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Original Research

Use of Cultured Cells of Mammal and Insect Origin to Assess Cytotoxic Effects of the Pesticide Chlorpyrifos

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Abstract

In the present study four different cell cultures, derived from rabbit kidney (RK13), rat, and murine liver (WBF344 and Hepa 1c1c7) and insect origin (Sf21) were used to examine the effects of chlorpyrifos. Sf21 cells were the most sensitive to chlorpyrifos, with significant suppression of their proliferative activity ranging from 10^{-1}-10^{-5} M. However, significant suppression of proliferative activity also was recorded in mammalian cell cultures Hepa 1c1c7 (10^{-1}-10^{-3} M), WBF344 (10^{-1}-10^{-2} M), and RK13 (10^{-1} M). A cytopathic effect and LDH leakage into the medium was observed in RK 13 (10^{-1}-10^{-3} M) > WBF344 and Hepa 1c1c7 cells (10^{-1}-10^{-2} M) > Sf21 (10^{-1} M) compared to solvent control.

Our results indicate that chlorpyrifos exposure caused a species-dependent decrease in cell proliferation and cell membrane damage.

Keywords: insecticide, chlorpyrifos, cell cultures, cell proliferation, LDH

Introduction

Organophosphates are chemicals that inhibit cholinesterase and are employed widely as pesticides in residential settings and in agricultural practice to increase crop yields. Because the use of organophosphate pesticides has been and remains pervasive in both developed and developing nations, concerns are increasing regarding the relative safety of these chemicals for the environment, humans, and animals [1]. One such organophosphate that has spurred renewed interest is chlorpyrifos, the most widely used insecticide in the world.

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Chlorpyrifos [O,O-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothionate is utilized extensively in agriculture and for residential pest control. Today it is registered in about 100 countries worldwide and more than 50 crops are protected from insect infestation with its products. It is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice in grain, cotton, fruit, nut, and vegetable crops, as well as on lawns and ornamental plants. It is also registered for direct use on sheep and turkeys, for horse site treatment, dog kennels, domestic dwellings, farm buildings, storage bins, and commercial establishments [2].

LD_{50} for chlorpyrifos is 60 mg/kg in mice, 1000 mg/kg in rabbits, 32 mg/kg in chickens, 500 to 504 mg/kg in

guinea pigs, and 800 mg/kg in sheep. Dermal LD_{50} is greater than 2000 mg/kg in rats, and 1000 to 2000 mg/kg in rabbits. The 4-hour inhalation LC_{50} for chlorpyrifos in rats is greater than 0.2 mg/l [3]. Chlorpyrifos is highly toxic to amphibians [4].

Chlorpyrifos is absorbed by all routes of exposure, distributed throughout the body. Although some chlorpyrifos may be stored in fat tissue, bioaccumulation is not expected to be significant due to an elimination half-life in humans of less than three days. Following oral exposure, rats excreted 90% of ingested chlorpyrifos through urine and 10% in feces [5]. Chlorpyrifos is a neurotoxin and suspected endocrine disruptor [6], and it has been associated with asthma, acute, reproductive, and developmental toxicity [1, 7]. For acute effects, the EPA classifies chlorpyrifos as Class II: moderately toxic. Symptoms of poisoning include brain activation, epileptiformic convulsions, muscular tremors (which lead ultimately to flaccid paralysis), increased sweating, salivation, profound bronchial secretion, bronchoconstriction, increased activity of the intestine and diarrhoea, miosis, hypertension, lowered body temperature, and hyperglycemia [8]. There are also some literature data about such tissue-specific toxicities as hepatotoxicity and nephrotoxicity of chlorpyrifos after *in vivo* exposure [9-11].

The aim of this study was to screen for a direct effect of chlorpyrifos in mammalian kidney and liver cell cultures in comparison with insect cell cultures under *in vitro* conditions.

Material and Methods

The Insecticide Tested

Chlorpyrifos was dissolved in dimethylsulfoxide (DMSO, Lachema, Brno, Czech Republic), with final concentration in the maintenance medium of 1%. DMSO is a commonly used solvent for test materials prepared for the treatment of cells in culture. The basic molar concentrations of the insecticide, freshly prepared before each experiment, were 10^{-1}-10^{-5} M. These insecticide concentrations were added to cell cultures at a rate of 1% of total cell volume (i.e. the actual dose was 100× lower than the basic preparation).

