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CHROMOSOMAL ABERRATIONS INDUCED BY THE INSECTICIDE ENDOSULFAN IN SHEEP PERIPHERAL LYMPHOCYTES *in vitro*

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The chromosomal aberration assay is the most common and sensitive method for detecting the biological damage induced by a great variety of mutagenic, carcinogenic and environmental agents. In this study an organochlorine insecticide, endosulfan, was investigated for its ability to induce chromosomal aberrations in vitro using sheep peripheral lymphocytes. Lymphocyte cultures from two donors were exposed to four different concentrations of insecticide (4×10^{-3}, 4×10^{-4}, 4×10^{-5} and 4×10^{-7} M) for 48 h. At the lowest concentration (4×10^{-7} M) the percentage of aberrant cells was not significantly different (4.5 % versus 1.5 % in DMSO control, $p<0.05$). Higher concentrations of insecticide (4×10^{-3}, 4×10^{-4} and 4×10^{-5} M) resulted in significant increases of the chromosome aberration frequency (11 %; 11 % and 9 % versus 1.5 % in DMSO control, $p<0.05$). The mitotic index was significantly decreased at the concentrations of 4×10^{-3}; 4×10^{-4} M ($p< 0.01$) as well as 4×10^{-5} M ($p<0.05$).

In this species the insecticide tested induced a mutagenic effect at the higher concentrations which should not be attainable under agricultural conditions.

Key words: Endosulfan, chromosomal aberration, mitotic index, sheep peripheral lymphocytes

INTRODUCTION

Pesticides together with heavy metals from emissions are dominant compounds of the chemical load on the environment of man and animals. Farm animals may often be exposed to them on pasture. An accumulation of residues in meat, an important component of the food chain, poses an additional hazard to man (Kacmar *et al.*, 1999; Korenekova *et al.*, 1999).

Chlorinated hydrocarbon insecticides were used extensively from the mid-1940s until the 1960s and are now applied only on a limited basis as many have been banned or severely restricted. One still available is endosulfan, an insecticide and acaricide of the cyclodiene group.

Endosulfan (1, 4, 5, 6, 7, 7-hexachloro-8, 9, 10-trinorborn-5-en-2, 3-ylenedi-methyl-sulphite) is a brown crystalline substance consisting of α- and β-isomers in the ratio of approximately 70:30. It has gained significance as a potential environmental pollutant due to its widespread use in the control of over 100 agricultural insect pests attacking 60 food and non-food crops (Anonymus, 1994). Endosulfan is a fairly persistent insecticide with a biological half-life ranging from a few months to up to 2 years in soil and 1 - 6 months in water; whereas in plants 50 % residues are lost in 3 - 7 days. Unlike other organochlorines, endosulfan is not accumulative *in vivo,* besides marine animals (Naqvi and Vaishnavi, 1993). Its residues were detected in samples of slaughterhouse bovine serum and whole blood (Waliszewski and Szymczynski, 1991). However there is very little information available regarding the genotoxicity of endosulfan in livestock. Its genotoxic effect on somatic cells was demonstrated by the induction of chromosome aberrations in human lymphocytes (Yoder *et al.*, 1973) and rat bone marrow (Dikshith and Datta, 1978); sister chromatid exchanges and micronuclei in a $HepG_2$ cell line (Lu *et al.*, 2000); sister chromatid exchanges in blood lymphocytes from pesticide applicators (Ruppa *et al.*, 1991) as well as micronuclei in mouse bone marrow (Usha Rani *et al.*, 1980) and germ cells (Sylianco, 1978; Pandey *et al.*, 1990). Velazquez *et al.* (1984) reported it to be mutagenic in germ cells of *Drosophila melanogaster.* On the other hand, according to WHO data (1984) endosulfan was essentially non-genotoxic in several mammalian and bacterial cell assays.

The present paper reports the induction of chromosomal aberrations by endosulfan in sheep peripheral lymphocyte cultures *in vitro*.

MATERIALS AND METHODS

The tested pesticide

Endosulfan (1, 4, 5, 6, 7, 7-hexachloro-8, 9, 10-trinorborn-5-en-2, 3-ylenedi-methyl-sulphite, purity: 99 % mix of isomers, Supelco, USA) was dissolved in dimethylsulfoxide (DMSO, LA CHEMA, Brno) and freshly prepared before each experiment at concentrations of 4×10^{-3}, 4×10^{-4}, 4×10^{-5} and 4×10^{-7}M. The final concentration of DMSO in the cultures was 0.5 %. The tested substance was added after culture initiation and left until the end of cultivation (48 h treatment time). The features of the pesticide are indicated in Table 1 as reported by Gupta and Gupta (1979) and Anonymus (1994).

Table 1. The features of pesticide endosulfan (Gupta and Gupta,1979; Anonymus, 1994).

