CHROMOSOME DAMAGE IN CULTURED BOVINE PERIPHERAL LYMPHOCYTES INDUCED BY HERBICIDE CHLORIDAZON

K. ŠIVIKOVÁ, J. DIANOVSKÝ, E. PIEŠOVÁ

Department of Veterinary Genetics, University of Veterinary Medicine,
Košice, Slovak Republic

Abstract

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Herbicide chloridazon was tested for its ability to induce micronuclei (MN), chromosome aberrations (CA) and sister chromatid exchanges (SCE) as well as for its influence on cell cycle kinetics in cultured bovine peripheral lymphocytes. To detect possible metabolic modifications in herbicide genotoxicity, the cultures for the SCE determination were also treated with S9 fraction.

Significant elevations of CA and MN were found in cultures treated with chloridazon at a dose of 7×10^{-4} mol·l⁻¹ ($P < 0.05$ or $P < 0.01$, respectively) but no increase of these endpoints were seen with both lower doses (7×10^{-5} and 7×10^{-6} mol·l⁻¹). Clear dose dependence and statistically significant increases in induced chromosome damage were observed in the SCE assay without use of S9 fraction. The highest concentration tested caused also a reduction of the proliferation index and a weak proliferation delay.

These positive results provide information about the possible genetic risk increase for humans through the food chain.

Chloridazon, bovine peripheral lymphocytes, sister chromatid exchanges, chromosome aberrations, micronuclei

In recent years pesticide toxicity has been extensively investigated. The reason for this is not only their important role in agriculture but especially findings that certain pesticides showed carcinogenic and mutagenic properties in laboratory animals and exposed humans (Dolara et al. 1992; Hrelia et al. 1996; Woo et al. 1996).

Among pesticides, herbicides are widespread in the environment because most of them have been usually applied directly into the soil. Chloridazon is a herbicide currently used for selective control in beets. It belongs to the class of Hill reaction inhibitors (Cremlyn 1978; Corbett et al. 1984). Several reports deal with the genotoxic effects of commonly used herbicides, e. g. alachlor, trifluralin and maleic hydrazide (Ribas et al. 1996ab) but no evidence of cytogenetic or genotoxic effects of chloridazon has been shown.

Previously we reported our results from cytogenetic studies with chloridazon treated sheep blood lymphocytes (Šiviková and Dianovský 1997). We confirmed the sensitivity of sheep lymphocytes to chloridazon exposure in the sister chromatid exchange assay.

To obtain additional data and to extend the information on the genotoxicity of chloridazon we report here our results on the induction of chromosomal aberrations (CA), micronuclei (MN) and sister chromatid exchanges (SCE) in cultured bovine peripheral lymphocytes. There are mentions that cattle is a very sensitive species of domestic animals with regard to exposure and accumulation of chemical agents (Hadač 1989). Ruminants can be exposed to various environmental mutagens via pasture. Thus they represent a suitable model for evaluating the genotoxicity of environmental pollutants with predictive value for human health risk.

Address for correspondence:

RNDr. Katarína Šiviková, CSc.
Department of Veterinary Genetics,
University of Veterinary Medicine,
Komenského 73, 041 81 Košice, Slovak Republic

Phone: +421 95 63 321 11, (ext. 482)
Fax: +421 95 63 236 66
E-mail: sivikova@uvm.sk
http://www.vfu.cz/acta-vet/actavet.htm

Materials and Methods

Chloridazon (5-amino-4-chloro-2-phenylpyridazin-3 (2H)-one, Istrochem, Slovak Republic, 92,6% purity) was soluble in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and freshly prepared at concentrations of $7x10^{-6}$, $7x10^{-5}$ and $7x10^{-4}$ mol·l^{-1} before each experiment. The final DMSO concentration in treated and control cultures was 0.05%.

Mitomycin C (MMC, Sigma, St. Louis, MO, USA), ethylmethanesulfonate (EMS, Sigma, St. Louis, MO, USA) and cyclophosphamide (CPA, Jenapharm, Ankerwerk, Rudolstadt, Germany) were used as positive control agents in the absence and in the presence of the metabolic activation (S9 mix), respectively.

Experiments were carried out with two healthy cow donors (Slovak spotted cattle, 3-months-old). For SCE assays, experiments were made both in the presence and in the absence of S9 mix. A freshly prepared S9 fraction (10% of the culture volume) from Aroclor 1254 (Supelco, Bellefonte, PA, USA) induced male mice was prepared according to the method of Maron and Ames (1983) and used in control and experimental cultures with glucose-6-phosphate cofactors (Aldrich, Milwaukee, WI, USA) and NADP (Sigma, St. Louis, MO, USA).

