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RESEARCH ARTICLE

Proliferation and LDH Leakage in Cell Cultures of Animal and Insect Origin Exposed to Insecticide Endosulfan [1]

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Summary

In the present study three different cell cultures, derived from rabbit kidney (RK13), rat liver (WBF344) and insect origin (Sf21), were used to examine the cytotoxic effect of the insecticide endosulfan. Cytotoxicity was determined on the basis of cell proliferation activity; the cellular damage was assessed by evaluation of cytopathic effect and lactate dehydrogenase (LDH) leakage. Endosulfan treatment suppressed proliferative activity in cell cultures as follows: insect Sf21 cells (10^{-1}–10^{-5} M; $P<0.01$) > WBF344 (10^{-1}–10^{-4} M) > RK13 cells (10^{-1}–10^{-3} M). LDH leakage into the medium was increased in WBF344 cells at 10^{-1}–10^{-3} M ($P<0.01$), whereas in RK13 and Sf21 cells at 10^{-1}–10^{-2} M ($P<0.05$) compared to solvent control. These results indicate cell type-dependent sensitivity to endosulfan exposure. Endosulfan caused a more pronounced decrease in insect cell proliferation in comparison with mammalian cell cultures; however, the LDH leakage and microscopical signs of cellular damage were the most intensive in liver cells.

Keywords: *Insecticide, Endosulfan, Cell cultures, Cytotoxicity, LDH, Cell proliferation*

İnsektisit Endosülfana Maruz Kalan Hayvansal ve Böcek Kökenli Hücre Kültürlerindeki Proliferasyon ve LDH Fazlalığı

Özet

Bu çalışmada, tavşan böbrek (RK13), sıçan karaciğer (WBF344) ve böcek (Sf21) kökenli üç farklı hücre kültürü, insektisit endofulfanın sitotoksik etkisini incelemek için kullanıldı. Sitotoksisite hücre proliferasyonu aktivitesine bağlı olarak belirlendi; hücre hasarı, sitopatik etkinin artması ve laktat dehidrogenaz (LDH) fazlalığı ile değerlendirildi. Endosulfan tedavisi hücre kültürlerinde proliferatif aktiviteyi baskılama değerleri; böcek kökenli Sg21 (10^{-1}–10^{-5} M; $P<0.01$) > WBF344 (10^{-1}–10^{-4} M) > RK13 hücre kültürü (10^{-1}–10^{-3} M) şeklinde belirlendi. Ortamda LDH fazlalığı, kontrol ile kıyasla WBF344 türündeki hücrelerde 10^{-1}–10^{-3} M ($P<0.01$) miktarında arttarken RK13 ve Sf21 hücre kültürlerinde 10^{-1}–10^{-2} M ($P<0.05$) miktarında belirlendi. Bu sonuçlar endosülfan maruziyetine karşı hücre tipine bağlı duyarlılığı işaret etmektedir. Endosülfan, memeli hücre kültürüne göre böcek kökenli hücre kültürlerindeki hücre sayılarında daha belirgin bir düşüşe neden olsa da LDH fazlalığı ve hücredeki mikroskobik hasar belirteçleri en belirgin olarak karaciğer hücrelerinde görülmektedir.

Anahtar sözcükler: *: İnsektisit, Endosülfan, Hücre kültürleri, Sitotoksisite, LDH, Hücre proliferasyonu*

INTRODUCTION

Organochlorine pesticides continue to be used in several developing countries despite concerns regarding their toxicity profile. One such organochlorine compound is an insecticide and acaricide endosulfan. This colourless

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solid has emerged as a highly controversial agrichemical due to its acute toxicity [1], persistence in the environment, potential for bioaccumulation, and role as an endocrine disruptor [2]. Banned in more than 63 countries, it is still used extensively to control insect pests including whiteflys, aphids, leafhoppers, Colorado potato beetles and cabbage worms in vegetables, cotton, and fruits in some other countries including India and Brasil. Because of its unique mode of action, it is useful in resistance management [3]. On the other hand, endosulfan is one of the most toxic pesticides on the market today, responsible for many fatal pesticide poisoning incidents in humans and animals around the world. It is absorbed through the intestinal tract, the lungs, and the skin. Toxicokinetics of ^{14}C-endosulfan in rats was described by Chan et al.[4]. Monitoring of the residue levels of insecticide revealed its accumulation in various tissues and fluids [5,6]. The commonest manifestations of endosulfan intoxication are neurological although other organ dysfunction also occurs [1]. Hepatotoxicity and nephrotoxicity in humans and animals exposed to endosulfan have been documented in many studies [7-9].