After chlorpyrifos exposure, cell viability, proliferation, and lactate dehydrogenase release were evaluated in various cell cultures.

Cell Cultures

Cell lines RK13 (rabbit kidney), WBF344 (rat liver), Hepa 1c1c7 (murine hepatoma cell line), and IPLBSF-21 (the pupal ovarian cells of the fall army worm, *Spodoptera frugiperda* – Sf21) were used in the study. RK13 were cultured in minimal essential medium (MEM) supplemented with 10% (v/v) foetal calf serum (FCS) and 1% (v/v) antibiotic solution of Penicillin G Sodium Salt and Streptomycin Sulphate (Gibco, Invitrogen, Corp., USA) at 37ºC. WBF344 and Hepa 1c1c7 cells were cultured in Dulbecco´s modified Eagle's medium (D-MEM) with 10% (v/v) FCS, 1% (v/v) antibiotic solution of Penicillin G Sodium Salt and Streptomycin Sulphate (Gibco, Invitrogen, Corp., USA) in humidified 5% CO_2 at 37ºC. Sf-900 II SFM medium was used for the insect cell line (Gibco, Invitrogen, Corp., USA), supplemented with 1% (v/v) antibiotic solution of Penicillin G Sodium Salt and Streptomycin Sulphate (Gibco, Invitrogen, Corp., USA). Sf21 cells were cultured at 27ºC.

Cell Density and Cytopathic Effect

Cell density and cytopathic effect, determined on the basis of microscopic signs of cellular damage (granulation and vacuolization of cytoplasm, rounding off and detachment of cells from the bottom of the cultivation vessel, rupture of cells), were evaluated by standard counting technique using an inverted microscope (Carl Zeiss, Germany) at magnification of 400× after 24-hour exposure to chlorpyrifos.

Proliferation Test (PT)

Cells were seeded in 100 µl of cell culture medium in a 96-multiwell culture plate (Corning, Inc., USA) at a density of 2×10^5/ml. After 24-hour incubation, different concentrations of the tested insecticide, diluted in DMSO, were added in the amount of 1% of culture volume and treated cells were incubated for 48 hours. There were five replicates of each treatment. After the exposure period a colorimetric immunoassay was used to quantify cell proliferation (Cell Proliferation ELISA Kit, BrdU-colorimetric, Roche Diagnostics, GmbH, Germany).

The mean optical densities were converted into a percentage of residual cell viability expressed as a percentage of proliferative activity (% PA):

$$\%\,PA = \frac{OD^{insecticide}}{OD^{DMSO}} \times 100$$

...where $OD^{insecticide}$ is the mean value of OD of cells treated with the insecticide and OD^{DMSO} is the mean value of OD of cells treated with the solvent control (DMSO), measured at 450 nm.

Cytotoxicity Assay

Cells were seeded in 100 µl of complete medium in a 96-multiwell culture plate (Corning, Inc., USA) at a density of 2×10^5/ml and incubated for 24 hours. The growth medium was changed to a maintenance medium with 1% (v/v) FCS, and different concentrations of tested insecticide diluted in DMSO were then added in the amount of 1% of culture volume and cells were incubated for an additional 24 hours. There were five replicates of each treatment. After the exposure period a non-radioactive colorimetric assay was used to quantify cytotoxicity/cytolysis by mea-

Table 1. Cytotoxic effect of chlorpyrifos insecticide on cell cultures determined by the proliferation test.

Cell culture	Test parameters	Concentration of chlorpyriphos in M (mean values)					Solvent control (DMSO)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
RK13	OD_{450}±S.E.	0.03±0.006	0.25±0.04	0.34±0.03	0.28±0.03	0.27±0.01	0.33±0.02
	%PA±S.E.	9.1±6.1**	75.8±36.4	103±33.3	84.9±24.3	81.8±3	-
WBF344	OD_{450}±S.E.	0.04±0.02	0.06±0.01	0.29±0.02	0.27±0.02	0.28±0.02	0.27±0.03
	%PA±S.E.	14.8±22.2**	22.2±3.7**	107.4±25.9	92.6±25.9	111.1±3	-
Hepa 1c1c7	OD_{450}±S.E.	0.08±0.01	0.14±0.01	0.23±0.01	0.25±0.02	0.27±0.01	0.3±0.02
	%PA±S.E.	26.7±10**	46.7±13.3**	76.6±6.7**	83.3±16.7	90±10	-
Sf21	OD_{450}±S.E.	0.008±0.001	0.0006±0.001	0.28±0.007	0.25±0.003	0.27±0.004	0.32±0.01
	%PA±S.E.	2.5±0.6**	0.2±0.7**	87.5±6**	78.1±3**	71.9±3**	-

