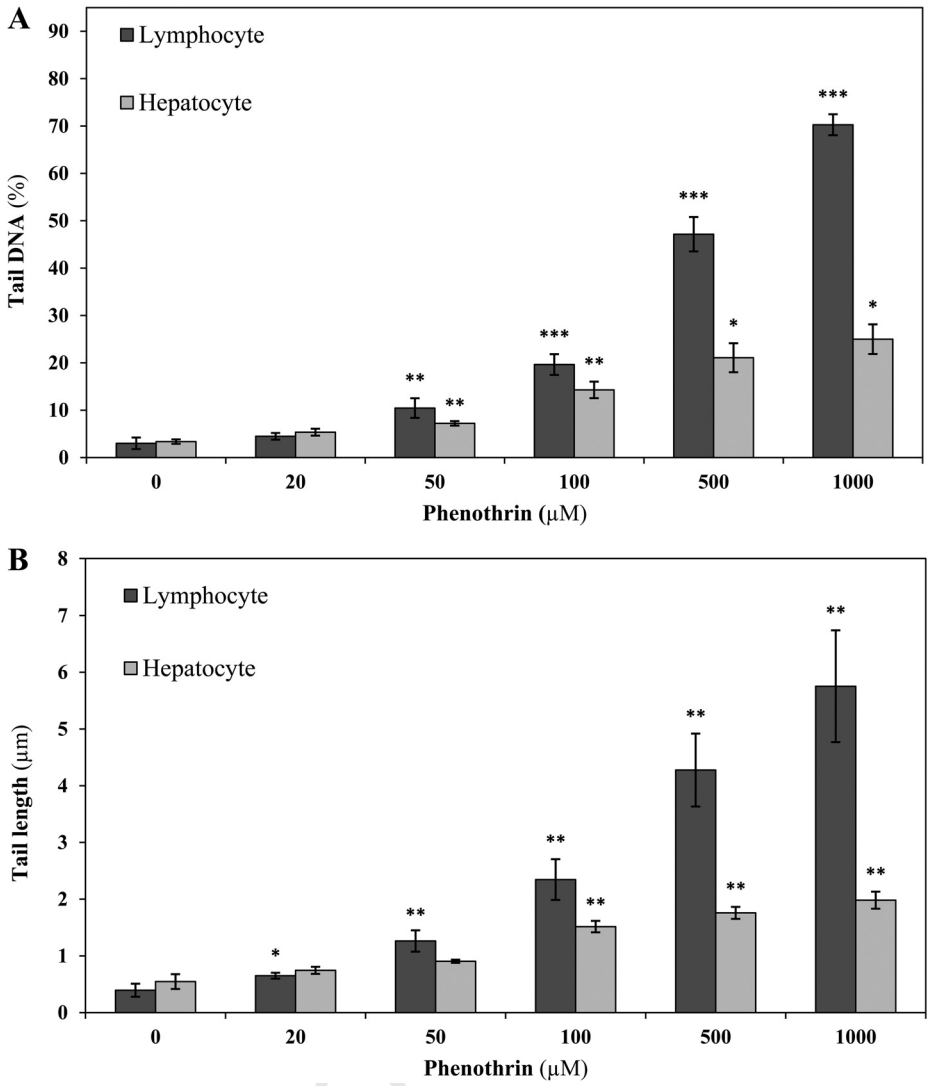

Fig. 3. DNA damage induced by phenothrin in human peripheral blood lymphocytes and cultured hepatocytes measured as % tail DNA (A) and tail length (B) in the comet assay. Data are means of median values of repeated experiments (± SEM indicated by error bars). Statistically significant increase ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) was determined by comparing the values of DNA damage induced by various doses of phenothrin with the background level in untreated cells by Student's *t*-test.

