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EVALUATION OF CYTOTOXIC AND GENOTOXIC EFFECTS OF PERMETHRIN USING IN VITRO MICRONUCLEUS TEST

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The objectives of this investigation were to evaluate the effects of the synthetic pyrethroid insecticide permethrin on mitotic activity and appearance of micronuclei in cultured human lymphocytes. In the standard cytokinesis-blocked micronucleus test in vitro protocol, four experimental concentrations were used: 1.2, 3, 6 and 12 mg/ml. The concentration of 6 mg/ml was calculated to correspond to the LD_{50} dose obtained in rats. The cytokinesis block proliferation index (CBPI), as a parameter of cell cycle kinetics, was decreased at all concentrations of permethrin except the lowest one. The analysis of micronucleus frequencies, however, point to the absence of genotoxic effects.

Key words: permethrin, lymphocyte, cell proliferation, micronucleus

INTRODUCTION

The pyrethroids are derivatives of natural pyrethrins (Elliot et al., 1967) recognized as very efficient insecticides due to their high potency and selectivity against various arthropods (Casida et al., 1983). The basic molecular mechanism of pyrethroid action is similar in insects and mammals and involves interference with the nerve membrane sodium channels, that causes prolonged depolarization and induction of repetitive activity (Narahashi, 1985).

Permethrin is a synthetic pyrethroid insecticide developed by Elliot et al. (1973). Permethrin, as a highly potent neurotoxic insecticide with the extreme low toxicity of the 25:75 cis:trans ratio isomer to homeothermic organisms, proved to be suitable in public health and industrial pest control, as well as for use in agriculture and veterinary medicine against arthropod ectoparasites (ticks, lice, fleas, mites) (Bouma et al., 1996). In addition, it has been reported that permethrin-impregnated bed nets lowered the incidence of malaria (Kroeger et al., 1995; Mbogo et al., 1996). Since DDT spraying was much less effective (Kere et al., 1996), use of permethrin is recommended in the control of insect vectors of diseases.

Although toxicological experiments on different animal species (mouse, rabbit, dog) confirmed low mammalian toxicity (Anadón et al., 1991), it is still not completely clear whether permethrin can influence genetic material leading to an

increased rate of mutations. Gupta et al. (1990) found permethrin to be nonmutagenic in a sex-linked recessive lethal test on *Drosophila melanogaster.* On the other hand, the effects of permethrin on mammalian cells varied depending on the cell type and applied experimental concentrations. Thus, Barrueco et al. (1992) observed genotoxic effects at the cytogenetic level after *in vitro* analysis of the effects of permethrin on human lymphocytes. At much higher concentrations in the same test-systems permethrin exhibited only cytotoxic effects without significant changes of chromosome abberration level (Djelić et al., 1997a) or sister chromatid exchange rate (Djelić et al., 1997b).

The aim of the present study was to evaluate possible effects of permethrin on the appearance of micronuclei and changes in cell cycle kinetics in cultured human lymphocytes.

MATERIALS AND METHODS

Human peripheral blood lymphocyte cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan (1975). Heparinised blood samples (0.8 ml) obtained by venipuncture from healthy men under 35 years of age, were added to vials with 9.2 ml of RPMI 1640 medium containing 30% of inactivated calf serum (Serva) and 0.04 mg/ml of phytohaemagglutinin (Murex Diagnostics Ltd.). Cultures were incubated for 72 h at $37\pm0.5^{o}C$.

Exactly 47 h and 30 min. after the beginning of incubation permethrin (cis:trans isomer ratio 25:75, CAS number 52645-53-1) dissolved in acetone was added to the cultivation vials in such amounts to obtain final experimental concentrations of 1.2, 3, 6 and 12 mg/ml. The concentration of 6 mg/ml was chosen as it corresponds to the LD_{50} dose obtained for rats. The solvent (acetone) was used as the negative control and two positive controls were included: a well known mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) at a final concentration of 10^{-6} M and 10^{-8} M human recombinant insulin (Inutral® (HM 100, ICN) as a compound able to elevate mitotic activity of human lymphocytes. In order to obtain cytokinesis block cytochalasine B (Cyt-B, Sigma Chemical Co., final concentration 6 μg/ml) was also added at 47 h and 30 min. after the PHA stimulation.

