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Fundamental and Molecular Mechanisms of Mutagenesis

Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro

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Abstract

The genotoxic activity of the pesticides gliphosate, vinclozolin and DPX-E9636 was studied in in vitro cultures of bovine lymphocytes, using chromosome aberration (CA) and sister chromatid exchange (SCE) frequencies as genetic end-points and a variation of glucose 6-phosphate dehydrogenase (G6PD) enzyme activity as a marker of changes in the normal cell redox state. Results indicated a statistically significant increase of structural aberrations, sister chromatid exchanges and G6PD activity, suggesting that the pesticides tested induce either oxidative stress or a mutagenic effect in this species. The evaluation of both mitotic index and cell viability, after pesticide exposure, demonstrates a high cytotoxic effect which is always associated with the observed genotoxic effect.

Keywords: Bovine; Pesticide; Chromosome aberration; Sister chromatid exchange; Oxidative stress; Glucose 6-phosphate dehydrogenase

1. Introduction

Pesticides are chemical compounds which have to comply with two requirements: a high degree of toxicity and a high target specificity.

Even though the presence of pesticide residues has been demonstrated in raw bovine milk and in different farm animal tissues at maximum or higher levels than allowed by the international tolerance levels [1–3], little is known about the possible effects and molecular consequences of pesticide exposure in livestock. It has been reported that the exposure to some pesticides determines both a serious decrease of progesterone secretion in in vitro cultures of bovine granulosa cells from preovulatory follicles [4] and an increase in the number of sister chromatid exchanges (SCEs) in chick embryo cells [5].

In the present study we have analysed the genotoxic potential of the pesticides gliphosate, vinclozolin and the recently introduced DPX-E9636 in in vitro bovine lymphocyte cultures, from three healthy bovines, using as genetic end-points the frequency of chromosomal aberrations (CAs) and of SCEs. We have also evaluated the induction of oxidative stress,

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following pesticide treatment, by measuring G6PD activity, an enzyme catalysing the first and rate-limiting step of the pentose phosphate pathway. G6PD action provides reducing power, in the form of NADPH, which is used by cells to drive enzymatic reactions required to remove reactive oxygen intermediates (ROIs) [6] and necessary to maintain the intracellular content of the prevalent antioxidant molecule present in the cell cytoplasm: reduced glutathione (GSH) [7]. Recent reports have clearly demonstrated that cell oxidative stress conditions and thus GSH depletion, are always followed by increased G6PD activity, indicating that G6PD is functioning as an antioxidant enzyme [8,9]. Furthermore, it has been observed that nucleated cells, bearing genetically determined G6PD null mutation, are extremely sensitive to oxidative stress [10]. We therefore examined both the cytogenetic effects and G6PD enzyme activity in bovine lymphocytes after pesticide exposure.

Our data indicate a mutagenic effect of the tested chemicals in bovine species as well as an induction of oxidative stress and thus a change in the cell redox state.

2. Materials and methods

2.1. Test chemicals

The following chemical agents were tested: gliphosate (*N*-(phosphonomethyl)glicine) and vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-5- vinyloxazolidine-2,4-dione) from Lab Service Analytica (Bologna, Italy) and DPX-E9636 (1-(4,6-dimethoxypyrimidin-2-yl)-3-(ethylsulfonyl-2-pyridysulfonyl)urea) from Du Pont (Paris, France) (purity of all active ingredients $\geq$ 98%). The features of pesticides assayed were indicated in Table 1 as certified by the supplying firms and reported by Worthing and Walker [11] and Perucci et al. [12]. Gliphosate was dissolved in sterile water whereas vinclozolin and DPX-E9636, because of their low water solubility, were dissolved in dimethyl sulfoxide (DMSO, Fluka, Buchs, Switzerland) 0.3% and ethanol (Sigma, St. Louis, MO, USA) 0.1% final concentration in culture, respectively. An equal concentration of DMSO or ethanol-containing culture medium was utilised as solvent positive controls.