Common name	Molecular formula	Mol. wt.	CAS registry number	Activity	Field doses (kg/ha)	Toxicity in rats LD_{50} per oral (mg/kg)
Endosulfan	$C_9H_6Cl_6O_3S$	406.9	115-29-7	Insecticide Acaricide	0.2-1.5	70 in water 110 in oil

Lymphocyte cultures

For chromosome analysis, heparinised blood (100 IU/ml) was obtained by jugular vein puncture from two healthy donors (11-month-old female Merino sheep). Peripheral blood lymphocytes were processed by the modified method of Moorhead *et al.* (1960): 0.4 ml of heparinised whole blood was added to 7 ml of chromosome medium serumfree PANSERIN 1 (MDC) (PAN SYSTEMS GmbH, Biotechnologische Producte) supplemented with 100 IU/ml penicillin G, 100 μg/ml streptomycin and phytohaemagglutinin (PHA, Sigma, USA) at the final concentration of 85 μg/ml. Lymphocyte cultures were divided into solvent controls and treated cultures and incubated at 37 ^{0}C for 48 h. Two h prior to harvest the cultures were treated with the spindle inhibitor colchicine (Fluka BioChemika) at the concentration of 10 μg/ml to arrest cells in metaphase. The slides were stained with 10 % Giemsa-Romanowski in Sorensen phosphate buffer pH 6.8 for 3 - 6 min.

Cytogenetic assays

For each concentration and from each donor, 100 well spread metaphases bearing 54 chromosomes were scored for chromosomal aberrations (magnification 1000 x). Structural aberrations were classified according to the criteria suggested by Savage (1975). Gaps were reported but not included in the calculation of the percentage of aberrant cells.

The mitotic index was evaluated by counting at least 1000 cells per treatment: the number of dividing cells (prophases and metaphases) was divided by the total number of cells (Preston *et al.*, 1987).

Statistical analysis

The comparison between the exposed and control cultures was performed by means of Student's t-test.

RESULTS

Table 2 shows the percentage of aberrant cells observed in sheep peripheral lymphocytes cultured in the presence of different concentrations of endosulfan (48 h treatment time). At the lowest concentration (4×10^{-7}M) the chromosomal aberration frequency was not significantly different from the control. Higher concentrations of endosulfan (4×10^{-3}, 4×10^{-4} and 4×10^{-5} M) resulted in significant increases in the percentage of aberrant cells ($p<0.05$). Chromatid breaks were the dominant type of aberrations while no chromatid or isochromatid exchanges were found. Other aberrations in endosulfan treated cells were represented mainly by fragments (1.0 - 2.5 %) and associations of acrocentric chromosomes (0.5 - 3.0 %). Gaps induced by endosulfan were mainly of the chromatid type.

A decrease of mitotic index was observed at the concentration of 4×10^{-3}, 4×10^{-4} M ($p<0.01$) as well as 4×10^{-5}M ($p<0.05$).

Table 2. The percentage of aberrant cells and mitotic index observed in sheep peripheral lymphocytes cultured in the presence of different concentrations of endosulfan (48 h treatment time)

Treatment	Dose (M)	No. of cells scored	Chromosomal aberrations					No. of AB. C.	% of AB.C.	B/C	G/C	Other aberr. %	Mitotic index MV ±SD
			B_1	B_2	E_1	E_2	G_1 G_2						
DMSO	0.5%	200	2	1	-	-	3 2	3	1.5 ±0.707	0.015	0.025	-	1.95 ±0.07
ENDO-SULFAN	4×10^{-3}	200	11	11	-	-	15 6	22	11 ±0.000*	0.110	0.105	Assoc. 3 Fragm. 2.5	0.88 ±0.00**
	4×10^{-4}	200	19	3	-	-	19 9	22	11 ±1.314*	0.110	0.140	Assoc. 1 Fragm. 2	1.05 ±0.40**
	4×10^{-5}	200	15	3	-	-	10 2	18	9 ±8.485*	0.090	0.060	Assoc. 2 Fragm. 1.5	1.13 ±0.22*
	4×10^{-7}	200	6	3	-	-	9 4	9	4.5 ±2.121	0.045	0.065	Assoc. 0.5 Fragm. 1	1.20 ±0.40

* $p < 0.05$; ** $p < 0.01$
AB.C. aberrant cells
% AB.C. - total number of aberrations without gaps/ total number of cells analysed, x 100
Mitotic index - cells in mitotic divisions/ 1000 cells examined, x 100
MV ±SD - mean value ± standard deviation
B_1, E_1, G_1 - chromatid breaks, exchanges, gaps
B_2, E_2, G_2 - isochromatid breaks, exchanges, gaps
B/C -number of breaks per cell; G/C ±number of gaps per cell

DISCUSSION

In the past, risk assessment of pesticides was limited to the danger to human health, but nowadays ecological risks are also taken into account. The study of their genotoxicity in animals is of particular concern, because they are immediately exposed to and most affected by the harmful influence of pesticides, especially on pasture. We used sheep as model animals which are close to wild ruminants.

