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EFFECT OF N-METYLCARBAMATE PESTICIDE BENDIOCARB ON CATTLE LYMPHOCYTES AFTER *IN VITRO* EXPOSURE

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Bendiocarb is a carbamate broad-spectrum insecticide used to control disease vectors such as mosquitoes and flies, as well as household and agricultural pests. Nowadays, only few papers reporting cytogenetic or possible genotoxic effect of this insecticide on mammalian cells are available. In the present study 24-hour exposure to bendiocarbamate at concentrations ranging from 20 to 160 μg/ml was used for investigation of unstable chromosomal aberrations (CA), sister chromatid exchanges (SCE) and stable chromosomal aberration induction in cultured bovine peripheral lymphocytes. The slight but no significant increase of chromatide breaks frequency was observed after the exposure of lymphocytes to 80 μg/ml of bendiocarb. At the highest concentration added to the cell cultures (160 μg/ml) mitotic index decrease was shown in both donors ($p < 0.05$; $p < 0.01$). Both statistically significant elevation of SCEs ($p < 0.05$) and a reduction of proliferative indices (PI) ($p < 0.01$) were shown at a dose of 80 μg/ml. By means of two fluorescent-labelled whole chromosome-painting probes, stable aberrations such as bovine chromosome 1 and 5 translocation as well as numerical aberrations (polyploidies, heteroploidies) were visualised under fluorescent microscope in some examined metaphases.

Keywords: Carbamate pesticides – bovine lymphocytes – chromosomal aberrations – sister chromatide exchanges – fluorescence *in situ* hybridisation

INTRODUCTION

Pesticides are a group of chemicals with high biological activity that are worldwide introduced into the environment and expose large populations of living organisms. Pesticide exposure is recognized as an important environmental risk factor associated with an increase in biomonitoring indices of genotoxicity [4] and with cancer development in humans [9].

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The large proportion of pesticides widely used for crop protection in agriculture is included in carbamate insecticides (N-methylcarbamates, NMCs). The NMCs residues may appear in fruits, vegetables, ground and surface waters and pose a potential hazard for consumers [16]. Carbamates are not only the reversible inhibitors of an essential nervous system enzyme-acetylcholine esterase [20], but according to Klotz et al. [10] they may act as general endocrine modulators in mammalian cells. N-methylcarbamates were preferred for their relatively short persistence in the environment, but under the conditions of mild acidity and suitable temperature (37 °C), NMCs are converted to N-nitrosometabolites, which are supposed to be carcinogenic and mutagenic.

Many authors have examined genotoxicity of N-methylcarbamates particularly in humans, but often with contradictory results. In animal study, Bueno et al. [3] revealed that carbamate fungicide benomyl induced aneuploidy and polyploidy in wild rodents living in pesticide exposed areas. Earlier experiments with Chinese hamster V79 cell lines showed that N-methylcarbamate insecticides and their N-nitroso derivatives have the potential to act through mediation of epigenetic and genotoxic mechanisms in the multiple stages of chemical carcinogens [23].

In addition to the most common N-methylcarbamates, such as carbaryl, promecarb and proxopur, a broad-spectrum insecticide bendiocarb is also included in this group, which is widely used in some countries to control disease vectors (mosquitoes, flies) [2, 13–14, 24] as well as pests in households and agriculture. At present, only limited data are available in relation to the cytogenetic effect of bendiocarb on human and animal cells, particularly that of grazing animals.

In this paper, the potential cytogenetic damage after bendiocarb exposure of cultivated bovine peripheral lymphocytes was investigated by means of cytogenetic endpoints such as Giemsa stained chromosome aberrations (CA), sister chromatid exchanges (SCE) as well as chromosome aberrations detected by fluorescent *in situ* hybridisation technique using whole chromosome painting probes (FISH-WCP). The effects on cell cycle was also examined by mitotic and proliferation indices.

MATERIALS AND METHODS

Cultivation of lymphocytes

The whole blood specimens from two clinically healthy bull donors (Slovak spotted cattle, 6–8-month old) were cultivated for 72 h at 38 °C in 5 ml of RPMI 1640 medium supplemented with L-glutamine and 15 mmol/l HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum (Sigma, Chemical Co. St. Louis, MO, USA), antibiotics (penicillin 250 U/ml and streptomycin 250 μg/ml) and phytohaemagglutinin (PHA, 180 μg/ml Welcome, Dartford, England).

Bendiocarbamate (CAS 22781-23-3, chemical name 2,3-isopropylidenedioxyphenyl methyl carbamate) was added to the lymphocyte cultures at concentrations of 20, 40, 80 and 160 μg/ml for the last 24 hours. The doses were chosen refer-

ring to the highest dose causing a reduction in the mitotic index (MI) of more than 50%. Ethylmethane sulfonate (EMS, Sigma, St. Luois, MO, USA, 250 μg/ml) and mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 mmol/l) were used as the positive control agents.

