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MITOTIC INDEX AND CELL PROLIFERATION KINETICS AS ADDITIONAL VARIABLES FOR ASSESSMENT OF GENOTOXIC EFFECT OF THE HERBICIDE MODOWN

K. ŠIVIKOVÁ, J. DIANOVSKÝ

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

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Abstract

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The *in vitro* effect of the herbicide Modown (with active component bifenox) was tested for the ability to influence cell proliferation of PHA-stimulated bovine peripheral lymphocytes. Mitotic (MI) and proliferation (PI) indices were determined as an alternative for the screening of the cytostatic activity. The herbicide Modown exerted a clear effect on the inhibition of MI and PI over a concentration-tested range of 25 μg/ml to 1000 μg/ml. An expressive proliferation delays was found after the treatment with herbicide at a dose of 250 μg/ml ($P < 0.001$), while the higher doses of 500 and 1000 μg/ml had caused nearly complete mitotic inhibition in each donor ($P < 0.05$ and $P < 0.001$, respectively). A correlation between the PI and MI inhibition refers rather to cytostatic than cytotoxic effects of the herbicide. The results support the possibility of immunosuppression by herbicide exposure.

Herbicide Modown, bovine peripheral lymphocytes, cell proliferation, cytostatic effect

Nitrodiphenylether herbicides are persistent and lipophilic environmental contaminants may contribute to the impairment of animal health and production. The presence of pesticide residues has been demonstrated in raw bovine milk and in different domestic animal tissues (Lioi et al. 1998).

All nitrodiphenylether herbicides exert their phytotoxic activity in chlorophyll of plants by the reduction itself to radical species, which initiate destructive reactions in membrane lipids, leading to the cell leakage (Corbett et al. 1984). Members, as acifluorfen and oxyfluorfen, e.g. are extremely potent inhibitors of protoporphyrinogen oxidase, a membrane–bound enzyme involved in the heme and chlorophyll biosynthesis pathways (Camadro et al. 1995). The later was studied for the induction of haematological diseases in human erythroid progenitor cells but a cytotoxic effect had been seen only at very high concentrations (Rio et al. 1997).

Bifenox is a selective herbicide used in control of annual broad-leaved weeds and some grasses, e. g. in cereals, maize, soya beans, rice and other crops.

Jinno et al. (1999) described the cytotoxic and porphyrogenic effects of bifenox in rat hepatocytes. They found the maximum porphyrin accumulation at 0.25 mM of the herbicide and an inhibion of protoporphyrogen oxidase, resulting in the accumulation of protoporphyrin IX.

Francis et al. (1999) evaluated maternal and developmental toxicity of ten diphenylether herbicides (including bifenox) in mice. They found a correlation among the position of chlorine substituents and the potential for inducing prenatal and postnatal syndroms. But no prenatal and postnatal embryotoxicity was shown.

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

We investigated the *in vitro* dose-dependent effects of commercial herbicide bifenox (principal tradename Modown) on the mitogenic activity of bovine peripheral lymphocytes stimulated with phytohaemagglutinin (PHA). Previously we reported the results on chromosome aberrations and sister chromatid exchanges in bovine blood lymphocytes exposed to the herbicide in different sampling times - for the last 2h, 24h and 48h of cultivation, respectively (Šiviková and Dianovský 1999). The statistically significant genotoxic effect was obtained in the sister chromatid assay after exposure to the herbicide for 24 h only. In the chromosome aberration assay no positive clastogenic effect was observed. From this point of view, references are quite scant. Considering the human and animal exposure to the herbicide, the results on lymphocyte proliferation kinetics can valuably complete data on the immmunotoxic potential of this agent possibly resulting in health consequences.

