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THE EFFECT OF GLYPHOSATE ON THE FREQUENCY OF MICRONUCLEI IN BOVINE LYMPHOCYTES *IN VITRO*

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Glyphosate is a widely used broad-spectrum herbicide that has expanded its applications on plant varieties that are genetically modified to tolerate glyphosate treatment.

The aim of this study was to determine the frequency of micronuclei (MNi) in bovine peripheral lymphocytes after exposure to glyphosate in vitro. The cytokinesis block micronucleus assay (CBMN) for estimation of genotoxic activity was used. The obtained results indicate that glyphosate weakly induced micronuclei in bovine peripheral lymphocytes. Significant elevations of MNi ($p < 0.05$) were observed at concentrations of glyphosate of 280 μM and 560 μM, respectively. Treatment of bovine lymphocytes did not result in the induction of micronuclei in a dose-dependent manner. From cytotoxicity data it is evident that CBPI does not reflect the reduction of cell proliferation.

The influence of metabolic activation on the genotoxic activity of glyphosate was investigated, too. When lymphocyte cultures were treated with glyphosate together with a liver membrane fraction (S9) from Aroclor 1245-induced rat liver, the number of micronuclei in binucleated cells did not increase significantly.

Key words: bovine peripheral lymphocytes, glyphosate, micronucleus, S9

INTRODUCTION

Glyphosate is a broad-spectrum, non-selective systemic herbicide. Its herbicidal activity is expressed through direct contact with the leaves with subsequent translocation throughout the plant. Today, a variety of glyphosate-based formulations are registered under different trade names such as: Roundup, Rodeo, Accord, Sting, Spasor, Muster, Tumbleweed and other. In pure chemical terms glyphosate is an organophosphate, however it does not affect the nervous system in the same way as organophosphate insecticide, and is exploited for its anticholinesterase effects (Marrs, 1993).

Glyphosate inhibits plant growth through interference with the production of essential aromatic amino acids by inhibition of the enzyme enolpyruvylshikimate phosphate synthase, which is responsible for the biosynthesis of choristmate,

which is an intermediate in phenylalanine, tyrosine, and tryptophan biosynthesis (Williams *et al*. 2000). The plant varieties have been inserted with a gene from a bacterium that makes them resistant to the herbicide glyphosate and then the weeds are killed, leaving the crop unaffected.

While glyphosate itself may be relatively harmless (Haughton *et al.* 2001; Smith and Oehme, 1992) some of the products with which contain it have a less benign reputation. Marketed formulations of glyphosate generally contain a surfactant. The purpose of this is to prevent the chemical from forming into droplets thus rolling off leaves that are sprayed. The most widely used type of surfactants in glyphosate formulations are known as ethylated amines. Members of this group of surfactants are significantly more toxic than glyphosate. In a recent study Adam *et al.* (1997) compared the toxicities of Roundup and its component chemicals following administration to rats. They found that POEA (polyoxy-ethyleneamine) and preparations that contained POEA were more toxic than glyphosate alone.

The acute toxicity of glyphosate itself is very low. According to the World Health Organisation, for pure glyphosate the oral LD_{50} in the rat is 4.320 mg/kg. In spite of low toxicity, some laboratory studies have reported adverse effects in each standard category of testing (subchronic, chronic, carcinogenicity, mutagenicity, and reproduction). These signs included eye and skin irritation (Temple and Smith, 1992), cardiac depression (Tai, 1990; Lin *et al.* 1999) vomiting (Lee *et al.* 2000; Burgat *et al.* 1998), and pulmonary edema (Lee *et al.* 2000; Martinez *et al.* 1990). Hietanen *et al.* (1983) reported that glyphosate could disrupt functions of enzymes in animals. In rats it was found to decrease the activity of cytochrome P-450 and monoxygenase activities as well as the intestinal activity of aryl hydrocarbon hydrolase. Other studies have shown some reproductive problems after glyphosate exposure (Savitz *et al.*1997). A study was undertaken to investigate the effect of chronic treatment of glyphosate on body weight and semen characteristics in mature male New Zealand white rabbits. Yousef *et al.* (1995) reported that glyphosate effects included reduced ejaculate volume, and increased abnormal and nonviable sperm. The potential of glyphosate to cause non-Hodgkin's lymphoma has been analyzed by Hardell *et al.* (2002). A variety of organisms have shown that glyphosate-containing products cause genetic damage: in *Salmonella bacteria*, in onion root cells (Rank *et al.* 1993) and in human lymphocytes (Vigfusson and Vyse, 1980). In other studies glyphosate was not mutagenic in the mouse bone marrow, *Salmonella* and Allium anaphase-telophase tests (Rank *et al.* 1993).

A primary purpose of short-term tests for mutation is to provide information on the production of heritable changes (mutations) that could lead to further adverse biological consequences. In the present study, the ability of glyphosate to induce genetic damage was evaluated by the cytokinesis block micronucleus (CBMN) assay.