The aim of our study was to compare the direct effect of different endosulfan concentrations on proliferative activity and cellular damage of mammalian and insect cell cultures.

MATERIAL and METHODS

The Insecticide Tested

Chemical data on endosulfan are depicted in *Fig. 1* [3]. Endosulfan was dissolved in dimethylsulfoxide (DMSO, Lachema, Brno, Czech Republic), of which the final concentration in the maintenance medium was 1%. The basic molar concentrations of insecticide, freshly prepared before each experiment, were 10^{-1}–10^{-5} M and added to cell cultures at the rate of 1% of total cell volume; i.e. the actual doses were 100x lower than the basic ones. After endosulfan exposure, cell proliferation, lactate dehydrogenase release and cell desintegration were evaluated in cell cultures of mammalian and insect origin.

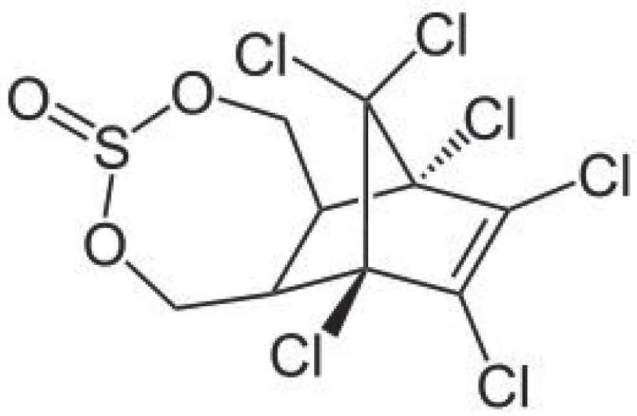

Fig 1. Chemical data on endosulfan [3]

CAS No. 115-29-7; Molecular formula: $C_9H_6Cl_6O_3S$; Mol. wt.: 406.9; Chemical family: organochlorines; Use: insecticide, acaricide; Producer, Purity: Supelco, Bellefonte, USA; 99%

Şekil 1. Endosulfan'ın kimyasal bilgileri [3]

CAS No. 115-29-7; Moleküler formül: $C_9H_6Cl_6O_3S$; Mol. ağırlık: 406.9; Kimyasal aile: organochlorines; Kullanım: insektisit, akarisit; Üretici, Saflık: Supelco, Bellefonte, ABD; 99%

Cell Cultures

Cell lines (kindly provided from Virological Institute, Bratislava, Slovakia) RK13 (rabbit kidney), WBF344 (rat liver) and the IPLBSF-21 (the pupal ovarian cells of the fall army worm, *Spodoptera frugiperda* – Sf21) were used in the study. RK13 cells 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 (PNC) and Streptomycin Sulphate (STM) (Gibco, Invitrogen, Corp., USA) at 37°C. WBF344 cells were cultured in Dulbecco´s modified Eagle´s medium (D-MEM) with 10% (v/v) FCS, 1% (v/v) PNC and STM in humidified 5% CO_2 at 37°C. Sf-900 II SFM medium was used for insect cell line (Gibco, Invitrogen, Corp., USA) supplemented with 1% (v/v) PNC and STM. Sf21 cells were cultured at 27°C.

Cell Density and Cytopathic Effect

Cell density and cytopathic effect determined on the basis of microscopical signs of cellular damage (granulation and vacuolisation of cytoplasm, rounding off and detachment of cells from the bottom of cultivation vessel, rupture of cells) were evaluated by standard counting technique using an inverted microscope (Carl Zeiss, Germany) at magnifications of 400 x after 24 h exposure to endosulfan.