Materials and Methods

The commercial herbicide Modown (consists of bifenox, $C_{14}H_9Cl_2NO_5$, 42%, and 58% inert components - composition not specified, Rhône – Poulenc, Toulouse, France) was dissolved into dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and applied into culture flasks. Concentrations of 25, 50, 250, 500 and 1000 μg/ml (except for the highest dose for cell proliferation kinetics assays) were used for treatments. The herbicide top doses were chosen on basis of the agent solubility and reduction in mitotic index (MI) > 50%. The final DMSO concentration in the treated and control cultures was 0.1%.

Experiments were carried out on two healthy cow donors (Slovak spotted cattle, 2- and 3-years-old).

Lymphocyte viability

Lymphocyte viability was studied by trypan blue exclusion test (Brusick 1984; Marzano et al. 1999). Cells were treated with the herbicide for the last 24 h and 48 h, then incubated with 0.25% trypan blue (Sigma, St. Louis, MO, USA) and 10% foetal serum, for 4 min. Viable cells were analysed at all the concentrations tested determining their ability to exclude dye.

Cell cultivation

Whole blood cultures (0.5 ml, 2.8×10^6 cells/ml) were cultivated for 72 h at 38 °C in 5ml 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, Darford, England).

Lymphocyte cultures were exposed to the herbicide Modown for the last 24 h of cultivation and slides were obtained by the standard cytogenetic method; 2 h before harvest, colchicine (Merck, Darmstadt, Germany) was added at the concentration of 5 μg/ml.

For 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. Slides were stained with the FPG (fluorescence plus Giemsa) technique to differentiate cell cycles (Perry and Wolff 1974).

One hundred metaphases per donor and concentration were analysed for determination of M_1 (darkly stained chromatids), M_2 (harlequin chromatids) and M_{3+} (part of metaphase with dark and light stained chromatids).

Proliferation index (PI) was calculated according to the formula: $PI = \frac{M_1+2M_2+3M_3}{100}$ (Lamberti et al. 1983).

Mitotic index was determined scoring the number metaphases in total number of 2000 cells. The inhibition of the mitotic index was calculated as 100 – [MI treated x 100/ MI control] (Rojas et al. 1993).

Statistical analysis of results was performed from analysed cells using the Student's *t*-test for estimation of the cell cycle delay and χ^2 test for reduction of MI.

Results

The dose - dependent effects of the herbicide Modown on the mitotic activity in bovine lymphocyte are shown in Fig. 1 and 2.

A statistically significant inhibition of the MI was detected after treatment with the highest herbicide concentrations of 500 μg/ml and 1000 μg/ml in both donors ($P < 0.05$ and $P < 0.001$, respectively). A more expressive decrease of mitotic activity was obtained with donor 2; so that a weak inhibition of the MI ($P<0.05$) was also seen after a dose of 250 μg/ml (Fig. 1).

The changes in the cell cycle proportions (M_1, M_2 and M_{3+}) were reflected in the proliferation delay (Fig. 2). The herbicide induced a cell cycle delay over a concentration

range of 25 µg/ml to 500 µg/ml with a statistical significance at all the doses, except for the initial dose ($P < 0.05$ and $P<0.001$, respectively). Even though an apparent decrease of total cell number was seen in comparison to the controls, no cytotoxicity had been confirmed with trypan blue dye test for 24 and 48 h of cultivation, respectively (data not shown). At the concentrations of 50 µg/ml and 250 µg/ml, the number of lymphocytes declined to about 50-70 %, whereas the highest concentrations (500 and 1000 µg/ml) resulted in nearly complete mitogenic inhibition. For 48 h, similar results of viability were shown.

A correlation among the percentage of the MI and the PI inhibition demonstrated a negative slope line, characteristic of a cytostatic effect of the herbicide (Fig. 3).