OD_{450} – optical density, S.E. – standard error, % PA – percentage of proliferative activity, ** – statistical significance <0.01, * – statistical significance <0.05, RK13 – Rabbit Kidney cell line, WBF344 – epithelial rat liver cell line, Hepa 1c1c7 – murine hepatoma cell line, SF21 – *Spodoptera frugiperda* pupal ovarian tissue, M – molar concentration, DMSO – dimethylsulfoxide

suring LDH activity released from damaged cells (Cytotoxicity Detection Kit^{PLUS}, Roche Diagnostics, GmbH, Germany).

To calculate percent cytotoxicity in each plate a low control (LC) and a high control (HC) were set up and the percentage of cytotoxicity was calculated according to the formula:

$$\text{Cytotoxicity (\%)} = \frac{OD^{insecticide} - OD^{LC}}{OD^{HC} - OD^{LC}} \times 100$$

...where $OD^{insecticide}$ is the mean value of OD of cells treated with insecticide, OD^{LC} (low control) is the mean value of OD cells treated with the solvent control (DMSO), and OD^{HC} (high control) is the mean value of OD cells treated with the lysis buffer (the maximum releasable LDH activity in the cells) measured at 450 nm.

Statistical Analysis

Data were analysed by ANOVA, followed by Dunnett's test. Results are presented as mean±SD, $p<0.05$ was considered to be statistically significant.

Results

The cytotoxic effect of the insecticide chlorpyrifos on cell cultures determined by proliferation activity is shown in Table 1. Proliferative activity of mammalian cell cultures was significantly suppressed as follows: liver cells Hepa 1c1c7 (10^{-1}-10^{-3} M), WBF344 (10^{-1}-10^{-2} M) and kidney cells RK13 (10^{-1} M, Table 1). Sf21 cells were the most sensitive to this insecticide, with significant suppression of their proliferative activity ranging from 10^{-1}-10^{-5} M ($p<0.01$), with PA = 0.2±0.7 – 87.5±6% (Table 1).

The cytopathic effect, determined on the basis of microscopical signs of cellular damage and LDH leakage into the medium, was observed in RK13 in the concentrations of 10^{-1}-10^{-3} M, in Hepa 1c1c7 and WBF344 cells in the concentrations of 10^{-1}-10^{-2} M compared to the solvent control. On the other hand, in the insect cell line Sf21 these effects were only observed in a concentration of 10^{-1} M (Table 2). After exposure to lower concentrations of pesticide, non-significant differences in percentages of cytotoxicity in studied cell cultures in comparison with control were determined.

Discussion

Traditionally, animal models have been used to examine the toxic potential of pesticides. With the discovery of a greater number of new pesticides, the feasibility of using *in vivo* models to screen for toxicity becomes impractical with respect to time, ethical, and cost considerations. *In vitro* cell culture systems have proved to be useful for toxicity prediction on target organs by chemicals or drug exposure [12]. Cell-based systems have become popular for studying interactions of chemicals with intact cells because it offers high-level integration. Various *in vitro* cell systems have been developed for studies of toxicity [12, 13]. The liver is known to be the main site of xenobiotic biotransformation due to the ability of this organ to express a plethora of enzymes, both quantitatively and qualitatively. Therefore, cell lines of liver origin are widely used in biomedical research involving xenobiotic metabolism [14]. For our study we used a WBF344 established from a single cloned non-parenchymal epithelial cell isolated from a normal male adult rat liver and Hepa 1c1c7, murine hepatoma cell line. The kidney is the main organ for elimination of xenobiotics from the body and that is why an RK13 cell line was used in the present study.

Table 2. Cytotoxic effect of chlorpyrifos insecticide determined by evaluation of LDH activity released from cells.

