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EVALUATION OF CHROMOSOMAL DAMAGE INDUCED BY THIACLOPRID-BASED INSECTICIDE IN BOVINE PERIPHERAL LYMPHOCYTES *IN VITRO*

Martina GALDÍKOVÁ, Katarína ŠIVIKOVÁ, Ján DIANOVSKÝ, Beáta HOLEČKOVÁ, Monika DRÁŽOVSKÁ

University of Veterinary Medicine and Pharmacy, Department of Biology and Genetics, Košice, Slovak Republic; Tel: +421 915 984 775; Fax: 055/6711674; Email: galdikova@uvm.sk

Abstract

Thiacloprid-based insecticide was evaluated for its ability to induce chromosome aberrations in cultured bovine peripheral lymphocytes. The cultures were treated with the insecticides at the concentrations ranged from 30 to 480 $\mu g.ml^{-1}$ for the last 24 and 48 h of incubation. Dose dependence in the increase of CAs was observed after exposure to thiacloprid formulation ranged from 120 to 480 $\mu g.\ ml^{-1}$. The highest concentration of the insecticide also reflected in reduction of mitotic index in donor 2. For detection of structural and numerical aberrations, three whole chromosome painting probes (WCPs) were used in our experiments. We observed numerical aberrations, but without statistical significance.

Introduction

Thiacloprid, a neurotoxic insecticide, belongs to the new and commercially very successful family of the neonicotinoids; relatively new class of synthetic organic insecticide, which are now widely used to control piercing and sucking insect pests around the world (Pandey et al., 2009). Thiacloprid is an acute contact and stomach poison, with systemic properties. Neonicotinoid insecticides act as agonists on the insect nicotinic acetylcholine receptors (nAChRs), which plays an important role, in synaptic transmission, in the central nervous system (Muccio et al., 2006).

Because of widespread application of thiacloprid, the assessment of its possible cytotoxic, genotoxic and/or carcinogenic potential effects is very important. Despite massive use, only manufactory's reports about its mutagenic potential are available. It has been described not to be mutagenic in *in vitro* bacterial mutation and *in vivo* cytogenetic mammalian assays (Pesticide residues in food, 2006).

We report here the cytogenetic effect of the thiacloprid-based insecticide (Calypso 480 SC) on the induction of chromosome aberrations (CA) in bovine peripheral lymphocytes *in vitro*. Chromosome aberrations are generally considered as biological endpoints to determine the level of genetic damage. Our interest was to detect also the frequency of stable aberrations, which do not result in the loss of chromosome material and it is assumed to be heritable. For this purpose, fluorescence in situ hybridization technique (FISH) was applied. In addition, changes in expression of bovine GSTA2 and GSTM3 after exposure to insecticide were investigated using real-time PCR method. Glutathione S-transferase (GST) family is considered as one of the most important detoxification enzymes groups (Isgor *et al*., 2010).

Keywords: thiaclopri-based insecticide, bovine peripheral lymphocytes, fluorescence *in situ* hybridization

Materials and Methods

Chemicals

The thiacloprid-based insecticide, (trade name Calypso 480 SC, with active agent N-{3-[(6-Chloro-3-pyridinyl)methyl]-1,3-thiazolan-2-yliden}cyanamide (Bayer AG, Germany), was solved in water and used in the experiments. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4µmol) and ethyl methanesulfonate (EMS, Sigma, St. Louis, MO, USA) at a concentration of 250 $\mu g.ml^{-1}$ were used as positive control agents.

Cell cultivation

0,5 ml of heparinised whole blood of 2 healthy young bulls (Slovak spotted cattle, 6-8 months old) was added to 5 ml of chromosome medium RPMI 1640 supplemented with L- glutamine, 15 µmol/L HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum (BOFES, Sigma, Chemical Co. St. Louis, MO, USA), antibiotics (penicillin 250 U/ml and streptomycin 250 µg. ml^{-1} and phytohaemagglutinin (PHA, 180 µg. ml^{-1}, Welcome, Dartford, England). Lymphocyte cultures were incubated at 37^{o}C for 72 h Lymphocyte cultures were treated with 30, 60, 120, 240 and 480 µg. $ml^{-1}$of thiacloprid insecticide for the last 24 and 48 h of the incubation. One and half hour before the end of cultivation, colchicine (Merck, Darmstadt, Germany) was added at the final concentration of 5 µg/ml. For the standard cytogenetic analysis the slides were stained with Giemsa solution. One hundred well-spread metaphases were analysed for the CA including chromatid, isochromatid breaks (CB, IB) and chromatid, isochromatid exchanges (CE, IE). Gaps (G) were examined separately. The mitotic index (MI) was calculated as the metaphase ratio of the total number of 3000 cells.