Cell cultivation

Whole blood cultures (0.5 ml) were cultivated for 72 h at 38 °C in 10 ml of RPMI 1640 medium supplemented with 15% fetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250 m g/ml), and phytohaemagglutinin (PHA, 180 m g/ml, Welcome, Darford, England).

Chromosome preparations for CA and SCE assays were obtained by the standard cytogenetic method; 2 h before harvest colchicine (Merck, Darmstadt, Germany) was added at a concentration of 5 m g/ml. The lymphocyte cultures without S9 fraction were exposed to chloridazon for the last 24 h of cultivation, while cultures with S9 were treated with herbicide for 1.5 - 2 h, then the cells were removed and replaced in fresh medium.

For the MN assay Cytochalasin B (Cyt. B, Sigma, St. Louis, MO, USA) was added at 44 h after start of the culture at a concentration of 3 m g/ml. The tested substance was added 24 h after culture initiation and was present until the end of cultivation (Watt and Stephen 1986; Surrallés et al. 1995). The herbicide treatment for 48 h is recommended to achieve an accumulation of MNi (Almássy et al. 1987).

For the SCE assay and the cell cycle kinetics bromodeoxyuridine (8 m g/ml, BrdUrd, Sigma, St. Louis, MO, USA) was added to all cultures 24 h after initiation of division. Slides were stained with Giemsa for CAs and MNi or FPG technique to differentiate sister chromatids and cell cycles (Perry and Wolff 1974).

One thousand of binucleated (BN) cells per donor and concentration were analysed for MNi and one hundred well-spread metaphases were determined for CAs; chromatid and isochromatid breaks (CB, IB), as well as chromatid and isochromatid exchanges (CE, IE) were determined. Gaps were not included among the aberrations.

Fifty differentially stained metaphases per donor and concentration were examined for SCE and 100 metaphases were analysed for determination of M_1, M_2 and M_{3+} mitotic divisions.

Statistical analysis of results were performed using the simple analysis of variance (ANOVA) and then Student's t-test was applied to evaluate SCEs occurrence between treated and untreated groups and also for estimation of the cell cycle delay. A c^2 test was appropriated to estimate the induction of CAs, MNi and induced heterogeneity of the frequencies of the first, second and third metaphases. The proliferation indices (PI) were calculated according to Lamberti et al. (1983).

Results

The frequency of chromosomal aberrations induced by herbicide in peripheral cow lymphocyte *in vitro* is shown in Table 1. Chloridazon treatments in concentrations ranging from $7 \yen 10^{-6}$ to $7 \yen 10^{-4}$ mol·l^{-1} for 24 h was found to induce a slight clastogenic effect in linear dose-dependent manner, reaching a statistical significance only at the highest concentration ($7 \yen 10^{-4}$ mol·l^{-1}, $p < 0.05$, c^2 test). Chromatid and isochromatid breaks were among the most common types of chromosomal alterations.

The ability of chloridazon to induce micronuclei in cytokinesis blocked cells is reflected in Table 2. A positive influence in induced MN frequencies was attained at the highest concentration of chloridazon ($7 \yen 10^{-4}$ mol·l^{-1}, $p < 0.01$, c^2 test); both lower doses had no significant effect in the induction of MN differences compared to the controls.

The results obtained from the SCE assays and the lymphocyte proliferation kinetics with and without microsomal fraction are summarised in Table 3.

A 24 h exposure to chloridazon in the absence of S9 showed statistically significant elevations of SCE at all concentrations tested ($p < 0.05$ to 0.001, respectively, ANOVA and Student's t- test). The highest concentration ($7 \yen 10^{-4}$ mol·l^{-1}) is also reflected in the reduction of the PI and in the induction of lymphocyte proliferation delays (Student's t-test,

Table 1
Induction of chromosome aberrations (CA) in cow peripheral lymphocytes exposed to
the herbicide chloridazon from two donors

Group	Types of chromosomal aberrations				% aberrant cells	% breaks (± SD)
	CB	IB	CE	IE		
Control (DMSO)	5	-	-	-	2.5 ± 0.15	2.5 ± 0.15
Chloridazon (mol·l^{-1})						
7x10^{-6}	9	-	-	-	4.0 ± 0.23	4.5 ± 0.23[a]
7x10^{-5}	12	-	-	-	5.5 ± 0.24	6.0 ± 0.26[a]
7x10^{-4}	17	-	-	-	8.0 ± 0.34	8.5 ± 0.27*
250 m g/ml, EMS	13	4	7	3	13.0 ± 0.65	18.5 ± 0.78***

A total of 200 well-spread metaphases of each group were determined.
*, *** , (P < 0.05, P < 0.001) -c^2 test,
a - n. s.
CB, IB - chromatid, isochromatid breaks, CE, IE - chromatid, isochromatid exchanges