Finally. after 72 h of incubation, culture content was centrifuged at 200 g for 10 min., resuspended in 0.9% NaCl, recentrifuged, and the cell pellet was treated with hypotonic solution (2-3 min. in 0.075 M KCl at room temperature). After the standard procedure of three repetitive cycles of fixation in methanol-acetic acid solution (3:1, v/v), the cell suspension was dropped on chilled, grease-free microscopic slides and air-dried. Staining was achieved by 2% Giemsa (Kemika) in Gurr buffer solution (pH=6.8). At each experimental point, at least 1000 binucleated cells were analysed for the presence of micronuclei. Cytotoxicity was evaluated on 500 or more cells, by the cytokinesis block proliferation index (CBPI) according to the following formula:

$$CBPI = [M_I + 2M_{II} + 3(M_{III+IV})] / 100$$

where M_I to M_{IV} represent the percentage of cells with one to four nuclei (Surralés et al., 1994).

Statistical analysis was performed by the χ^2 test.

RESULTS

Table 1. shows experimental values for cytokinesis block proliferation index (CBPI) as an indicator of cell cycle kinetics. The CBPI was calculated by counting the cells with one to four nuclei, and therefore it expresses the ratio of cells in different mitotic cycles (Fig. 1). In cultures treated with the solvent as a negative