Fluorescence in situ hybridization

Orange-red labelled whole chromosome painting probes (WCPs), specific for the bovine chromosome 1 and 7, and green labelled WCP, specific for the bovine chromosomes 5 (Kubičková et al., 2002) were used for hybridization, simultaneously. The painting probe in hybridization mixture (50% formamide in 2xSSC, 10% dextran sulphate, salmon sperm DNA, competitor DNA) was denatured at 72˚C for 10 min and reannealed at 37˚C for 90 min. The denaturation of slides was performed in 70% formamide in 2xSSC (pH 7.0) at 72˚C for 2 min and followed by a dehydration procedure (70, 80, 90% ethanol, -20˚C and 96% ethanol, RT) for 2 min. After overnight hybridization at 37˚C, the slides were washed in 2xSSC (3-5 min, room temperature), in 0, 4 xSSC with 0, 3% Igepal at 72˚C for 2 min and again in 2xSSC at room temperature from 5 sec to 2 min and TNT (Tris-NaCl-Tween 20 buffer) (2 x 5 min, RT). The slides were counterstained in DAPI/Antifade (4´, 6´-diamidino-2-phenylindole, Q-BIOgene, UK). One thousand of well spread metaphases were analysed for each and all experimental cultures and controls. Aberrations were scored according to the PAINT nomenclature (Tucker *et al*., 1995). A fluorescent microscope NIKON Labophot 2A/2, equipped with a single (Texas Red and Fluorescein isothiocyanate - FITC) and dual band pass filters (FITC/Texas Red; DAPI/FITC) was used for visualisation of painting probes. Statistical analysis was performed using the χ^2 test for the estimation of CAs and MI in both experimental conditions.

Real time PCR

RNA was extracted using the Aurum™ Total RNA Mini Kit (BioRad, USA). cDNA was prepared by iScript™ cDNA Synthesis Kit (BioRad, USA). Real time PCR was performed using CFX96 Touch™ Real-Time PCR Detection System (BioRad). Relative expression values were calculated according to comparative threshold cycle method.

Results

The frequency of chromosomal aberrations induced by thiacloprid-based insecticide in bovine lymphocyte cultures can be seen in Tables 1 and 2. When compared with controls a weak higher percentage of chromosomal breaks were found after treatment with the fungicide for 24 h (Table 1). A dose dependence of CAs was found after treatment with the insecticide at concentrations ranging from 120 to 480 µg.ml^{-1} ($p<0.05$ or $p<0.01$). A slightly decrease in mitotic indices was seen in each donor, with statistical significance at the highest concentration of the insecticide in donor 2 ($p<0.05$). In the CA assay for 48 h, no statistically significant increases in the induction of chromosome aberrations were obtained (Table 2). After the exposure to the

Table 1.

Induction of CA in bovine lymphocytes exposed to several concentrations of commercial insecticide thiacloprid for 24h