Proliferation Test (PT)

Cells were seeded in 100 ml of cell culture medium in 96-multiwell culture plate (Corning, Inc., USA) at a density of 2x10^5/ml. After 24 h incubation different endosulfan concentrations were then added and treated cells were incubated for 48 h. 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). This was based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis. Twenty four hours before the end of the cultivation, 10 µM BrdU was added and the cells were reincubated. After removing the culture medium, denaturation of DNA and fixation of the cells on the bottom of wells, 100 ml of anti-BrdU-peroxidase labelled conjugate was added and allowed to react for 90 min at 25°C. The immune complexes were detected by the subsequent substrate reaction (100 ml substrate solution) for 30 min at room temperature. The reaction was stopped by 25 ml 1 M H_2SO_4 and the optical density (OD) was measured in an ELISA-multiwell reader (BIO-RAD Laboratories, Inc., USA) at 450 nm (OD_{450}). The mean optical densities were converted into percentage of

residual cell viability expressed as percentage of proliferative activity (% PA): %PA = [$OD^{endosulfan}$ / OD^{DMSO}] x 100; where $OD^{endosulfan}$ is the mean value of OD of cells treated with insecticide and OD^{DMSO} is the mean value of OD of cells treated with the solvent control, measured at 450 nm.

Cytotoxicity Assay

Cells were seeded in 100 ml of complete medium in 96-multiwell culture plate (Corning, Inc., USA) at a density of $2x10^5$/ml and incubated for 24 h. Growth medium was changed to maintenance medium with 1% (v/v) FCS and different endosulfan concentrations were then added and cells were incubated for additional 24 h. There were five replicates of each treatment. After the exposure period a colorimetric assay was used to quantify cytotoxicity/cytolysis by measuring LDH activity released from damaged cells (Cytotoxicity Detection Kit^{PLUS}, Roche Diagnostics, GmbH, Germany). To each well on the 96-well plate 100 µl reaction mixture was added and plate was incubated for 30 min at room temperature. After incubation 50 µl of stop solution was added to each well. Optical density (OD) was measured in an ELISA-multiwell reader (BIO-RAD Laboratories, Inc., USA) at 450 nm (OD_{450}). To calculate percent cytotoxicity in each plate low control (LC) and high control (HC) were set up and the percentage of cytotoxicity was calculated according to the formula: Cytotoxicity (%) = [$OD^{endosulfan}$ – OD^{LC} / OD^{HC} – OD^{LC}] x 100; where $OD^{endosulfan}$ 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

Cytotoxic effect of endosulfan on cell cultures determined by proliferation activity is shown in *Table 1*. Proliferative activity of mammalian cell cultures was significantly suppressed in liver WBF344 (10^{-1}–10^{-4} M) and kidney cells RK13 (10^{-1}–10^{-3} M). Insect 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 = 1.6–65.6% *(Table 1)*.

Cytopathic effect, determined on the basis of microscopical signs of cellular damage and the LDH leakage into the medium, was observed in WBF344 in the concentrations

Table 1. *Cytotoxic effect of insecticide endosulfan on cell cultures tested determined by proliferation test*
Table 1. *İnsektisit endosulfan'ın hücre kültürleri üzerinde proliferasyon testi ile belirlenen sitotoksik etkileri*

Cell Culture	Test Parameters	Concentration of Endosulfan in M (Mean Values)					Solvent Control (DMSO)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
RK13	OD_{450}±SD %PA±SD	0.04±0.02 12.1±6**	0.02±03 6.1±9**	0.25±0.16 75.8±48.5*	0.37±0.07 115.2±21.2	0.35±0.04 75.8±6.0	0.33±0.05 -
WBF344	OD_{450}±SD %PA±SD	0.02±0.005 7.4±1.9**	0.03±0.01 11.1±3.7**	0.11±0.07 40.7±25.9**	0.19±0.03 70.4±11.1*	0.32±0.07 118.5±25.9	0.27±0.09 -
Sf21	OD_{450}±SD %PA±SD	0.005±0.003 1.6±0.96**	0.005±0.004 1.6±1.3**	0.006±0.004 1.9±9.5**	0.13±0.03 40.6±9.4**	0.21±0.007 65.6±2.2**	0.32±0.04 -