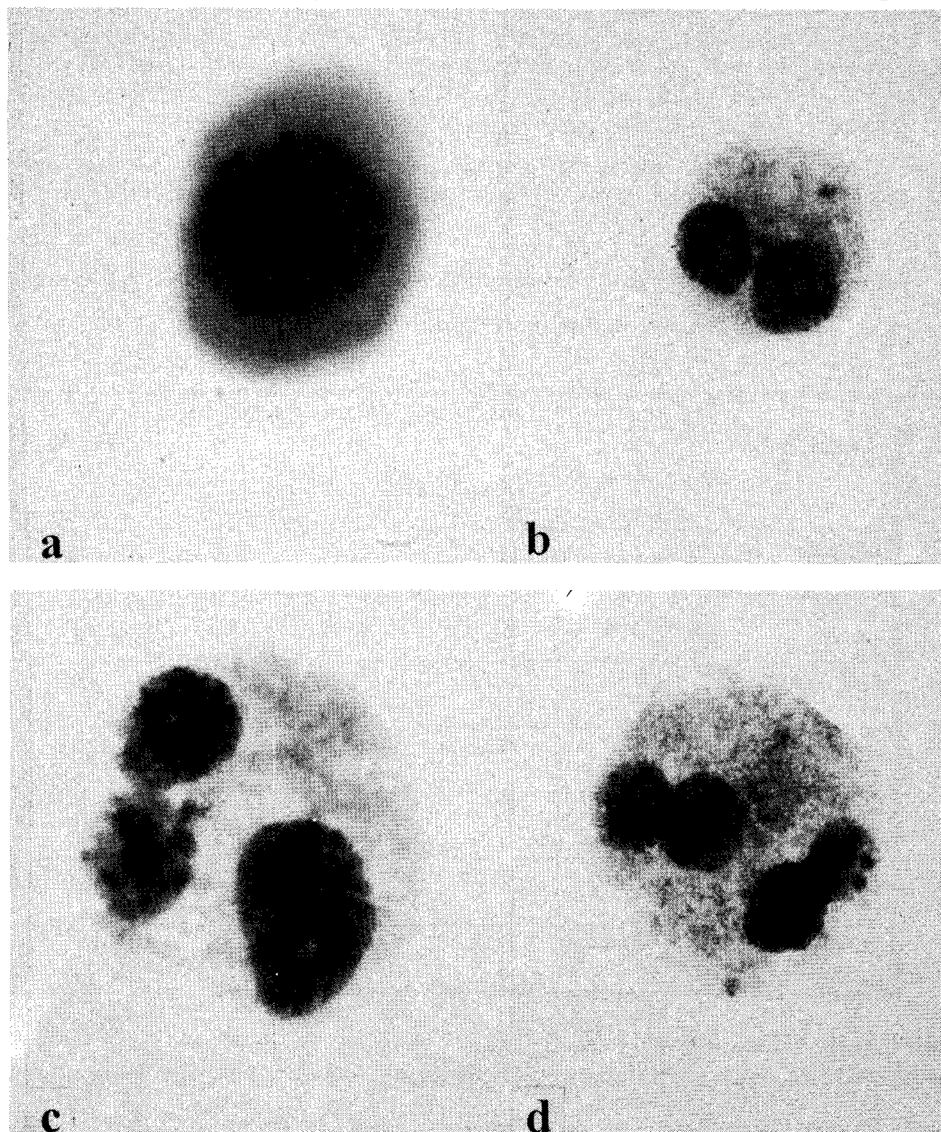

Figure 1. The appearance of lymphocytes with one (a), two (b), three (c) and four (d) nuclei.

control CBPI was 1.65. Treatment with the well known mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) as a positive control lowered the CBPI by 15.7 % (CBPI=1.39; $p<0.01$) in comparison to the negative control. Human recombinant insulin used as another positive control proved to be an efficient mitogen for human lymphocytes (Djelić, 1997). Thus, the experimental concentration of 10^{-8} M insulin caused a 10.3 % elevation of CBPI (CBPI = 1.82; $p<0.05$). Statistical analysis (χ^2 test) revealed a significant decrease of CBPI values at all concentrations of permethrin used in this investigation, except the lowest one. The concentration of 6 mg/ml which corresponds to the LD_{50} for rats, suppressed CBPI by 12.1 % ($p<0.01$), while the highest concentration applied (12 mg/ml) caused a more profound effect (CBPI = 1.40; $p<0.001$).

The genotoxic effects of permethrin were evaluated on the same microscopic slides by analysis of micronucleus induction in binucleated cells

Table 1 - Cytokinesis block proliferation index (CBPI) in cultures of human lymphocytes treated with permethrin

Treatment	No. of analysed cells	Distribution of cells according to No. of nuclei (%)			CBPI
		M_I	M_{II}	$M_{III}+M_{IV}$	
negative control	1024	45.1	44.6	10.3	1.65
10^{-6} M MNNG	1016	67.8	25.6	6.6	1.39**
10^{-8} M insulin	1103	32.0	54.1	13.9	1.82*
permethrin 1.2 mg/ml	1086	48.3	42.2	9.5	1.61
permethrin 3 mg/ml	1052	57.4	35.4	7.2	1.50*
permethrin 6 mg/ml	1008	61.3	32.2	6.5	1.45**
permethrin 12 mg/ml	1074	66.0	27.8	6.2	1.40***

$CBPI = (M_I + 2M_{II} + 3(M_{III} + M_{IV})) / 100$
where M_I to M_{IV} represent the percentage of cells with one to four nuclei

*$p < 0.05$; **$p < 0.01$; ***$p< 0.001$ (χ^2 test)

Table 2 - Evaluation of the genotoxic effects of permethrin in cytokinesis-blocked micronucleus assay on human peripheral blood lymphocytes

Treatment	BN cells scored	Distribution of BN cells according to no. of MN				MN/cell (%o)
		0	1	2	3	
negative control	2061	2033	20	8	0	17.5
10^{-6} M MNNG	2014	1946	51	13	4	44.2***
10^{-8} M insulin	2103	2068	30	5	0	19.0
permethrin 1.2 mg/ml	2076	2042	28	6	0	19.3
permethrin 3 mg/ml	2034	2000	31	3	0	18.2
permethrin 6 mg/ml	2009	1971	32	5	1	22.4
permethrin 12 mg/ml	2115	2079	30	6	0	19.9

BN- binucleated; MN-micronuclei;

***$p < 0.001$ (χ^2 test)

(Table 2). The control value for micronucleus frequency was 17.5 ‰. In permethrin treated cultures the frequency of MN was in the range from 16.9 ‰ to 22.4 ‰. Evidently only the positive control (10^{-6} M MNNG) significantly ($p<0.001$) increased the frequency of MN in binucleated cells, whereas permethrin did not influence this genotoxic endpoint significantly.