MATERIALS AND METHODS

Chemicals

Isopropylamine salt of glyphosate, Monsanto, Antwerp, Belgium

Components	CAS No.	EINECS/ELINCS No.	% by weight (approximate)
Isopropylamine salt of glyphosate	38641-94-0	254-056-8	62
Inert ingredients			38

Glyphosate was dissolved in sterile water and added to the lymphocyte cultures at concentrations of 28, 56, 140, 280, and 560 μM. The highest dose of glyphosate was chosen on the basis of the reduction in mitotic index by >50%. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 μM), cyclophosphamide (CP, Jenapharm, Ankerwerk, Rudolstadt, Germany, 0.1 mM) and ethylmethanesulphonate (EMS, Sigma, St. Louis, MO, USA, 250 μg/ml) were used as positive control agents in the assays in both the absence and presence of the metabolic activator (S9 mix).

Lymphocyte cultures

Peripheral blood was drawn from the jugular vein of two clinically healthy donors, 5 months old. Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood to 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 μM HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250 μg/ml) and phytohaemagglutinin (PHA, 180 μg/ml, Welcome, Dartford, UK).

For the CBMN test the cultures were incubated at 37° C for 72 h and 44 h from the start, cytochalasin B (Cyt. B) at a final concentration of 6 μg/ml was added to arrest cytokinesis. The test chemical was added 24 h after PHA stimulation.

The cultures treated for 2 h with S9 mix and those without S9 mix were set up without heat inactivated fetal calf serum. After the treatment, cultures were washed twice with PBS and reconstituted in the same way as cultures treated for 48 h.

For MN assay in the presence of S9 mix, a freshly prepared S9 mix (10% of the culture volume) from Aroclor 1254 (Supelco, Bellefonte, PA, USA) was prepared according to the method of Maron and Ames (1983).

Standard cytogenetic method was used for the obtained slides.

MN analysis

A total 1000 binucleated cells (BN) with well-preserved cytoplasm were examined for each experimental concentration and donor. The cytokinesis block

proliferation index (CBPI) was evaluated by classifying 500 cells according to the number of nuclei (Surrallés *et al.* 1995).

The statistical evaluation of the results was carried out using Fisher's exact test for micronucleated cells and χ^2 test for CBPI.

RESULTS

Table 1 and 2 show frequencies of binucleated cells with micronuclei (BNMN) and cytotoxicity index (CBPI) obtained after treatment with glyphosate. In each table, the data obtained from the different experimental conditions is shown: treatment lasting forty-eight hours without microsomal fraction and treatment for two hours with and without S9 microsomal fraction, respectively.

Table 1. Induction of micronuclei in bovine lymphocyte cultures treated with glyphosate-donor A

Treatment	Concentration μM	CBPI	Total BNMN
48 h	Control	1.63	20
	28	1.60	19
	56	1.69	24
	140	1.68	22
	280	1.51	39*
	560	1.66	27
MMC (0.4 μM)		1.52	55***
2h (-S9)	Control	1.54	21
	28	1.48	17
	56	1.48	14
	140	1.53	19
	280	1.57	20
	560	1.55	20
EMS (250 μg/ml)		1.48	48***
2 h (+S9)	Control	1.52	20
	28	1.44	23
	56	1.65	20
	140	1.72	21
	280	1.71	19
	560	1.58	28
CP (0.1 mM)		1.49	51***

1000 binucleated cells of each concentration were determined;
Statistical significance: *$p<0.05$, ***$p<0.001$

Table 2. Induction of micronuclei in bovine lymphocyte cultures treated with glyphosate-donor B

Treatment	Concentration μM	CBPI	Total BNMN
48 h	Control	1.50	13
	28	1.40	16
	56	1.43	12
	140	1.41	19
	280	1.50	23
	560	1.47	26*
MMC (0.4 μM)		1.48	36***
2h (-S9)	Control	1.54	12
	28	1.58	12
	56	1.62	14
	140	1.53	18
	280	1.57	11
	560	1.55	20
EMS (25 μg/ml)		1.44	35***
2 h (+S9)	Control	1.51	15
	28	1.58	14
	56	1.54	23
	140	1.45	20
	280	1.60	27
	560	1.62	22
CP (0.1 mM)		1.48	40***

1000 binucleated cells of each concentration were determined;
Statistical significance: *$p<0.05$, ***$p<0.001$

The positive controls were MMC (0.4 μM) in the experiments without microsomal activation, CP (0.1 mM) in the experiments with S9 and EMS (250 μg/ml) in the experiments lasting 2 h without S9. Glyphosate treatments lasting for forty-eight hours appear to induce a very slight but statistically significant increase in BNMN frequency in cultures at higher tested concentration (280 μM and 560 μM, respectively). However, none of donors tested was able to induce a dose-dependent increase on micronuclei frequencies.