Dose	Metaphase number	G	Types of CA				% Breaks (±SD)	% Aberrant cells (±SD)	% MI
			CB	IB	CE	IE			
Donor 1									
Control	100	5	1	–	–	–	1.0±0.10	1.0±0.10	2.6
	Concentrations of thiacloprid (μg.ml^{-1})								
30	100	7	3	1	-	-	4.0±0.20[a]	4.0±0.20[a]	2.2[a]
60	100	7	4	-	-	-	4.0±0.20[a]	3.0±0.17[a]	2.3[a]
120	100	7	3	4	-	–	7.0±0.25*	7.0±0.25*	1.9[a]
240	100	8	7	2	-	-	9.0±0.32**	8.0±0.27*	2.2[a]
480	100	8	8	2	-	-	10.0±0.30**	10.0±030**	1.5[a]
EMS (250μg. ml^{-1})	100	8	14	2	1	-	18.0±0.55***	12.0±0.38**	1.6[a]
Dose	Metaphase number	G	Types of CA				% Breaks (±SD)	% Aberrant cells (±SD)	% MI
			CB	IB	CE	IE			
Donor 2									
Control	100	5	2	–	–	–	2.0±0.14	2.0±0.14	2.5
	Concentrations of thiacloprid (μg.ml^{-1})								
30	100	8	4	1	-	-	5.0±0.22[a]	5.0±0.22[a]	2.0[a]
60	100	5	5	-	-	-	5.0±0.22[a]	5.0±0.22[a]	2.1[a]
120	100	5	8	1	-	-	9.0±0.29*	9.0±0.29*	1.8[a]
240	100	7	9	-	-	-	9.0±0.32*	8.0±0.29[a]	1.9[a]
480	100	13	7	5	-	-	12.0±0.38**	10.0±0.30**	1.3*
EMS (250μg. ml^{-1})	100	8	16	4	1	-	22.0±0.48***	19.0±0.40***	1.2*

a-statistically non significant data, *, *** -statistical significance ($p<0.05$, $p<0.001$, respectively: χ^2 test), CB, IB-chromatid, isochromatid breaks, CE, IE-chromatid, isochromatid exchanges

Table 2.

Induction of CA in bovine lymphocytes exposed to several concentrations of commercial insecticide thiacloprid for 48h

Dose	Metaphase number	G	Types of CA				% Breaks (±SD)	% Aberrant cells (±SD)	% MI
			CB	IB	CE	IE			
Donor 1									
Control	100	4	2	-	-	-	2.0±0.14	2.0±0.14	3.3
	Concentrations of thiacloprid (μg/ml)								
30	100	7	1	1	-	-	2.0±0.14[a]	2.0±0.14[a]	3.3[a]
60	100	7	1	1	-	-	2.0±0.14[a]	2.0±0.14[a]	3.2[a]
120	100	9	2	1	-	-	3.0±0.17[a]	3.0±0.17[a]	2.9[a]
240	100	5	2	2	-	-	4.0±0.20[a]	4.0±0.20[a]	1.9*
480	100	4	-	3	-	-	3.0±0.17[a]	3.0±0.17[a]	1.1***
MMC (4μM)	100	8	19	4	-	-	23.0±0.54***	18.0±0.42***	1.0***
Dose	Metaphase number	G	Types of CA				% Breaks (±SD)	% Aberrant cells (±SD)	% MI
			CB	IB	CE	IE			
Donor 2									
Control	100	4	2	-	-		2.0±0.14	2.0±0.14	3.1
	Concentrations of thiacloprid (μg/ml)								
30	100	8	2	-	-	-	2.0±0.14[a]	2.0±0.14[a]	2.9[a]
60	100	9	1	1	-	-	2.0±0.14[a]	2.0±0.14[a]	2.7[a]
120	100	4	4	1	-	-	5.0±0.22[a]	5.0±0.22[a]	2.5[a]
240	100	4	3	3	-	-	6.0±0.24[a]	6.0±0.24[a]	1.7*
480	100	4	3	-	-	-	3.0±0.17[a]	3.0±0.17[a]	1.0***
MMC (4μM)	100	4	15	1	2		20.0±0.51***	16.0±0.40***	0.9***

a-statistically non significant data, *, *** -statistical significance ($p<0.05$, $p<0.001$, respectively: χ^2 test), CB, IB-chromatid, isochromatid breaks, CE, IE-chromatid, isochromatid exchanges

insecticide at concentration of 240 and 480 μg.ml^{-1}, an apparent or total inhibition in mitotic activity was shown ($p<0.05$ and $p<0.001$, respectively).