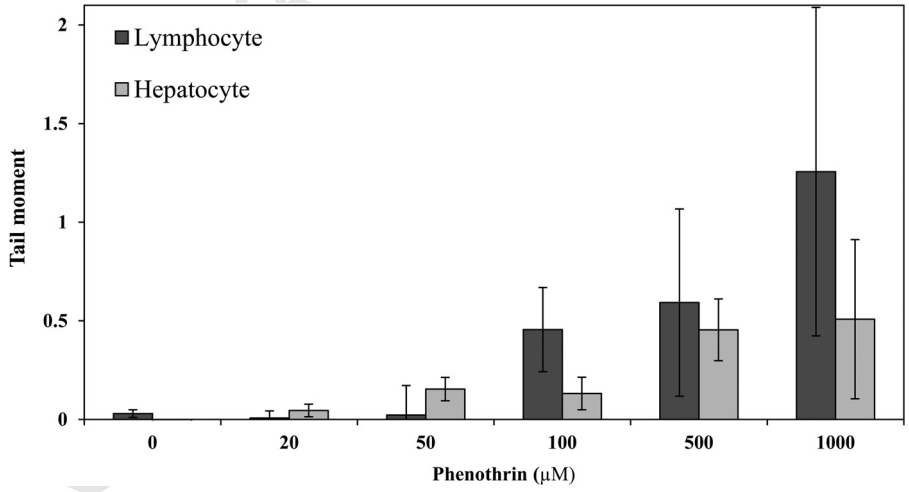

Fig. 4. Oxidative DNA damage induced by phenothrin in human peripheral blood lymphocytes and cultured hepatocytes measured as tail moment in the comet assay. The bars represent additional damage detected by incubation with restriction endonuclease Fpg. Data are means of differences of corresponding median values detected with and without Fpg modification of the comet assay (±SEM indicated by error bars).

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strand-breaks in human peripheral blood lymphocytes and cultured human hepatocytes.

Our findings point at the potential of this pesticide to induce genotoxic insult. Acute exposure to phenothrin led to a significant, concentration-dependent increase in DNA damage in the absence of marked cytotoxicity in the cell types examined, under the conditions applied. The lowest concentrations of phenothrin that resulted in a statistically significant DNA-damage induction were 20 μM in human peripheral blood lymphocytes and 50 μM in hepatocytes. Hepatocytes proved to be less sensitive towards the genotoxic action of phenothrin than lymphocytes, a finding that may be explained by the abundant detoxification systems of HepG2 cells [14]. The oxidative potential of phenothrin could be shown in both cell types, although the effect did not reach statistical significance. Detailed information on the genotoxic properties of phenothrin is provided by this study for the first time, and it may have important consequences, since significant DNA-damaging effects were observed at relatively low (micromolar) doses.

The general population is exposed to phenothrin primarily from incidental ingestion of residues after residential pest control or use of commercial pediculicides, but significant exposure may occur in certain occupational settings as well [2]. Measurements of urinary metabolites provide useful biomarkers of exposure, but there is insufficient information at this time to allow for correlation of the amount of metabolites measured in the urine with the body burden or with the level of environmental exposure to phenothrin. Results of a single study in humans following inhalation exposure to pyrethroid sprays containing phenothrin as one of the components indicate that the mean concentration of the pyrethroid metabolite *trans*-CDCA in urine was 1.1 μg/l urine, despite the fact that the proportion of phenothrin in the pyrethrum mixture was unknown [5]. It would be speculative to estimate the amount of urinary metabolites produced by the doses applied in our experimental system in the absence of applicable toxicokinetic models of phenothrin metabolism in humans. Data exist only for some other pyrethroid pesticides, the residues of which have been measured in the urine and blood plasma of industrial workers. Concentrations of fenvalerate and cypermethrin were reported to reach 0.044 μM in urine and 1.08 μM in plasma, respectively [16,17]. However, the toxicokinetic properties of these pesticides may differ from those of phenothrin.

Toxicity studies on the direct DNA-damaging effect of phenothrin are lacking. The mutagenicity of commonly used insecticides containing 0.2% phenothrin was previously investigated with the Ames spot forward mutation assay and yielded negative results [8]. Based on animal studies, exposure to phenothrin has been associated with the development of liver cancer in mice and rats, although the increase in cancer incidence was statistically non-significantly different between the control and exposed groups [9,10]. Another investigation has linked phenothrin with breast cancer. Phenothrin has been shown to mimic estrogenic activity in MCF-7 human breast-carcinoma cells by increasing the level of WNT10B proto-oncogene expression [18]. Other studies do not support the cancer-causing ability of this insecticide [4,19].

In conclusion, our findings provide the first evidence that the pyrethroid insecticide phenothrin has pronounced genotoxic potential. The effective doses observed in this study are in the low micromolar range, which is close to, although higher than the internal dose of phenothrin estimated by extrapolation from the limited data on endogenous concentrations of other synthetic pyrethroids. Nevertheless, due to the stochastic nature of a genotoxic effect without a threshold of safety, the DNA-damaging potential cannot be ruled out at lower concentrations. The limited data available on the toxic effects of phenothrin and the positive results reported here, with the agent used on primary and secondary cell cultures, call for further studies to characterize the genotoxic properties of this pesticide. In the light of our findings, it is advisable to reconsider the health hazards of phenothrin, especially in regard to its DNA-damaging potential. First and foremost, in order to minimize the risk of exposure and related health effects, cautious use of phenothrin-containing products can be recommended, in particular the avoidance of direct human applications.

Conflict of interest statement

The authors declare that there are no conflicts of interests.

Uncited reference

[15].

Acknowledgements

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