For the SCE assay and the cell cycle kinetics, bromodeoxyuridine (8 μg/ml, BrdUrd, Sigma, St. Louis, MO, USA) was added to all cultures 24 h after initiation of division.

Chromosomal aberrations and sister chromatid exchange analysis

For CA, the standard cytogenetic method was used to prepare slides with Giemsa stained chromosomes. One hundred well-spread metaphases per donor and concentration were analysed for 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 between number of cells in mitosis and the total number of 2000 cells.

SCE analysis was performed using FPG staining technique to differentiate sister chromatids and cell cycles [17]; fifty differentially stained metaphases were examined per donor and concentration. One hundred metaphases were analysed for the determination of M_1, M_2 and M_3 mitotic divisions. The proliferation index (PI) was calculated according to Lamberti et al. [11].

Fluorescence in situ *hybridisation*

For fluorescence *in situ* hybridisation analysis (FISH) green and orange-labelled whole chromosome painting probes, specific for the bovine chromosomes 1 and 5 (prepared in Veterinary Research Institute, Brno, Czech Republic) were used. The painting probes in hybridisation mixture (50% formamide, 2×SSC, 10% dextran sulphate, salmon sperm DNA, competitor DNA) were denatured at 72 °C for 10 min and reannealed at 37 °C for 80 min. The denaturation of slides was performed in 70% formamide, 2×SSC (pH 7.0) at 72 °C for 2 min and following by dehydration procedure (70, 90, 96% ethanol, –20 °C). After overnight hybridisation at 37 °C, the slides were washed in 50% formamide, 2×SSC (pH 7.0) at 42 °C, in 0.1×SSC (pH 7.0) at 42 °C and in TNT (Tris-NaCl-Tween 20 buffer, pH 7.0) at 42 °C. The slides were counterstained in DAPI/Antifade (4′,6′-diamino-2-fenolindol, Q-BIOgene, Middlesex, UK). Fluorescent microscope Nikon Labophot 2A/2, equipped with dual band pass filter FITC/TRITC was used for probe visualisation. A 250 metaphases were scored per donor and concentration. Chromosome aberrations were described according to PAINT nomenclature [21] and recorded by means of Nikon digital camera (Coolpix 4500, Nikon).

Statistical evaluation

The analysis of variance (ANOVA) and the Student's *t*-test were applied to compare SCE occurrence between treated and untreated groups. The induction of CA, reduction of MI, the cell cycle delay and the statistical analysis of FISH results were performed using χ^2 test.

RESULTS

The results of chromosome aberrations analysis in bovine peripheral lymphocytes after 24 h exposure to bendiocarb are shown in Table 1. A small elevation of induced chromosome damage (chromatid breaks, CBs) was shown in cells in relation to the increasing concentrations ranged from 20 to 80 μg/ml in each donor. However, no statistically significant dose-dependence proving the clastogenic effect of bendiocarb was observed. The highest bendiocarb concentration tested (160 μg/ml) caused a significant inhibition of mitotic activity in both donors ($p < 0.05$, $p < 0.01$, respectively),

Table 1
Induction of CAs in bovine peripheral lymphocytes exposed to bendiocarb for 24 h

Dose	Types of chromosomal aberrations					% breaks (±SD)	% MI
	G	CB	IB	CE	IE		
Donor 1			Treatment for 24 h				
Control	2	1	–	–	–	1.0 ± 0.10	2.8
Bendiocarb (μg/ml)							
20 μg/ml	4	1	–	–	–	1.0 ± 0.10[a]	2.9[a]
40 μg/ml	4	2	–	–	–	2.0 ± 0.14[a]	2.9[a]
80 μg/ml	7	5	–	–	–	5.0 ± 0.22[a]	2.8[a]
160 μg/ml	3	3	–	–	–	4.0 ± 0.20[a,b]	1.3*
250 μg/ml, EMS	14	12	–	5	2	26.0 ± 0.64***	1.0**
Donor 2			Treatment for 24 h				
Control	5	2	–	–	–	2.0 ± 0.14	3.0
Bendiocarb (μg/ml)							
20 μg/ml	3	3	–	–	–	3.0 ± 0.17[a]	2.7[a]
40 μg/ml	5	3	–	–	–	3.0 ± 0.17[a]	2.6[a]
80 μg/ml	9	6	–	–	–	6.0 ± 0.24[a]	2.0[a]
160 μg/ml	4	4	–	–	–	6.7 ± 0.25[a,c]	1.2**
250 μg/ml, EMS	11	14	2	9	1	36.0 ± 0.97***	0.8***

A total of 100 well-spread metaphases of each concentration was determined, if it was possible.
*, **, *** Statistical significance ($p < 0.05$, $p < 0.01$, $p < 0.001$, $\chi 2$ test).
[a] No statistically significant data.
[b,c] Insufficient number of cells (b75, c60 analysed metaphases).
CB, IB – chromatid, isochromatid break; CE, IE – chromatid, isochromatid exchange; G – gaps, not included into statistic data.