2.2. Lymphocyte cultures

Peripheral blood was drawn from the jugular vein of three clinically healthy unrelated cows, nearly two years of age, by using heparinized vacutainers. Lymphocytes were separated by Ficoll–Hypaque gradient density following standard techniques [13]. One milliliter of buffy coat was cultured in 9 ml of RPMI 1640 medium (Dutch modification) supplemented with 15% heat inactivated fetal calf serum (FCS), 10 μg/ml L-glutamine and 10 μg/ml Pokeweed mitogen, all obtained from Gibco (New York, NY, USA). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. To eliminate variable sources due to culture conditions, exposed and control cultures were concurrent; the same batches of culture medium and an equal number (1×10^6) of cells/plate were used. For CA and SCE analysis pesticides were added immediately after Pokeweed stimulation and left throughout the whole culture period.

2.3. Cytogenetic assays

Cells were cultured for 72 h at 37°C for CA studies. After 70 h of incubation, colcemid (Gibco, 50 μl for 10 ml cultures) was added and left during the last 2 h of cell growth. Lymphocytes were collected by centrifugation, resuspended in prewarmed hypotonic solution (0.075 M KCl) for 20 min, fixed

Table 1
Pesticides tested

Common name	Molecular formula	CAS registry number	Activity	Field doses	Toxicity in rats, LD_{50} (mg/kg)
Gliphosate	$C_3H_8NO_5$	1071-83-6	herbicide	0.34–2.24 kg a.e/ha	4320
Vinclozolin	$C1_2H_9Cl_2NO_3$	50471-44-8	fungicide	0.75–1.0 kg a.i/ha	> 10 000
DPX-E9636	$C_{14}H_{17}N_5O_7S_2$	122931-48-0	herbicide	15 g/ha	> 5000

in methanol/acetic acid (3:1) for 10 min and stained with a 5% Giemsa solution (pH 6.8) for 10 min.

For SCE analysis, 30 h prior harvesting, BrdU (Sigma) was added to each culture (10 μg/ml final concentration). Samples were protected from light. Colcemid was added during the final 2 h (50 μl for 10 ml cultures); harvested cells were treated with hypotonic solution and fixed with methanol/acetic acid (3:1). Air dried slides were stained with a 0.2% acridine orange solution in phosphate buffer (pH 6.8) and sealed with paraffin.

From each concentration and from each subject, 50 well spread metaphases bearing 60 chromosomes were scored on blindly coded slides for CAs and for SCEs. Structural aberrations (isochromatid and chromatid breaks, gaps, fragments and chromosomal rearrangements) were classified according to the criteria suggested by Savage [14]. Gaps were reported but not included either in the calculation of aberration frequency or in the percentage of aberrant cells.

The MI was evaluated counting at least 1000 cells per treatment: the number of dividing cells (prophases and metaphases) was divided by the total number of all cells [15].

2.4. G6PD enzyme assay

Mitogen stimulated cells were cultured for 48 h at 37°C in order to analyse a larger number of cells. An equal number of lymphocytes from each subject were plated and, except for the controls, treated for 6 h with increasing concentrations of each pesticide. Cells were collected and centrifuged, G6PD activity was determined, as already described [16], by measuring the rate of increase in absorbance at 340 nm due to the conversion of $NADP^+$ to NADPH. Since 6-phosphogluconate dehydrogenase (6PGD), the second enzyme of the pentose phosphate pathway, also produces NADPH, both 6PGD and total dehydrogenase activity (G6PD plus 6PGD) were determined separately as previously reported, in order to obtain accurate enzyme activity for G6PD [17,18].

Antioxidant *N*-acetyl-cysteine (NAC, Sigma) treatment was performed adding 40 mM final concentration of this substance dissolved in PBS (Gibco) in both exposed and control cultures 1 h prior to pesticide exposure and left during the incubation period [19].

Cell survival was determined by the Trypan-blue (0.5% solution, Serva, Heidelberg, Germany) exclusion technique.

2.5. Statistical analysis

The comparison between the exposed and control groups for each pesticide and for each dose was performed by means of the two-tailed paired Student's *t*-test.

3. Results

3.1. CA, SCE and MI analysis

In order to assess the mutagenic potential of pesticides gliphosate, vinclozolin and DPX-E9636 in in vitro bovine lymphocyte cultures after 72 h exposure, we first carried out a preliminary study to determine the higher doses at which we were still able to observe a sufficient number of metaphases either for CAs or for SCEs. We chose concentrations ranging from 17 to 170 μM for gliphosate and vinclozolin; DPX-E9636 used at the same concentrations was extremely cytotoxic. The fixed number of cells scored were observed in the range 0.0012–12 μM (Table 2).