Table 2
Induction of micronuclei (MN) in cow lymphocyte cultures exposed to chloridazon from two donors

Concentration of chloridazon (mol·l^{-1})	Number of BN	Number of MN in BN cells	Distribution of MN		% MN ± SD
			1 MN	2 MN	
Control (DMSO)	2 081	39	31	4	1.7 ± 0.14
7x10^{-6}	2 031	40 [a]	40	-	2.0 ± 0.14
7x10^{-5}	2 011	49 [a]	49	-	2.4 ± 0.15
7x10^{-4}	2 034	67 **	57	5	3.5 ± 0.20
MMC (0.4 m mol·l^{-1})	2 032	96 ***	82	7	5.0 ± 0.23

, * - (P < 0.01, P < 0.001, c^2 test)
a - n. s.
BN - binucleated cells

Table 3
Frequency of sister chromatid exchanges (SCE) and proliferation indices in cultured cow lymphocytes
exposed to the herbicide choridazon from two donors

Group	SCE / cell	PI	Group	SCE / cell	PI
without S9			with S9		
Control (DMSO)	7.08 ± 2.04	2.1	Control (DMSO)	7.02 ± 1.73	1.87
Chloridazon (mol·l^{-1})			Chloridazon (mol·l^{-1})		
7x10^{-6}	7.74 ± 1.72 *	2.03 [a]	7x10^{-6}	7.13 ± 1.42 [a]	1.89 [a]
7x10^{-5}	8.28 ± 2.33 **	2.16 [a]	7x10^{-5}	7.58 ± 2.20 [a]	1.83 [a]
7x10^{-4}	8.91 ± 2.54 ***	1.92 *	7x10^{-4}	8.0 ± 2.45 *	1.8 [a]
Positive control 0.04 m g/ml, MMC	17.7 ± 8.2	1.47***	Positive control 4 m g/ml, cyclophosphamid	21.77 ± 1.43***	1.75 [a]

A total of 100 second - division metaphases of each group were analysed for SCE.
*, **, *** (P < 0.05, P < 0.01, P < 0.001, ANOVA, Student's *t*-test)
a - n. s.

p < 0.05).
  A similar dose-dependence was found in the SCE assay with S9 microsomal fraction. Treatments with chloridazon for 1.5 - 2 h caused lower frequencies of SCE than those for

24 h. A significant increase of exchanges was observed only at the highest concentration (7×10^{-4} mol·l^{-1}, p < 0.05), while both lower doses (7×10^{-6} and 7×10^{-5} mol·l^{-1}) had no positive effect in SCE increases. Also, no reduction in cell cycle kinetics was found.

Discussion

Our results give evidence of the genotoxicity of chloridazon in cultured bovine peripheral lymphocytes. A greater sensitivity in the response of lymphocytes to chloridazon exposure was obtained in the SCE assay without S9. In cultures with metabolic activation chloridazon induced lower frequencies of exchange than those seen without S9. This decrease is probably due to the short time of exposure to the herbicide. SCEs have been commonly employed in cytogenetic studies. They are considered to be available and sensitive bioindicators or biomarkers of chemical exposure, but their prediction value for assessment of adverse health consequences has been limited (Morimoto et al. 1985; Tucker and Preston 1996). Taking the sensitivity of the SCE technique into account, cultures for SCE determination were also treated with S9 fraction to detect possible metabolic modifications in herbicide genotoxicity.

To our knowledge there is no information on these endpoints in human or animal cells exposed to this herbicide.

The results presented here correspond to our previous finding from CA and SCE assays with chloridazon treated sheep peripheral lymphocytes *in vitro* (Šiviková and Dianovský 1997). In the CA assay with cow and sheep lymphocyte cultures, respectively, a corresponding dose dependence of induced chromosome aberrations was observed. Partial differences in the SCE assay were found. In bovine peripheral lymphocytes the lowest dose of herbicide caused a significant increase in SCE frequencies (7×10^{-6} mol·l^{-1}, P < 0.05), but damage induced by the same dose in sheep lymphocytes show no statistical significance. The higher test concentrations had the same positive effect in lymphocytes of both species. With regard to the cell cycle kinetics, exposure to herbicide for 24 h at a concentration of 7×10^{-4} mol·l^{-1} caused a slight cytotoxic effect in cow, measured as proliferation delay (P < 0.05, Student's *t*-test) that was not seen in sheep cultures.

Significant increase in MN frequencies after treatment with the highest dose of chloridazon (P < 0.01) was probably due to a longer time of exposure to herbicide.