Treatments with glyphosate for 2 h did not show any positive response, probably due to the short time of exposure to the herbicide.

The results from the experiments for two hours in the presence of S9 mix showed no significant increase in the MN levels.

One of the well known cytotoxicity indexes CBPI was used. From our cytotoxicity data it is obvious that the herbicide did not induce the reduction of cell proliferation.

In conclusion, our results indicate that glyphosate is able to exert a very weak effect on frequency of micronuclei in bovine peripheral lymphocytes *in vitro*.

DISCUSSION

Environmental risk assessments require multidisciplinary knowledge to study the mechanisms of action, metabolism, genetic damage and detoxification. Optimal integration of chemical measurements and biomarker responses could lead to an improved understanding of adverse effects in both human and ecological assessment (Eason and O'Halloran, 2002). The formation of micronuclei in peripheral blood lymphocytes is a valuable cytogenetic biomarker in human populations exposed to genotoxic compounds (Bolognesi *et al.* 2004).

To our knowledge, there is a small number of available reports describing the cytotoxicity or genotoxicity effects of glyphosate on domestic animal cells even though many of environmental mutagens are associated with reduced productive and reproductive efficiency of livestock. The purpose of this study is to provide evidence of the genotoxic potential of glyphosate on *in vitro* cultures of bovine lymphocytes using CBMN assay.

Lioi *et al.* (Lioi *et al.* 1998a; Lioi *et al.* 1998b) reported that glyphosate produced an increased frequency of chromosomal aberrations in both cultured human and bovine lymphocytes. In experiments with bovine lymphocytes they chose herbicide concentrations ranging from 17 to 170 μM and applied on lymphocytes separated by Ficoll-Hypaque gradient density that were cultured for 72 h. Purity of tested glyphosate was = 98%. Their results indicate a statistically significant increase of structural aberrations and sister chromatid exchanges. However, according to data by Li and Long (1988) administration of glyphosate to rats did not produce an increase in frequency of chromosomal aberrations. Similarly, from the results of the studies by De Marco *et al.* (1992) and Rank *et al.* (1993) it seems evident that glyphosate alone was not responsable for chromosomal damage.

Both the mouse and rat bone marrow micronucleus assays were used to study the effects of exposure to glyphosate on dividing red blood cells. The micronucleus assay appears to be sensitive enough to detect both clastogenicity and aneuploidy.

Bolognesi *et al.* (1997) obtained in Swiss/CD-1 mice a weak positive glyphosate-induced increase in the bone marrow micronucleus assay. Their results were in contrast with those of Kier *et al.* (1997) that reported no increased micronucleus formation.

The information about genotoxic effects of glyphosate is both large and heterogeneous. Their primary goal is to determine whether the chemical interacts directly or indirectly with DNA and thus could lead to adverse biological consequences, including cancer.

In conclusion, glyphosate only weakly increased the frequency of micronuclei in bovine lymphocyte cultures. Further studies are needed in this area, as genetically modified plant varieties will be likely used more extensively throughout the food chain. The presence of herbicides in animal diets could affect not only the health of livestock but also the quality of animal products. Development of glyphosate resistance in weeds species could be also a serious risk from now. It will make farmers more dependent on other pesticides and will probably lead to their increased use.

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EFEKTI GLIFOZATA NA FREKVENCIJU POJAVLJIVANJA MIKRONUKLEUSA U LIMFOCITIMA GOVEDA *IN VITRO*

PIEŠOVÁ ELENA

SADRŽAJ

Glifozati su herbicidi širokog spektra, čija se primena proširuje i na razne vrste biljaka, koje su genetski modifikovane tako da mogu tolerisati tretman glifozatima.

Cilj ovog rada je bio utvrđivanje frekvence pojavljivanja mikronukleusa (MNi) u perifernim limfocitima goveda, nakon njihovog izlaganja glifozatima *in vitro*. Analizirana je blokada citokineze mikronukleusa (CBMN) u cilju procene genotiksične aktivnosti. Dobijeni rezultati ukazuju da glifozati vrlo slabo utiču na pojavljivanje mikronukleusa u perifernim limfocitima goveda. Značajno povećanje MNi ($p<0.05$) je utvrđeno pri koncentraciji glifozata od 289 μM i 560 μM. Pri ovom tretmanu govođih limfocita nije utvrđen dozno-zavisni efekat na indukciju pojave mikronukleusa. Podaci o citotoksičnosti govore da se CPBI ne odražava na redukciju stepena ćelijske proliferacije.

Takođe je ispitivan i uticaj metaboličke aktivacije na genotoksičnu aktivnost glifozata. Kada se kultura limfocita tretira istovremeno sa glifozatom i frakcijom membrane jetre (S9) iz jetre pacova, tretirane Aroklorom 1245, ne dolazi do statistički značajnog povećanja broja mikronukleusa u ćelijama sa dva jedra.