DISCUSSION

Short-term cultures of PHA-stimulated peripheral blood lymphocytes are extensively used in cytogenetic assays. Application of cytochalasine B affects the cell membrane and prevents cytokinesis, though kariokinesis still occurs. Therefore, in the presence of cytchalasine B, binucleated cells can be observed after the completion of one cycle of nuclear division. If the cell contains 3 or 4 nuclei, however, it indicates the existence of a second nuclear division after the addition of cytochalasine B. Cells with only one nucleus have not completed nuclear division (chromosomes in metaphase plate) or it has not happened at all. Since the lymphocytes were not synchronized in this experiment, under the microscope the cells appeared with one to four nuclei (Fig. 1). The cytokinesis

block proliferation index (CBPI) actually reflects the ratio of cells in three different cycles of nuclear division. The numerical values of CBPI can vary from 1.00 to 3.00 (the first extreme CBPI =1.00, expresses the theoretical situation when 100% of the cells contain only one nucleus; whereas CBPI=3.00 should reflect the theoretical situation of 100% cells with 3 or 4 nuclei).

The decreased values of CBPI obtained after treatment with permethrin suggest possible cytostatic effects. Also, the percentage of cells with one nucleus gradually increased with concentration of permethrin in the cultures, while the percentages of cells with two or more nuclei decreased. These observations are consistent with previous data (Djelić, 1998) showing that permethrin decreases proliferation index in human lymphocyte cultures established with 5-bromo-2'-deoxyuridine and successively stained metaphase spreads using the fluorescence-plus Giemsa (FPG) procedure (Perry and Wolff, 1974).

The suppression of mitotic activity detected by analysis of CBPI in this study, may have been caused by interference of normal mitosis. This assumption is supported by the observed capability of permethrin to exert aneugenic potential (Surralés et al., 1995). It must be kept in mind, however, that high concentrations of permethrin may also exhibit cytotoxic effects enhancing the overall decrease in mitotic activity.

As for the evaluation of genotoxic effects of permethrin, analysis of the micronucleus frequency should have indicated possible aneugenic and/or clastogenic effects. Namely, the cytokinesis-blocked micronucleus assay is used to detect acentric chromosome fragments or whole chromosomes left upon nucleus division and visible as small additional nuclei (micronuclei) in the cytoplasm (Fenech and Morley, 1985). Thus, micronuclei are a cytologically detectable manifestation of the effects of agents capable of causing chromosome breakage (clastogens) or disruption of the mitotic spindle and aneuploidies (aneugens).

The negative results obtained in this study are in accordance with absence of genotoxic effects of permethrin on *Drosophila melanogaster* (Gupta et al., 1990) and human lymphocytes *in vitro* (Djelić et al., 1997a; 1997b).

As far as we aware genotoxicological characterization of permethrin was performed mainly by short-term *in vitro* and *in vivo* test-systems. It would be interesting, therefore, to examine further possible genetic changes after chronic or subchronic exposure of laboratory rodents by monitoring various genotoxic endpoints (chromosome aberrations, sister-chromatid exchange, micronuclei, heritable translocations, dominant lethal mutations, analysis of DNA adducts, changes in DNA sequence etc.).

REFERENCES

1. *Anadön A., Martinez-Larrañaga M.R., Diaz M.J., Bringas P.* 1991: Toxicokinetics of permethrin in the rat. *Toxicol. Appl. Pharmacol., 110,* 1-8
2. *Barrueco C., Herrera A., Caballo C., de la Peña E.* 1992.: Cytogenetic effects of permethrin in cultured human lymphocytes. *Mutagenesis, 7,* 433-437
3. *Bouma M.J., Parvez S.D., Nesbit R., Sondrop H.E.* 1996: Rapid decomposition of permethrin in the outer fly of an experimental tent in Pakistan. *J. Am. Mosq. Control. Assoc., 12,* 125-129
4. *Casida J.E., Gammon D.W., Glickman A.H., Lawrence L. J.* 1983: Mechanisms of selective action of pyrethroid insecticides. *Annu. Rev. Pharmacol. Toxicol., 23,* 413-438