Cell culture	Test parametres	Concentrations of chlorpyriphos in M (mean values)				Solvent control (DMSO)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	
RK13	OD_{450}±S.E.	2.38±0.18	2.71±0.06	1.67±0.07	1.46±0.06	1.24±0.16
	% Cytotoxicity± S.E.	99.1±20.8**	121.4±7.6**	53.3±6.6*	37.1±4.1	22.27±8.3
WBF344	OD_{450}± S.E.	1.44±0.03	1.33±0.04	0.9±0.01	0.9±0.01	0.94±0.06
	% Cytotoxicity± S.E.	54.5±2.6**	46.9±3.9**	17.2±0.3	17.2±1	23.43±4
Hepa 1c1c7	OD_{450}±S.E.	2.6±0.02	2.54±0.02	1.85±0.11	1.70±0.04	1.83±0.17
	% Cytotoxicity± S.E.	84.8±1.6**	81.4±1.9**	42.4±7.3	33.9±2.8	41.25±10.6
Sf21	OD_{450}± S.E.	0.87±0.04	0.71±0.04	0.66±0.04	0.67±0.03	0.62±0.08
	% Cytotoxicity± S.E.	22±3.5*	11.8±2	8.6±1.4	9.2±1	6±3

OD_{450} – optical density, S.E. – standard error, ** – statistical significance <0.01, * – statistical significance <0.05, RK13 – Rabbit Kidney cell line, WBF344 – epithelial rat liver cell line, Hepa 1c1c7 – murine hepatoma cell line, SF21 – *Spodoptera frugiperda* pupal ovarian tissue, M – molar concentration, DMSO – dimethylsulfoxide

The cell lines used in this study have also been used as model systems for testing of various xenobiotics, e.g. WBF344 for food and perfume additives [15], RK13 for mycotoxins [16], Hepa 1c1c7 for many environmental contaminants [17], industrial chemicals [18], plant extracts and pigments [19], new cosmetic ingredients [20], and also pesticides [21-23]. Continuous cell line Sf21 developed from ovarian tissue of the *Spodoptera frugiperda*, a moth species that is an agricultural pest on corn and other grass species, was chosen for evaluation of the direct effect of the insecticide chlorpyrifos on insect cells. Recently, this cell line has been used for testing of insect cell extracts [24] or novel insecticide [25].

There are a lot of literature data about cytotoxic effects of chlorpyrifos on various cells, e.g. oocytes [26], embryos [27], eggs [28], nervous system cells [1, 29], thyroid [30], peripheral blood lymphocytes [31, 32], bronchial epithelial cells [32], Jurkat human T cells [33], mesenchymal stem cells [34], and liver cells [35]. Several *in vivo* studies also showed that chlorpyrifos may damage liver and kidneys after long-term exposure [9-11, 36, 37].

Our results showed that chlorpyrifos significantly influenced *in vitro* proliferation of mammalian liver Hepa 1c1c7, WBF344, and kidney RK13 cells. Sf21 cells were the most sensitive to this insecticide with significant suppression of their proliferative activity ranging from 10^{-1}-10^{-5} M.

The quantitative relationship between LDH release and the loss of animal cell viability or proliferation was described by Wang et al. [38], Aly and Dominica [39], El-Shenawy [40], and Cuello et al. [41]. LDH is an important glycolytic enzyme, which is present in the cells of almost all body tissues, and changes in this enzyme activity may provide direct and indirect evidence of cellular damage and can indicate the toxic mechanism [42]. LDH release is a parameter that reflects membrane integrity. Correlation of LDH activity with loss of cell viability in Sf-9 insect cell cultures was observed by Wu et al. [43]. Our study showed a significant proliferative suppression of Sf21 cells, but without correlation with LDH leakage into the maintenance medium and cytopathic effect. Cell proliferation in these cells was significantly suppressed but without cell destruction (except exposure to 10^{-1} M of chlorpyrifos). On the other hand, a cytopathic effect was observed in RK13 > Hepa 1c1c7 and WBF344 cells. DMSO, a commonly used solvent for test materials designated for the treatment of cells in culture, did not cause substantial alterations in the studied cultures.

Conclusions

The present findings show that chlorpyrifos under *in vitro* conditions suppressed cell proliferation of mammalian cells at the concentration range 10^{-1} (kidney cell line) – 10^{-3} M (liver cell lines) and of insect cells at concentrations 10^{-1}-10^{-5} M. Our results indicate that chlorpyrifos exposure caused a species and cell-type dependent decrease in cell proliferation and cell membrane damage. These results also showed that kidney and liver cell cultures may be useful as a supplementary tool in toxicity screening before testing substances on animal *in vivo*. On the other hand, the insect cell line could be an optimal method for *in vitro* evaluation of insecticide potential.

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