Table 2
Frequency of SCEs and proliferation indices in cultured peripheral lymphocytes exposed to the insecticide bendiocarb for 24 h

Dose	SCE/cell	PI
Donor 1	Treatment for 24 h	
Control (DMSO)	6.40 ± 2.4	2.01
Insecticide (μg/ml) 24 h		
20	6.86 ± 2.91[a]	1.95[a]
40	7.18 ± 3.78[a]	1.96[a]
80	7.60 ± 2.68*	1.52**
160	ND	ND
Positive control, 0.4 μM MMC	9.64 ± 3.03***	1.85[a]
Donor 2		
Control (DMSO)	6.34 ± 1.98	2.06
Insecticide (μg/ml) 24 h		
20	6.80 ± 2.55[a]	1.98[a]
40	7.12 ± 3.07[a]	1.92[a]
80	7.56 ± 3.79*	1.60**
160	ND	ND
Positive control 0.4 μM, MMC	9.44 ± 3.06***	1.90[a]

A total of 50 second-division metaphases of each group were analysed for SCE, if it was possible.

*, **, *** Statistical significant data ($p < 0.05$, $p < 0.01$, $p < 0.001$, ANOVA, Student's *t*-test).

[a] No statistical significance; ND – not done.

reflected in the lower values of mitotic indices in comparison with control cultures. Thus only insufficient number of metaphases could be analysed to detect chromosome aberrations after treatment with the highest insecticide concentration.

Some positive results were recorded by investigation of the effect of bendiocarb on SCE induction. The evaluation of SCE frequencies revealed a statistically significant elevation of SCEs at a dose of 80 μg/ml ($p < 0.05$) in each donor when compared to the controls (Table 2). At the same concentration treatment, the significant reduction of the proliferation indices ($p < 0.01$) was determined in cultured cattle cells of both donors.

On the basis of the results of CA and SCE, two concentrations of insecticide (40 and 80 μg/ml) were chosen and added to the cell cultures to examine the stable chromosomal aberrations by FISH-WCP. In donor 1, one translocation between chromosome 5 and other non-painted chromosomes were observed at both concentrations used. In donor 2, only one translocation between bovine chromosome 1 (green labelled) and 5 (red labelled) was visualised by means of DNA probes at a concentration of 80 μg/ml in 250 cells analysed.

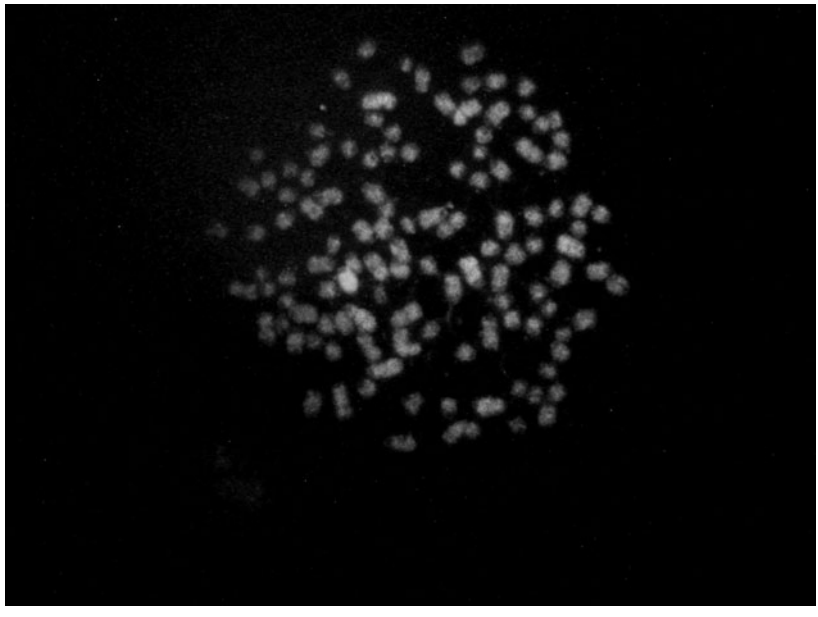

Fig. 1. Heteroploid bovine lymphocyte (4n) with seven green BTA1 and one red BTA 5 chromosomes fluorescently labelled with whole chromosome painting probes

Numerical aberrations such as polyploidies (mostly tetraploidies, 4n) were also detected after higher bendiocarb exposure. In addition to typical tetraploidies with four green painted chromosomes 1 (BTA1) and four red painted chromosomes 5 (BTA5), heteroploid cells were visualised under the fluorescent microscope. A typical picture was the metaphase plate with seven green chromosomes 1 and one red chromosome 5 (Fig. 1). However, the increased level of both translocations and numerical aberrations in exposed bovine lymphocyte cultures did not reach the statistically significant values.

