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Identification of chromosomes and aberrations after exposure to pesticide in bovine lymphocytes

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ABSTRACT

Tebuconazole belongs to the group of triazole fungicides. As a systemic foliar fungicide it was widely used in agriculture for crops protection, such as barley, wheat, peanuts and orchard fruits. Associations between exposure to pesticides and health outcomes in humans, including different kinds of cancer were reported by several authors. Effects in immune, hematological, nervous, endocrine and reproductive systems have also been described. Data about genotoxic effects of pesticides are rarely reported. We are interested in the detection of structural and numerical aberrations in bovine peripheral lymphocytes using fluorescence *in situ* hybridization (FISH) *in vitro*. Three whole chromosome painting probes (WCPs), BTA1 for the bovine chromosome 1 (red colour), BTA 5 for chromosome 5 (green colour) and BTA 7 for chromosome 7 (red colour), as well, were used in experiments. Aneuploidy and unstable structural aberrations were detected more precisely than those seen in conventional chromosome aberrations assays, but only on the marked chromosomes could be analysed.

Key words: tebuconazole-based fungicide, bovine peripheral lymphocytes, whole chromosome painting probes, fluorescence *in situ* hybridization

INTRODUCTION

Pesticides are among the most widely used chemicals throughout the world. They include a great variety of substances different both in composition and properties with the purpose to kill, destroy or repel undesirable living organisms.

Triazole fungicides are used agriculturally to control rust and mildew on fruit, vegetables, cereals and seeds, residential and commercial turf, and in pharmaceutical applications for the treatment of local and systemic fungal infections (Goetz *et al*., 2007). The fungicidal mode of action of triazoles involves disruption of fungal cell membranes and walls by the mechanism of inhibiting fungal lanosterol-14a-demethylase, a xenobiotic metabolizing enzyme in fungi that has a homolog in mammals known as CYP51 (Georgopapadakou, 1998; Ghannoum and Rice, 1999), which is evolutionarily conserved between plants, fungi, and animals. This enzyme is critical component for cholesterol synthesis and therefore steroid biosynthesis in animals (Zarn *et al*., 2003). Besides CYP51, triazoles also modulate the gene expression and enzyme activity of multiple cytochrome P450 (CYP) and other metabolic enzymes in mammalian liver and other tissues (Barton *et al*., 2006; Goetz *et al*., 2006; Ronis *et al*., 1994; Sun *et al*., 2005, 2006; Tully *et al*., 2006). In spite of the massive use of these pesticides, only few developmental toxicological studies of azole fungicides have been published. As far as we know, there is no information dealing with the clastogenic or genotoxic effects of this agent on human or animal cells.

We report here the cytogenetic effect of the fungicide tebuconazole (ORIUS 25

W) on the induction of chromosome aberrations (CA) in bovine peripheral lymphocytes *in vitro*. Our interest was directed towards the detection of 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, we have use fluorescence *in situ* hybridization technique.

MATERIAL AND METHODS

The tebuconazole-based fungicide, trade name Orius α- terc.butyl - α – (4-chlorophenylethyl) -1 H -1,2,4 - triazolyl-ethanol, 25% of active agent was used in the experiments.

Lymphocyte cultures were prepared by adding 0.5 ml of heparinized whole blood from healthy donor of cattle to 5 ml of chromosome medium RPMI 1640 supplemented with L- glutamine, 15 $\mu mol.l^{-1}$ 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 $\mu g.ml^{-1}$ and phytohaemagglutinin (PHA, 180 $\mu g\ ml^{-1}$, Welcome, Dartford, England). Lymphocyte cultures were incubated at 37°C for 72 h. Lymphocyte cultures were treated with 3, 6, 15, 30 and 60 $\mu g.ml^{-1}$ of tebuconazole fungicide for the last 24 hours. Ethylmethane sulfonate (EMS, Sigma, St. Luois, MO, USA, 250 $\mu g.ml^{-1}$) was used as the positive control agent. One and half hour before the end of cultivation, colchicine (Merck, Darmstadt, Germany) was added at the final concentration of 5 $\mu g.ml^{-1}$. 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 ratio metaphases of the total number of 2000 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 were used for hybridization, simultaneously. The painting probe in hybridization mixture (50% formamid 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 for 5 sec- 2 min. The slides were counterstained in DAPI/Antifade (4´, 6´-diamidino-2-phenylindole, Q-BIOgene, UK). Aberrations were scored according to PAINT nomenclature (Tucker *et al*., 1995). A fluorescent microscope NIKON Labophot 2A/2, equipped with dual band pass filter FITC/TRITC, was used for probe visualization.

Statistical analysis for the CA and MI for standard and fluorescence chromosomal analysis was performed using χ^2 test.