As shown in Table 2, each chemical compound produced a significant increase in the percentage of aberrant cells as well as in the aberration frequency (isochromatid breaks and chromatid breaks). No fragments or chromosomal rearrangements were found. The genotoxic effect evidenced in our experimental conditions was always associated with a considerable reduction of the mitotic index ($P < 0.05$ for all examined pesticides). These results showed that all tested pesticides induced a significant clastogenic effect and a dose-dependent decreasing trend of cell proliferation, which was mainly evident in DPX-E9636 and vinclozolin exposed lymphocyte cultures.

Table 3 shows the SCE frequencies obtained in pesticide-exposed lymphocyte cultures of the same donors' group. All pesticides induced a dose-dependent increase in the number of SCE/cell in exposed cultures compared to controls; furthermore, at the highest concentrations a slight but not significant reduction was evident.

Table 2
Chromosomal aberrations in controls and 72 h pesticide-exposed bovine lymphocyte cultures

Treatment	Dose (μM)	No. of subjects	No. of cells scored	Chromosomal aberrations			Aberration frequency[a]	% of cells with aberrations[b]	Mitotic index[c]
				Isochromatid breaks	Chromatid breaks	Gaps	MV ± SD	MV ± SD	MV ± SD
Control	–	3	150	2	5	1	4.7 ± 1.2	4.0 ± 2.0	18.0 ± 2.0
DMSO	(0.3%)	3	150	2	3	1	3.3 ± 1.2	2.0 ± 0.0	17.2 ± 1.6
Ethanol	(0.1%)	3	150	2	6	1	5.3 ± 1.2	3.3 ± 1.2	18.1 ± 2.7
Gliphosate	17	3	150	11	18	3	19.3 ± 4.2 *	12.7 ± 3.1 *	8.2 ± 1.4 *
	85	3	150	22	25	6	31.3 ± 6.1 *	21.3 ± 4.6 *	6.6 ± 0.8 *
	170	3	150	17	41	5	38.7 ± 7.0 *	27.3 ± 5.7 *	4.8 ± 1.3 *
Vinclozolin	17	3	150	18	27	8	30.0 ± 5.3 *	22.7 ± 4.2 *	7.5 ± 0.5 *
	85	3	150	16	42	9	38.7 ± 3.1 * *	26.0 ± 2.0 *	5.7 ± 0.8 *
	170	3	150	28	54	12	54.7 ± 2.3 * *	31.3 ± 6.4 *	4.3 ± 0.5 *
DPX-E9636	0.012	3	150	20	25	10	30.0 ± 3.5 *	24.0 ± 5.3 *	6.4 ± 0.5 *
	0.12	3	150	20	32	9	34.7 ± 7.6 *	28.7 ± 6.1 *	4.8 ± 0.3 *
	12	3	150	22	34	0	37.3 ± 10.3 *	29.3 ± 3.1 *	3.2 ± 0.3 *

[a] Total number of aberrations without gaps/total number of cells analysed, × 100.
[b] Total number of cells with at least one chromosome aberration but not gaps/total number of cells scored, × 100.
[c] Cells in mitotic division/1000 cells examined, × 100.
* $P < 0.05$, * * $P < 0.01$ vs. control.
MV ± SD: mean value ± standard deviation.

Table 3
Number of sister chromatid exchanges per cell (SCE/cell) in controls and 72 h pesticide-exposed bovine lymphocyte cultures

Treatment	Dose (μM)	No. of subjects	No. of cells scored	SCE/cell (MV ± SD)
Control	–	3	150	5.2 ± 0.6
DMSO	(0.3%)	3	150	3.9 ± 0.9
Ethanol	(0.1%)	3	150	5.7 ± 1.1
Gliphosate	17	3	150	9.4 ± 2.1 *
	85	3	150	10.9 ± 1.6 *
	170	3	150	8.5 ± 1.6 *
Vinclozolin	17	3	150	11.0 ± 1.1 *
	85	3	150	13.6 ± 1.5 *
	170	3	150	9.6 ± 1.0 *
DPX-E9636	0.012	3	150	10.0 ± 0.9 *
	0.12	3	150	15.6 ± 2.5 *
	12	3	150	8.8 ± 0.9 *

SCE/cell: total number of SCEs/total number of cells scored.
* $P < 0.05$ vs. control.
MV ± SD: mean value ± standard deviation.