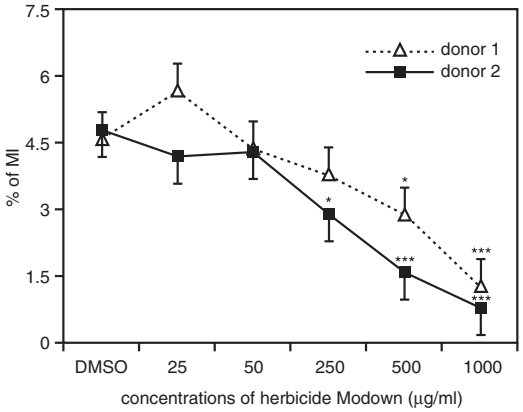

Fig. 1. Effect of the herbicide Modown on the mitotic index (MI) of cultured bovine lymphocytes. Data are presented as percentage of the MI in untreated and treated cultures. Calculations of the MI see in the methods. Positive effects of the herbicide were obtained at the doses started of 250 or 500 µg/ml per donor, respectively.

Discussion

For the *in vitro* assays, the concentration scale of chemical agents up to a cytotoxic dose is required. Cytotoxicity is estimated from several endpoints, e.g. as cell death or cell confluence, that could be caused by the membrane disruption, osmotic shock or pH imbalance (Brusick 1984). For the blood lymphocyte cultures, a determination of the MI inhibition has a practical meaning (Preston et al. 1987; Armstrong et al. 1992; Kirkland 1998). The MI is interpreted in terms of the cell death or arrest of cells at any moment during the interphase. The later case would lead to an increase of the first metaphases along with a delay in the cell cycle that would affect the PI values.

Our results demonstrate inhibitions of the MI and PI at all the herbicide concentrations tested. Three highest concentrations of the herbicide (250, 500 and 1000 µg/ml) induced a statistically significant reduction in mitotic activity associated with the PI decrease ($P < 0.05$, $P < 0.001$, respectively).

Guadaño et al. (1998) reported that a parallel dose dependent decrease of the MI and PI indicates a cytotoxic effect of the herbicide. To differentiate cytotoxic and/or cytostatic effects of the chemical agent Rojas et al. (1993) suggested using a correlation between the MI and PI inhibition, which could also be used

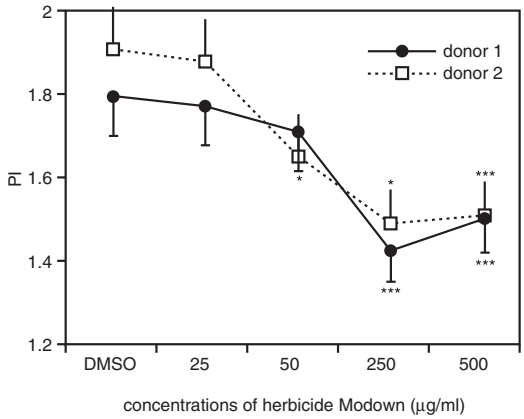

Fig. 2. Effect of the herbicide Modown on the proliferation index (PI) of cultured bovine lymphocytes (calculation of the PI see in methods). Positive effects of the herbicide were obtained at the doses started of 250 or 500 µg/ml per donor, respectively.