In the present study we evaluated the mutagenic potential of the insecticide endosulfan on *in vitro* cultures from two subjects. Endosulfan is toxic not only to insects, but also to fish, animals and humans. Perhaps because its carcinogenicity and genotoxicity have not been confirmed, it is still widely used and continues to pollute the environment in many countries.

Our *in vitro* experiment showed that endosulfan induced structural chromosomal aberrations in all exposed cultures compared to the control. A significant increase was present at the concentrations of 4×10^{-3}, 4×10^{-4} and 4×10^{-5} M ($p<0.05$).

The aberrations induced by endosulfan were mainly of the chromatid type. Most chemicals are S-dependent clastogens supporting the rise of chromatid-type aberrations which are largely unstable, and only symmetrical exchanges would be expected to be stable (Kirkland, 1998). The highest frequency of gaps was also recorded to be of the chromatid type. It is generally proposed that gaps should not be included among chromosomal aberration frequencies for statistical analysis. However, since some of the gaps could be deletions, it is appropriate to analyse the data excluding and including gaps (Preston *et al.*, 1987). According to Brogger (1982), gaps are a sensitive indicator of exposure to genotoxic drugs, because they serve mainly at low doses as a "guard" parameter.

The data regarding endosulfan genotoxicity are limited. The induction of chromosome aberrations was observed in human lymphocytes (Yoder *et al.*, 1973) and rat bone marrow (Dikshith and Datta, 1978) only. Here we report, for the first time to our knowledge, an induction of structural chromosomal aberrations by endosulfan in sheep lymphocytes *in vitro*. In our previous experiment with sheep lymphocyte cultures we investigated the induction of micronuclei by endosulfan at the same concentrations (4×10^{-3}, 4×10^{-4}, 4×10^{-5} and 4×10^{-7} M) after 48 h exposure. Higher concentrations of insecticide (4×10^{-3} and 4×10^{-4} M) resulted in a significant increase in the number of micronuclei (Kovalkovicova *et al.*, 2000), while in the chromosomal aberration assay a significant increase was also present at the concentration of 4×10^{-5} M ($p<0.05$). According to Savage (1988) the observed frequencies of micronuclei are often lower than the frequencies of chromosomal aberrations seen at the first post-treatment metaphase, because not all fragments necessarily form visible micronuclei.

Cytotoxicity is a phenomenon that may be (causally) associated with the induction of chromosomal damage for a number of compounds which are non-DNA reactive and thus thresholded *in vitro* clastogens (Müller and Kasper, 2000). One of the cytotoxicity parameters is mitotic index that gives direct information about the toxic effect on metaphases. Although depression of mitotic index may be the consequence of cell death, it is more often the result of cell cycle delay (Kuroda *et al.*, 1992). Mitotic index was decreased significantly at the concentration of 4×10^{-3}, 4×10^{-4} M ($p<0.01$) as well as 4×10^{-5} M ($p<0.05$).

According to our calculation one sheep may graze 5-8 kg of grass daily, while the practical application concentration of insecticide is 0.2-1.5 kg/ha (Gupta and Gupta, 1979). Our results thus demonstrated a weak genotoxic effect of endosulfan in sheep peripheral lymphocytes *in vitro*, because significant changes in the percentage of aberrant cells were detected at the higher concentrations which should not be attainable under agricultural conditions. Further studies should be performed in a larger group of animals.

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HROMOZOMSKE ABERACIJE U LIMFOCITIMA PERIFERNE KRVI OVACA IZAZVANE INSEKTICIDOM ENDOSULFANOM *IN VITRO*

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SADRŽAJ

Test za određivanje hromozomskih aberacija je široko primenjivan i osetljiv metod za detekciju bioloških oštećenja izazvanih brojnim mutagenim, karcinogenim i ambijentalnim faktorima. U ovom radu je ispitivana sposobnost organohlornog insekticida endosulfana da u *in vitro* uslovima dovede do hromozomskih aberacija ovčijih eritrocita iz periferne krvi. Limfociti su bili kultivisani *in vitro* i izloženi insekticidu koji je primanjivan u četiri različite koncentracije (4×10^{-3}, 4×10^{-4}, 4×10^{-5}, 4×10^{-7} M) u toku 24 časa. Pri najnižoj koncentraciji (4×10^{-7} M), procenat aberantnih ćelija nije bio značajno različit u odnosu na DMSO kontrolu (4.5% vs 1.5%, $p>0.05$). Veće koncentracije insekticida (4×10^{-3}, 4×10^{-4}, 4×10^{-5}) dovodile su do značajnog povećanja frekvence aberacija (11%, 11% i 9% vs 1.5% u DMSO kontrolama, $p<0.05$). Mitotički indeks je bio značajno smanjen pri koncentracijama od 4×10^{-3}, 4×10^{-4} ($p<0.01$) i 4×10^{-5} ($p<0.05$).

Kod ove životinjske vrste, ispitivani insekticid ima mutageni efekat u većim koncentracijama ali se one ne bi smele primenjivati u praksi.