3.2. G6PD activity

To study the possible induction of oxidative stress following pesticide treatment, we analysed G6PD activity in the three bovine subjects exposed to the same pesticides, since we have already obtained evidence demonstrating that an increased activity of this enzyme is always coupled to the pro-oxidant state of the cell [9]. Thus we exposed aliquots of lymphocyte cultures to the same cytogenetically-tested pesticide doses. G6PD activity was measured 6 h after treatment. Results are reported in Table 4; the exposure time chosen was optimal to determine G6PD variations under oxidative stress conditions [9].

A significant enhancement of G6PD activity was observed in all treated lymphocyte cultures compared to the controls. The highest induction of G6PD stimulation reached 118% and 89% over the control, in samples exposed to the lower concentrations of gliphosate and vinclozolin, while the effect was less evident at the highest dose of DPX-E9636 (see also Fig. 1).

Since elevated pesticide concentrations could be cytotoxic for the cells we also determined the percentage of cell killing at the end of the incubation period (6 h). Results (Table 4) showed an increasing but not statistically significant cytotoxicity in the exposed cultures.

We repeated the same experiments in non (Pokeweed) stimulated lymphocytes, in order to assess if there was any mitogen interference in the observed effect. In non-stimulated lymphocyte cultures we were unable to detect G6PD induction, since cells became extremely sensitive to the cytotoxic effects of the pesticide (more than 60% of cells were killed) (data not shown).

To ascertain whether the pesticide exposure involved G6PD as an antioxidant enzyme or as a key member of the hexose monophosphate shunt, we exposed stimulated lymphocytes from the subjects to

Table 4
Effects of pesticide exposure on G6PD enzyme activity and cell death in controls and 6 h treated bovine lymphocyte cultures

Treatment	Dose (μM)	G6PD activity[a]	% of cell killing[b]
		MV ± SD	MV ± SD
Control	–	0.10 ± 0.03	18.3 ± 0.2
DMSO	(0.3%)	0.07 ± 0.04	20.5 ± 3.2
Ethanol	(0.1%)	0.12 ± 0.05	16.1 ± 2.5
Gliphosate	17	0.21 ± 0.05 *	19.4 ± 8.0
	85	0.17 ± 0.04 *	22.5 ± 2.1
	170	0.14 ± 0.02 *	20.5 ± 6.4
Vinclozolin	17	0.22 ± 0.07 *	21.2 ± 0.7
	85	0.16 ± 0.04 *	39.3 ± 5.7
	170	0.13 ± 0.02 *	47.2 ± 8.7
DPX-E9636	0.012	0.17 ± 0.04 *	27.4 ± 3.4
	0.12	0.19 ± 0.02 *	37.9 ± 7.5
	12	0.13 ± 0.03	46.6 ± 6.5

[a] U/mg of protein.
[b] Total number of cells killed/total number of cells, × 100.
[c] $P < 0.05$ vs. control.
MV ± SD: mean value ± standard deviation.

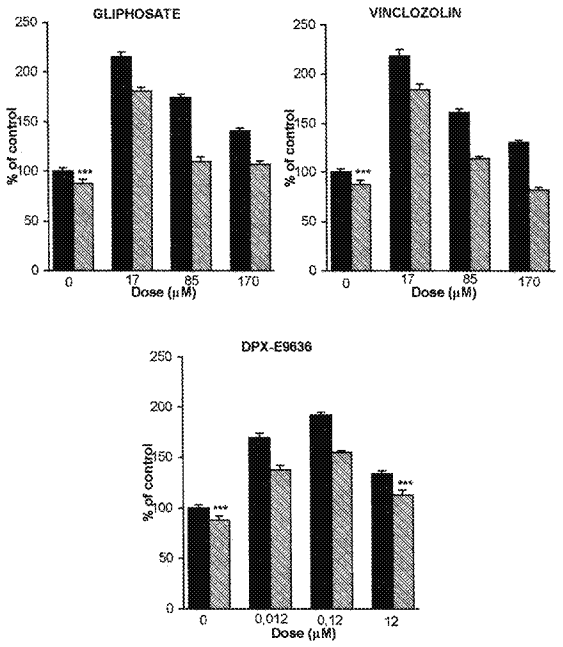

Fig. 1. G6PD enzyme activity in controls and 6 h pesticide-exposed lymphocyte cultures from three bovine subjects in the absence (black graph) or in the presence (bar graph) of the antioxidant NAC. (***) Not significant mean value of G6PD activity compared to the mean value obtained in the absence of NAC.

the same increasing concentrations of pesticides in the presence or in the absence of the antioxidant NAC, which is a GSH precursor. Results reported in Fig. 1 show that NAC buffers the pesticide-derived increase of G6PD activity thus indicating that the chemicals produced a reduction of intracellular GSH pool and a consequent pro-oxidant state.