DISCUSSION

In predominantly agricultural states as well as in the countries with the high incidence of insect disease vectors, exposure to pesticides is a common everyday occurrence. The experimental results show that a wide range of pesticides, including carbamate insecticides, induce genotoxic effects on different genetic end-points. As far as carbamate insecticides are concerned, it was shown that while some carbamates had no cytogenetic effect either in mitosis or meiosis [22], other group of carbamates include the genotoxic agent and a potential germ cell mutagen [8]. In our study, a cytogenetic and possible genotoxic potential of a broad-spectrum N-methylcarbamate insecticide bendiocarb was examined. Bendiocarb can be expected to move to

surface water through runoff [5], and despite its relatively short persistence in water and soil, adverse influence on non-target animals such as birds, fish, grazing animals and ruminants cannot be definitely excluded. Only few data have still been published concerning induction of chromosomal aberrations and sister chromatid exchanges in animal cells exposed to the insecticide. Piešová and Valočíková [18] recorded that *in vivo* exposure to bendiocarbamate did not cause a significant increase in the frequencies of micronuclei in rabbit bone marrow erythrocytes. Interestingly, decrease of bone marrow proliferation was evident in reduction of polychromatic erythrocytes. In our experiment, no significant concentration dependence was observed in relation to induction of chromosomal aberrations after treatment of cultured bovine lymphocytes with bendiocarb. Twenty-four-h application time was used, which is in general recommended in the guidelines as the basic period for the standard chromosome aberration assay *in vitro.* In aqueous solution with pH 7.0 half life of bendiocarb is 81 hours [5]. Therefore we suppose that the possible effect of bendiocarb degradation product in culture medium (pH 7.2) was eliminated during 24 h of insecticide treatment. Only chromatid type of aberrations was detected probably indicating the indirect mode of action of the insecticide. As reported by Natarajan [15] chemical mutagens which do not induce directly DNA strand breaks but cause other lesions, were shown to induce only chromatid type aberrations (irrespective of the DNA synthetic stage treated). The highest bendiocarb dose used (160 μg/ml) induced statistically significant decrease in the mitotic ability of both donor cultures when compared with controls. This suggests cytotoxic effect of the agent at this concentration treatment reflecting in the reduction of MI (>50%). However, as emphasised by Fellows and O'Donovan [6] for a variety of agents with different mode of actions, cytotoxicity varies considerably depending on the method used to estimate it. The authors claim that MI grossly underestimates cytotoxicity when compared with some other methods.

Our results are similar to those of presented by EPA [5]; in the absence of S9 activation there was no indication of clastogenicity at 143 μg/ml, which was a dose level, considered slightly below an excessively cytotoxic dose 170 μg/ml. In relation to the effect of bendiocarb on acetylcholine esterase activity *in vitro,* Smulders et al. [20] recorded that the insecticide inhibited this rat brain enzyme with 1 μmol (223 μg/ml) IC_{50} value. This dose is similar to the highest one used in our experiment (160 μg/ml) causing a significant inhibition of lymphocyte mitotic activity.

In SCE assay, which is the most widely used measure of exposure/dose [1], elevations of sister chromatid exchanges occurred at a dose of 80 μg/ml ($p < 0.05$). In addition, the significant reduction of proliferation indices were found at the same dose ($p < 0.01$) indicating the effect of bendiocarb on the cell cycle delay.

Chromosome translocations are the stable aberrations of choice for evaluation of many types of exposure as it is well known that stable chromosomal changes have been associated with different types of human cancer. Under conditions of our experiment, two types of translocations were visualised by means of fluorescently labelled probes: translocations between chromosome 5 and other non-labelled chromosomes as well a translocation of chromosome 1 and chromosome 5. These findings suggest

that the insecticide treatment probably had no potential to induce a sufficient number of breaks following the higher incidence of translocations in treated cells. Besides structural changes, numerical aberrations such as tetraploid and heteroploid lymphocytes with colour painted chromosomes 1 and 5 were distinguished by FISH WCP; at present it is assumed that one proposed route to aneuploid cancer is through unstable tetraploid intermediate [7].

Carbamate insecticides are broad spectrum pesticides that comprise the major proportion of agricultural pesticides used in today's agricultural industry [19]. Linn et al. [12] presented that N-methylcarbamate insecticides significantly inhibited gap-junctional intercellular communication, revealing their potential as non-genotoxic carcinogens. According to these authors, the importance of further studies of the above insecticides, especially the study of their genetic toxicology, is evident.

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