RESULTS

The frequency of chromosomal aberrations induced by tebuconazole in bovine lymphocyte cultures is shown in Table 1.

Dose	Metaphase number	G	Types of CA				% Breaks (±SD)	% MI
			CB	IB	CE	IE		
DMSO (control)	100	4	2	–	–	–	2.0±0.14	2.3
Concentration (μg/ml)								
3	100	6	5	1	–	–	6.0±0.24a	2.2a
6	100	12	7	1	1	–	10.0±0.33*	2.0a
15	100	12	10	2	2		16.0±0.42***	1.9a
30	100	11	8	2	1		12.0±0.35**	1.2*
60	31	3	1	–	–	–	3.2±0.17a,c	0.6***
EMS (250μg/ml)	100	15	20	5	3	–	31.0±0.70**	1,5*

Table 1: Induction of CAs in bovine peripheral lymphocytes exposed to tebuconazole-based fungicide for 24 h

a - statistically non significant data *, **, *** statistical significance ($p<0,05$, $p<0,01$, $p<0,001$, respectively: χ^2 test)

CB, IB - chromatid, isochromatid breaks, CE, IE – chromatid, isochromatid exchanges

Statistically significant elevations in the mean of chromosomal aberrations (CAs) was observed after 24 h exposure to the tebuconazole-based fungicide at the concentrations ranged from 6 to 30 $\mu g.ml^{-1}$ ($p<0.05$ or $p<0.01$ and $p<0.001$, respectively by χ^2 test). The highest concentration tested (60 $\mu g.ml^{-1}$) caused a significant inhibition of mitotic activity, and thus only insufficient number of metaphases could be analysed.

On the basis of the results of standard chromosomal analysis, a concentration of $6\mu g.ml^{-1}$ was chosen for the investigation of stable chromosomal aberrations using FISH technique. One thousand of metaphases were analysed for both the cultures - control and experimental. The frequencies of chromosome aberrations after using of FISH technique are summarised in Table 2. Monosomy and trisomy of bovine chromosome 5 were the most common type of numerical aberrations. Besides aneuplodies, several cells with polyploidy were detected. Under conditions of our experiments none reciprocal translocations were found.

Table 2: The frequency of chromosome aberrations in bovine peripheral lymphocytes exposed to tebuconazole-based fungicide 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
ORIUS 25 W	6 $\mu g.ml^{-1}$	1000	29	5	13	11	7 [a]	5	-	2	-	0,9±0,11**
EMS Positive control	250 $\mu g.ml^{-1}$	1000	15	2	8	5	9 [a]	16	1	1		1,9±0,14***

a - statistically non significant data

, * statistical significance ($p<0,01$, $p<0,001$, respectively: χ^2 test)

DISCUSSION

It is well-known from the literature that several among the investigated triazole fungicides influence the activity of various cytochrome P450 enzymes. One example is their inhibition of the activity of aromatase (CYP19) that converts androgens to estrogens (Sanderson *et al*., 2002; Vinggaard *et al*., 2000). Azole fungicides have the ability to act through multiple mechanisms and to induce various endocrine-disrupting effects (Vinggaard *et al*., 2005). Autors Taxvig *et al*. (2007) have reported that the predominant effect of *in utero* tebuconazole exposure in male offspring is a feminization and in female fetuses virilising effect. This virilising effect on female offspring is suggested to be a result of the increased progesterone levels in the dams (Willingham *et al*., 2006).

One developmental toxicity study in vivo showed that exposure of pregnant rats to tebuconazole at dose levels around the lowest observed effect level (U.S. EPA, 1999) resulted in disturbance of the reproductive system in the offspring. The males had reduced weight of epididymides, and females had reduced uterus weight after peri-and postnatal exposure (Moser *et al*., 2001). This study has shown that perinatal exposure to tebuconazole produced neurobehavioral deficits and neuropathology in rats, but did not alter immunological or reproductive function.

Numerous studies have been conducted on the genotoxicity of tebuconazole. No genotoxic activity was found in any study. Tebuconazole has low acute toxicity and has been classified by WHO (1994) as unlikely to present an acute hazard in normal use (slightly hazardous).

Our interest was directed towards the detection of stable aberrations, which do not result in the loss of chromosome material and which should be therefore heritable. Under conditions of our experiment no stable aberrations such as translocations or inzertions were observed. It is probably caused by the relatively low proportion of the painted bovine genome as well as insufficient numbers of analysed metaphases for the detection of stable aberrations.

We have predominantly observed numerical aberrations, aneuploidies and also polyploidies. It is widely recognised, that aneuploidy is an integral component in the development of human tumours and the acquirement of malignancy (Duesberg *et al*., 2000).

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