4. Discussion

The study of the genotoxicity of environmental mutagens in domestic animals is of particular concern because of their possible consequences on the productive and reproductive efficiency of livestock. In the past, risk assessment was limited to the danger to human health, but nowadays ecological risks are also taken into account. In this sense, further studies are required in order to determine the mechanism of action of pesticides, how they interfere with metabolism, genetic damage and detoxification mechanisms in domestic animals. From this point of view, the literature is quite scanty: the only available data on the chemicals herein tested are reported in human or in bacterial mutagenicity assays [20,21] but no specific effects are demonstrated in bovine or in other domestic species.

The present study provides evidence for the intrinsic mutagenic potential of the herbicides gliphosate and DPX-E9636 and of the fungicide vinclozolin on in vitro cultures of bovine lymphocytes from three subjects randomly chosen. Our results indicate a statistically significant increase of both structural CAs and SCEs in exposed cultures compared to the controls. The slight reduction of SCEs, observed at the highest pesticide concentration, could be a consequence of the treatment with highly cytotoxic doses. This observation is in agreement with the lower mitotic index found at these doses. Moreover, all doses tested of each pesticide produced cytogenetic damage and a change in the cell redox state; this finding corroborates the recently reported evidence that reactive oxygen species may be involved in pesticide toxicity [22,23].

In order to increase our knowledge about the genotoxicity of the tested environmental mutagens and to gain insight into their mechanism of action, we also analysed the change of the redox state of the cell following pesticide treatment by evaluating G6PD enzyme activity. We chose to detect the pesticides' effect on G6PD activity after 6 h treatment as we had already demonstrated that, in eukaryotic cell lines, G6PD activity stimulation persists for at least 6 h after the addition of oxidative agents, but that the stimulation decreases with longer incubation periods [9].

We observed a different but statistically significant enhancement of G6PD activity, induced by pesticides, comparable to the increase we found by treating bovine lymphocytes with the GSH oxidative agent, diamide (data not shown). The maximum enhancement of G6PD activity was reached at the lowest doses of gliphosate and vinclozolin and at the intermediate concentration of DPX-E9636. The observation that at the higher doses of the chemicals, G6PD activity decreases could be explained by G6PD enzyme instability consequent to an hyper oxidative cell condition, as already reported for other proteins [24]. Moreover, as dose of vinclozolin or DPX-E9636

increases, besides the decrease of G6PD activity, we also observed an increasing cytotoxic effect. This finding would indicate a threshold over which the normal cell circuits could be altered as well as cells becoming more sensitive to pesticide dependent cytotoxicity. The cytogenetic damage and the induction of pro-oxidant state of the cell seems to be a general response as we witnessed the same phenomenon in lymphocyte cultures exposed to atrazine, another chemical agent widely used in agriculture (data not shown).

In summary, our results indicate that the tested pesticides are genotoxic in in vitro cultures of bovine lymphocytes and support the hypothesis that cells exposed to these chemical compounds have altered cell metabolism, probably generating ROIs through a mechanism still unknown. However, rapid depletion of intracellular GSH and subsequent activation of G6PD would be expected to be a primary event after pesticide treatment. Furthermore, continuous exposure to these chemicals (for 72 h) would result in the inability of the hexose monophosphate shunt to replenish GSH intracellular pool and thus to protect the cells against oxidant injury. The evidence reported here has been strengthened by experiments carried out in human lymphocytes in which the pesticide induced genotoxicity resulted in a change in the cell redox state (manuscript in preparation).

Further studies are necessary in this research field as the presence of organic compounds such as pesticides in animal diets may affect not only the health of livestock themselves but also the qualitative and/or quantitative characteristics of animal productions.

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