RK13 *- Rabbit kidney cell line;* ***WBF344*** *- epithelial rat liver cell line;* ***Sf21*** *- Spodoptera frugiperda pupal ovarian tissue;* ***DMSO*** *- dimethylsulfoxide;* ***OD_{450}*** *- optical density;* ***SD*** *- standard deviation;* ***%PA*** *- percentage of proliferative activity;* ***M*** *- molar concentration; ** P<0.01; * P<0.05*

Table 2. *Cytotoxic effect of insecticide endosulfan determined by evaluation of LDH activity released from cells*
Table 2. *İnsektisit endosulfan'ın hücrelerden serbest kalan LDH aktivitsinin değerlendirilmesi ile baelirlenen sitotoksik etkileri*

Cell Culture	Test Parametres	Concentration of Endosulfan in M (Mean Values)				Solvent Control (DMSO)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	
RK13	OD_{450}±SD % Cytotoxicity±SD CPE	1.81±0.18 60.7±6.0* +	1.9±0.1 66.8±3.5* +	1.23±0.05 21.6±0.9 -	1.2±0.06 6.08±10.1 -	1.24±0.46 22.27±8.3 -
WBF344	OD_{450}±SD % Cytotoxicity±SD CPE	1.86±0.04 83.6±1.8** +	1.76±0.03 76.6±1.3** +	1.51±0.13 59.4±5.1** +	0.94±0.14 20.0±3.0 -	0.94±0.13 23.43±4.0 -
Sf21	OD_{450}±SD % Cytotoxicity±SD CPE	0.91±0.03 24.6±0.8** +	0.84±0.07 20.1±1.7* +	0.78±0.08 16.2±1.7 -	0.61±0.05 6.0±0.5 -	0.62±0.31 6.01±3.0 -

RK13 *- Rabbit Kidney cell line;* ***WBF344*** *- epithelial rat liver cell line;* ***Sf21*** *- Spodoptera frugiperda pupal ovarian tissue;* ***DMSO*** *- dimethylsulfoxide;* ***OD_{450}*** *- optical density;* ***SD*** *- standard deviation;* ***CPE*** *- cytopathic effect;* ***M*** *- molar concentration; ** P<0.01; * P<0.05*

of 10^{-1}–10^{-3} M, in RK 13 cells and the insect cell line Sf21 in the concentrations of 10^{-1}–10^{-2} M compared to solvent control *(Table 2)*. Cytopathic effect and LDH leakage into the medium were strongly limited in the insect cell line Sf21 and RK 13 cells in comparison to the inhibition of proliferative activity.

DISCUSSION

The indiscriminate and injudicious use of pesticides particularly endosulfan in agriculture and animal husbandry practices has considerably increased the risk of health hazard. Traditionally, animal models have been used to examine the toxic potential of pesticides. For screening purposes, there is increasing interest in the development of *in vitro* methods to replace conventional animal toxicity tests. Established mammalian and insect cell lines may contribute to rapid screening of pesticides and discover their modes of action or may be useful for toxicity prediction of target organs on chemicals or drug exposure [10].

The liver is known to be the main site of xenobiotic biotransformation, therefore, cell lines of liver origin are widely used in biomedical research involving xenobiotic metabolism and hepatotoxicity [11]. For our study we used WBF344 established from a single cloned non-parenchymal epithelial cell isolated from a normal male adult rat liver.

Kidney is the main organ for elimination of xenobiotics from the body and the use of the cultured cells of kidney origin to assess specific cytotoxic effects of nephrotoxins described Li et al.[12]. Rabbit kidney (RK13) cell line was used in the present study.

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 direct effect of endosulfan on insect cells. Cell lines used in this study were also used as model systems for testing of various xenobiotics, e.g. WBF344 for food and perfume additives [13], RK13 for mycotoxins [14], and the cell line Sf21 for testing of insect cell extracts [15] or novel insecticides [16]. Manji and Friesen [17] and Tseng et al.[18] studied induction of apoptosis and Takahashi et al.[19] cell cycle arrest induced by radiation in SF21 cell line. The present work was undertaken to evaluate the direct toxic effect of endosulfan on model cell cultures mentioned above.