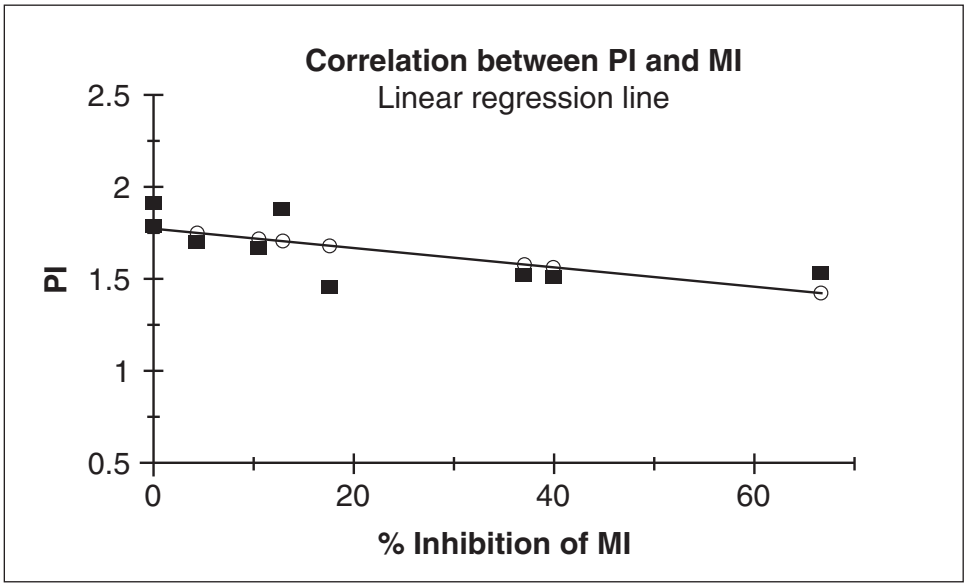

Fig. 3. Graphically analysed correlation between the inhibition of the PI and the MI (linear regression line). A negative slope of line is evident for agents with cytostatic activity. % of inhibition of MI see in methods.

to predict the action of unknown compounds. In accordance with the proposed method a negative slope of the regression line was obtained in our experiments indicating a cytostatic effect of the herbicide Modown.

Similarly, Jinno et al. (1999) found no dose-dependent induced cytotoxicity in rat hepatocytes treated with bifenox at concentrations up to 1 mM.

Other members of diphenylether herbicides, e.g. oxyfluorfen, showed a cytotoxic effect in human erythroid progenitor cells only at very high concentrations (10^{-4} M), but no adverse effect on cellular proliferation was seen (Rio et al. 1997).

Beside other toxic effects, the herbicides can also act on the immune system by stimulation or suppression of immune functions.

Blakley (1997) reported immunosupressive activity of 2,4 - dichlorophenoxyacetic-acid herbicide in mice. Rat lymphocyte exposure to the same herbicide did not alter blastogenesis (Blakley et al. 1998). No effects on mitogen-induced proliferation or immunoglobulin synthesis were observed in human peripheral lymphocytes after exposure to polybrominated biphenylethers up to the concentrations of 10^{-5} M (Fernlof et al. 1997). These authors presume that a certain function of human peripheral lymphocytes (proliferation and immunoglobulin synthesis) was intensive to the direct action of these agents.

Stelzer and Gordon (1984) documented the inhibition effects of pyrethroids (permethrin and cypermethrin) on human lymphocytes; similar results in the inhibition of mitogenic responses to concanavalin A and lipopolysaccharide were obtained, respectively. With respect to the similarity between the mitogenic inhibitory effect of permethrin and cypermethrin the authors take non-specific actions of these compounds.

Our results showed altered PHA mitogen responsiveness in the bovine peripheral lymphocytes. Involvement of membrane perturbing action of the herbicide in cellular function is not unexpected due to its high lipophilicity. More extensive experiments on immune suppression by the herbicide *in vivo* might support these results.

Mitotický index a bunková proliferačná kinetika ako prídavné varianty pri odhade genotoxického účinku herbicídu Modown

Testovali sme schopnosť herbicídu Modown (s aktívnou zložkou bifenoxom) ovplyvniť bunkovú proliferáciu PHA-stimulovaných periférnych lymfocytov hovädzieho dobytka in vitro. Ako alternatívu pre skríning cytostatickej aktivity látky sme zvolili mitotický (MI) and proliferačný (PI) index. Herbicíd Modown zapríčinil inhibíciu MI and PI vo všetkých testovaných koncentráciách od 25 μg/ml do 1000 μg/ml. Výrazné proliferačné oneskorenie sme zistili po pôsobení herbicídu v dávke 250 μg/ml ($P < 0.001$), kým vyššie dávky 500 and 1000 μg/ml zapríčinili takmer úplnú mitotickú inhibíciu u oboch donorov ($P< 0.05$, resp. $P < 0.001$). Korelácia medzi PI and MI poukazuje skôr na cytostatický než cytotoxický účinok herbicídu. Naše výsledky tiež podporujú možnosť imunosupresívneho účinku pri expozícii herbicídu.

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