5. *Djelić N.* 1997: Cytogenetic effects of estradiol, thyroxine, insulin and epinephrine on human lymphocytes in vitro. *Ph.D. thesis, Faculty of Biology, University of Beograd, pp. 121-124 (In Serbian)*
6. *Djelić N.* 1998: Effect of permethrin on mitotic activity of cultured human lymphocytes. *Pesticidi, 13, 233-238 (In Serbian)*
7. *Djelić N., Soldatović B., Djelić D.* 1997a: Analysis of chromosomal aberrations in human lymphocyte cultures treated with permethrin. *Vet. Glasnik, 51, 211-216 (In Serbian)*
8. *Djelić N., Soldatović B., Andjelković M., Milutinović R.* 1997b: The rate of sister-chromatid exchanges in cultured human peripheral blood lymphocytes treated with permethrin. *Genetika, 29,* 97-102
9. *Elliot M., Farnham A.W., Janes N.F., Needham P. H., Pearson B.C.* (1967): 5-benzyl-3-furylmethyl chrysanthemate: A new potent insecticide. *Nature (London), 213,* 493-494
10. *Elliot M., Farnham A.W., Jones N.F., Needham P.H., Pulman D.A., Stevenson J.H.* 1973: A photostable pyrthroid. *Nature (London), 246,* 169-170
11. *Evans H.J., O'Riordan M.L.* 1975: Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. *Mutat. Res., 31,* 135-148
12. *Fenech M., Morley A.A.* 1985: Measurement of micronuclei in lymphocytes. *Mutat. Res., 147,* 29-36
13. *Gupta R.K., Mehr Z.A., Korte D.W.Jr., Rutledge L.C.* 1990: Mutagenic potential of permethrin in the Drosophila melanogaster (Diptera: Drosophilidae) sex-linked recessive lethal test. *J. Econ. Entomol., 83,* 721-724
14. *Kere N.K., Arabola A., Bakoteé B., Qualo O., Burkot T,R., Webber R.H., Southgate B.A.* 1996.: Permethrin-impregnated bednets are more effective than DDT house- spraying to control malaria in Solomon Islands. *Med. Vet. Entomol., 10,* 145-148
15. *Kroeger A., Mancheno M., Alarcon J., Pesse K.* 1995: Insecticide-impregnated bed nets for malaria control varying experiences from Equador, Columbia and Peru concerning acceptability and effectiveness. *Am. J. Trop. Med. Hyg., 53,* 313-323
16. *Mbogo C.N., Baya N.M., Offulla A.V., Githure J.I., Snow R.W.* 1996.:The impact of permethrin-impregnated bednets on malaria vectors of the Kenyan coast. *Med. Vet. Entomol., 10,* 251-259
17. *Narahashi T.* 1985: Nerve membrane ionic channels as the primary target of pyrethroids. *Neurotoxicology, 6,* 3-22
18. *Perry P., Wolff S.* 1974.: New Giemsa method for the differential staining of sister chromatids, *Nature, 251,* 156-158
19. *Surrallés J., Antoccia A., Creus A., Degrassi F., Peris F., Tanzarella C., Xamena N., Marcos R.* 1994: The effect of cytochalasine B concentration on the frequency of micronuclei induced by four standard mutagens.Results from two laboratories. *Mutagenesis, 9,* 347-353
20. *Surrallés J., Xamena N. Creus A. Marcos R.* 1995: The suitability of the micronucleus assay in human lymphocytes as a new biomarker of excision repair. *Mutat. Res., 342,* 43-59

EVALUACIJA CITOTOKSIČNIH I GENOTOKSIČNIH EFEKATA PERMETRINA UPOTREBOM *IN VITRO* MIKRONUKLEUS TESTA

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SADRŽAJ

Cilj ovih ist'ivanje bio je da se izvrši evaluacija efekata sintetičkog piretroidnog insekticida permetrina analizom mitotske aktivnosti i pojave mikronukleusa u kulturama humanih limfocita. U standardnom *in vitro* mikronukleus testu upotrebljene su četiri eksperimentalne koncentracije permetrina: 1.2, 3, 6 i 12 mg/ml. Koncentracija od 6 mg/ml proračunata je da odgovara LD_{50} dozi kod pacova. Vrednosti proliferacionog indeksa pri blokiranoj citokinezi (CBPI) kao pokazatelja ćelijske kinetike, smanjene su pri svim koncentracijama permetrina osim najniže. Analize frekvenci mikronukleusa, međutim, ukazuju na odsustvo genotoksičnih efekata.