The cytogenetic analysis revealed that donor 2 had been a carrier of a relatively frequent aberration in several cattle breeds - the Robertsonian translocation 1/29 [59, XX, rob (1;29)]. There was no cause described to give rise to this aberration *de novo*.

The spontaneous frequency of SCEs in the donor with normal karyotype (donor 1) was a little different from those seen in the donor 2 - with the Robertsonian translocation (7.0 or 7,15 SCEs/cell in cultures without S9 and 6.93 or 7.12 SCE/cell in cultures with S9, respectively). Popescu (1978) described similar differences in cattle with normal karyotype and one bull with the same translocation. The basal mean of SCEs and CAs as well as the individual sensitivity of blood lymphocytes to herbicide exposure for 24 h between both donors is shown in Fig. 1. Differences in the MN frequency are not given in Figure because of prolonged sampling time. In spite of different donors' karyotypes variability in CA and SCE frequencies are not considered as substantial. The mean in SCE frequency and individual sensitivity to genotoxic agents is widely described by several investigators. Those can be caused by genetic and environmental factors (Catalan et al. 1995) and/or culture conditions (namely by the presence of BrdU in medium, Morales - Ramirez et al. 1987). Our cultures were performed with BrdU at a final concentration of 8 μg/ml of medium. Catalan et al. (1995) observed increased frequencies of SCE in cattle at 15 μg/ml of BrdU concentration. There is also a report confirming that chromosome 1 in cattle has been very sensitive to BrdU - labeling (Iannuzzi et al. 1991). Similarly,
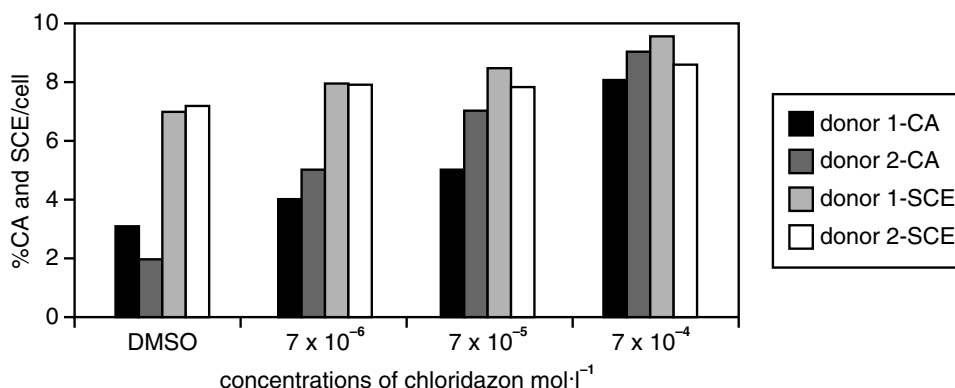
Fig. 1. Individual differences in CA and SCE frequencies between donors with normal and abnormal karyotype

Popescu (1978) demonstrated that the exchange frequency was not changed on the chromosome of the 1/29 translocation compared to those of free homologues. In conclusion, our results indicate genotoxic effects in cultured bovine lymphocytes. A greater sensitivity in detection of chloridazon genotoxicity was obtained in the SCEs assay. In comparison with sheep lymphocytes, experiments presented with bovine peripheral lymphocytes showed also a weak cytotoxic influence of the herbicide.

Chromozómové poškodenia indukované herbicídom chloridazonom v kultivovaných periférnych lymfocytoch hovädzieho dobytka

Hodnotili sme schopnosť herbicídu chloridazon zvýšiť frekvencie mikrojadier (MN), chromozómových aberácií (CA) a sesterských chromatidových výmen (SCE) v kultivovaných periférnych lymfocytoch hovädzieho dobytka, a tiež jeho vplyv na kinetiku bunkového cyklu. Možnú metabolickú modifikáciu v genotoxicite herbicídu sme v teste pre determináciu SCE študovali ovplyvnením kultúr S9 frakciou.

Signifikantné zvýšenie CA a MN sme zistili po aplikácii chloridazonu v koncentrácii 7×10^{-4} mol·l⁻¹ (P < 0.05, resp. P < 0.01), nižšie dávky herbicídu (7×10^{-5} a 7×10^{-6} mol·l⁻¹) neboli štatisticky významné. Jasnú dávkovú závislosť a štatisticky významné zvýšenie indukovaných chromozómových poškodení sme pozorovali v SCE teste bez použitia S9 frakcie. Najvyššia koncentrácia herbicídu sa prejavila redukciou proliferačného indexu a indukciou slabého oneskorenia proliferácie.

Tieto pozitívne výsledky tiež informujú o možnosti zvýšenia genetického rizika pre človeka prostredníctvom potravinového reťazca.

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