Cytotoxic effects of endosulfan were previously studied in various cell cultures. Holovská et al.[20] evaluated proliferative activity of cell lines Madin-Darby Bovine Kidney (MDBK), Rabbit Kidney (RK13), Porcine Kidney (PK15), and semi-continual line of Bovine Embryonic Pulmonary Cells (BEPC) after exposure to endosulfan. From these cell cultures cell proliferative activity was suppressed most intensively in PK15 culture at the concentrations of 10^{-1}–10^{-6} M of endosulfan. Krovel et al.[21] observed moderate cytotoxicity and steatosis in a dose-dependent manner in the primary cultures of hepatocytes isolated from Atlantic salmon exposed to concentrations of 0.001–100 microM endosulfan for 48 h. Skandrani et al.[22] investigated endosulfan toxicity in neuronal SH-SY5Y and pulmonary A549 cell lines exposed to increasing pesticide concentrations for 3 days. This experiment showed that neuronal cells were more sensitive than pulmonary cells. Insecticide was found to cause concentration-dependent (50-400 microM) apoptotic cell death in SH-SY5Y human neuroblastoma cells [23]. Kannan et al.[24] also recorded the ability of endosulfan to induce apoptosis in a human T-cell leukemic line. In our previous studies we determined the cytotoxic and immunotoxic effects of endosulfan on sheep peripheral blood phagocytes and lymphocytes on the basis of functional immunological assays under *in vitro* conditions [25,26]. Results of our present *in vitro* study showed that endosulfan significantly suppressed mainly proliferation of insect Sf21 cells, whereas from mammalian cell cultures WBF344 liver cells were the most sensitive to insecticide exposure.

The quantitative relationship between lactate dehydrogenase (LDH) release and the loss of animal cell viability or proliferation was described by several authors [27-30]. LDH is an important glycolytic enzyme, present in the cells of almost all body tissues, and changes in the enzyme activity may provide direct and indirect evidence of the cellular damage and can indicate the toxic mechanism [31]. The LDH release is a parameter, which reflects the membrane integrity. In our study the cytopathic effect of endosulfan and LDH leakage were the most intensive in WBF344 cell culture, whereas RK 13 and insect Sf21 cells showed higher resistance to endosulfan. Study of El-Shenawy [29] also showed that incubation of rat hepatocytes with 10 or 100 microM of endosulfan for 2 h induced cell membrane damage as indicated by increasing the leakage percentages of LDH. Correlation of LDH activity with loss of cell viability in Sf-9 insect cell cultures was observed by Wu et al.[32]. However, our study showed a highly significant proliferative suppression of insect Sf21 cells (10^{-1}–10^{-5} M), but without correlation with LDH leakage into the maintenance medium and cytopathic effect (10^{-1}–10^{-2} M).

The molecular mechanisms of endosulfan cytotoxicity have not yet been elucidated and require further studies. One of causes of cytotoxicity might be the result of the intrinsic toxicity of pesticides that could lead to the generation of reactive oxygen species (ROS) during the metabolisation of certain substances or interactions with secondary targets. Some reports showed that many organochlorine and carbamate insecticides are capable of inducing oxidative stress by overproduction of ROS in experimental systems. Cells possess enzymatic and nonenzymatic antioxidant system, which detoxify reactive oxygen species (ROS) that are generated during oxidative stress [33,34].

It can be concluded that endosulfan caused a more pronounced decrease in insect cell proliferation in comparison

with mammalian cell cultures; however the LDH leakage and microscopical signs of cellular damage were limited on the first two highest concentrations of insecticide. The most intensive decrease of cell proliferation and the LDH leakage of mammalian cells was observed in liver cells. Our results revealed different sensitivity of mammalian and insect cells to endosulfan exposure under *in vitro* conditions, probably caused by different metabolisation pathways of the insecticide in the studied cells.

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