On the basis of the results of standard chromosomal analysis, the lowest concentration (30 μg.ml^{-}1) of the insecticide was chosen for the investigation of stable structural and the numerical aberrations using FISH technique. The results after exposure of bovine peripheral lymphocytes to thiacloprid-based insecticide are shown in Table 3. Under condition of our experiment no translocation were detected. We have observed numerical aberrations, aneuploidies and polyploidies, without statistical significance.

Table 3.

The frequency of CA in bovine lymphocytes exposed to thiacloprid-based insecticide evaluated by WCP *in vitro*

			Numerical aberrations					Structural aberrations				
	Dose	No.	Aneuploidy				Polyploidy (4n)	CB	IB	CE	IE	Total %
			Total	1	5	7						
Control		1000	5	1	2	2	4	_	_	_	_	0,0±0,0
Calypso 480SC	30 $\mu g.ml^{-1}$	1000	9	5	3	1	6	4	3	-	-	7,0±0.08**
EMS	250 $\mu g.ml^{-1}$	1000	15	2	8	5	9 [a]	16	1	1		19±0,14***

a - statistically non significant data, **, *** statistical significance ($p<0,01$, $p<0,001$, respectively: χ^2 test

Discussion

In our study, the cytogenetic activity of thiacloprid-based insecticide *in vitro* was investigated using bovine peripheral lymphocytes. Under condition of our experiments, slight concentration dependence was observed in relation to induction of chromosomal aberrations for 24 h treatment. The highest concentration of the insecticide tested (480 $\mu g.ml^{-1}$) caused a weak significant inhibition of mitotic activity in donor 2 ($p<0.05$), reflected in the lower values of mitotic index in comparison with the control value. No statistically significant increase in CAs induction was observed after prolonged time of exposure (48 h). When compared with the controls the highest thiacloprid dose (480 $\mu g.ml^{-1}$) induced a significant decrease in the mitotic ability in both donor cultures. Thus, the cytotoxic effect of the insecticide was observed. Using WCP, only low levels of numerical aberrations, without statistical significance were found. No stable aberrations, such as translocations and insertions were detected. It is likely that the use of three WCP provides relatively low proportion of the painted bovine genome; thus detection of the total number of stable aberrations was not possible.

The activity of some detoxification enzymes does not only depend on the genotype; it changes as a consequence of exposure to chemicals or by many dietary components that could either induce or inhibit their enzyme activity (Lampe et al., 2000). In our study, we have investigated changes in expression of two GST genes in association with exposure to thiacloprid. Our data suggest that GSTM3 gene was underexpressed in lymphocytes treated with insecticide. GSTA2 displayed very low or udentedectable expression levels in cultured lymphocytes (unpublished data).

Thiacloprid has been classified by WHO (1994) as moderately hazardous. The EPA (2006) identified this chemical as “likely to be carcinogenic to humans” based on the occurrence of thyroid tumors in male rats, and uterine and ovarian tumors in rats and mice, respectively However there are few data in the literature on clastogenic and genotoxic effects of thiacloprid. No statistically significant increase in the number of cell with chromosome aberrations was observed in Chinese hamster V79 cells after *in vitro* exposure to thiacloprid with purity 96.8-97.2%. Cytotoxicity was described at the highest concentration (750 $\mu g.ml^{-1}$) (Pesticide residues in food, 2006). In contrast to these findings, Kocaman et al. (2012) reported significantly increase in the CA, SCE and MN (micronuclei) frequencies in the human peripheral lymphocytes treated with thiacloprid. Thiacloprid also caused reduction in the mitotic, proliferation and nuclear division indices. Cytogenetic effects of commercial formulations of deltametrin and/or thiacloprid in rat bone marrow cells were documented by

Sekeroglu et al. (2011). They found significantly increase in the CA and MN frequencies. Other authors (Calderón-Segura et al., 2012) have investigated neonicotinoid insecticides (thiacloprid, clothianidin and imidacloprid) *in vitro* in human peripheral lymphocytes using comet assay. They have demonstrated that commercial formulations, Jade, Gaucho, Calypso and Poncho directly induce DNA damage in a concentration-dependent manner.

In conclusion, our results indicated ability of thiacloprid formulation to induce clastogenic/genotoxic and/or cytotoxic effects in bovine peripheral lymphocytes.

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