

In vitro and *in vivo* studies demonstrate non-mutagenicity of the herbicide metolachlor

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ABSTRACT

The herbicide metolachlor was evaluated for genotoxic potential. Metolachlor did not induce micronuclei in mice, however at 40 mg/kg it significantly decreased the percentage of polychromatic erythrocytes, which is a cytotoxic effect. Metolachlor did not induce chromosomal aberrations in human lymphocytes *in vitro*, but 2.0 µg/ml culture medium resulted in cytotoxicity, decreasing the mitotic index significantly. The indirect exposure test was carried out by adding plasma from metolachlor-pretreated rats to the human lymphocyte cultures. There was no indication of clastogenicity by metolachlor metabolites. On the other hand, plasma of cyclophosphamide-pretreated rats had a significant clastogenic effect.

INTRODUCTION

Acetanilide herbicides, including metolachlor, have been widely used in agriculture for many years, but relatively few genotoxicological data are available for these moderately toxic compounds (Pintér *et al.*, 1992). Known mutagenic effects include the ability of metolachlor to induce mutations in *Salmonella* with and without S9 activation and in *Saccharomyces* with S9 activation (Plewa, 1978). Roloff *et al.* (1992) demonstrated metolachlor cytogenetic damage in human lymphocytes exposed *in vitro*.

The mutagenic and/or carcinogenic hazard of an organic xenobiotic depends on at least two factors: the induction of detoxifying mechanisms and the proficiency of DNA repair systems. Biotransformation increases solubility and facilitate subsequent excretion. Whereas biotransformation normally results in detoxification, some xenobiotics may also be converted into highly reactive electrophilic metabolites which can induce deleterious effects (Buhler and Williams, 1988).

MATERIAL AND METHODS

The metolachlor used was an emulsifiable formulation available commercially under the name DUAL 960 CE (lot 69000-02) obtained from CIBA-GEIGY of Brazil. It is liquid at room temperature and is moderately soluble in water (530 ppm). Metolachlor is a member of the chloroacetanilide class of herbicides. The chemical name of metolachlor is 2-chloro-6-ethyl-N-(2-methoxy-1-methylethyl) acet-0-toluidine.

Mouse micronucleus test

Lethal doses (LD 50%) for mice are 50 mg/kg body weight, previously determined in our laboratory. The Swiss mice, from the Central Animal Facility of Universidade de Brasília, were divided into 10 groups of six animals and treated with metolachlor dissolved in distilled water at concentrations of 10, 20 and 40 mg/kg body weight, which means 20, 40 and 80% of LD 50%, respectively. All mice were treated intraperitoneally (*ip*) for 24 and 48 h. They were fed Purina mouse chow and filtered water *ad libitum*. The bone marrow preparations for micronucleus analysis were made according to Schmid (1975). At least 2000 cells per animal were counted and classified as polychromatic erythrocytes (PCE) and normochromatic erythrocytes

(NCE). The results were evaluated using chi-square analysis. Each group was compared with the control group. According to Schmid (1975), the relationship of polychromatic to mature erythrocytes is fairly constant, at a proportion of 1:1. Cytotoxic effects on bone marrow cell proliferation was evaluated by scoring the populations of PCE and NCE, and calculating the percentage of PCE.

Lymphocyte test

Eight independent cultures of human lymphocytes were set up for each experimental *in vitro* test. Six blood samples (10 ml) were obtained from healthy non-smoking adults who were not on medication (three males and three females, 25-37 years old). Leukocyte-rich plasma was distributed among 48 culture tubes containing 5 ml RPMI 1640, enriched with 10% calf serum, 1% L-glutamine, penicillin-streptomycin, and 0.1 ml phytohemagglutinin. After 5 h of incubation at 37°C, the cultures were directly exposed to medium containing 0.5, 1.0 or 2.0 µg/ml metolachlor, 0.6 µg/ml cyclophosphamide, 0.5 ml of sterile rat plasma, 0.5 ml of plasma from a metolachlor-pretreated rat and 0.5 ml of plasma of cyclophosphamide-pretreated rats. The cultures were returned to the incubator for more 43 h at 37°C, with colchicine added during the last 2 h. The bioassay for indirectly acting mutagens was carried out after metolachlor rat metabolization. Metolachlor was administered to rats by *ip* injection, dissolved in water at a concentration of 50 mg/kg body weight (2 doses with 24-h interval). One hour after the last treatment, blood was drawn by cardiac puncture with a heparinized needle. Cyclophosphamide, 30 mg/kg body weight *ip*, was also used as a positive control for indirectly acting mutagens, according to Natarajan *et al.* (1983).

Harvesting and slide preparation

After 48-h incubation, the cultures were harvested by centrifugation at 200 g for 10 min, followed by 5 min in 0.075 M KCl for hypotonic treatment at 37°C. Next, the cells were centrifuged, and methanol/acetic acid (3:1) solution was added. This fixation step was repeated twice and the resulting cells were resuspended to prepare the slides, which were stained with 5% Giemsa (pH = 6.8) for 5 min and scored blindly.

RESULTS

Metolachlor did not significantly induce micronucleated polychromatic erythrocytes in mice (χ^2 test,

$P = 0.49$). The treatment also did not markedly reduce the proportion of young erythrocytes. Under metolachlor influence bone marrow proliferation was normal, except for the highest dosage after 48-h treatment, which significantly decreased the percentage of PCE (Table I).

In the human lymphocyte *in vitro* test, metolachlor did not induce chromosomal aberrations, but at 2.0 µg/ml it showed cytotoxic effects, significantly decreasing the mitotic index. At dosages of 1.0 and 2.0 µg/ml, the frequencies of chromosomal aberrations were at the same level as our historical laboratory control. The indirectly acting exposure test by *in vivo* rat metabolization did not show any metolachlor metabolite clastogenicity, although the plasma of cyclophosphamide-pretreated rats had a significant effect (Table II).

DISCUSSION

Roloff *et al.* (1992) found significant chromosomal damage with 1.0 µg/ml metolachlor in the human lymphocyte *in vitro* test, a concentration which was non-toxic based on mitotic indices analysis. However, we found no evidence for any clastogenic effect of metolachlor even at a higher concentration of 2.0 µg/ml medium, which was cytotoxic, decreasing the mitotic index (Table II).

In human lymphocyte tests we added plasma of a rat previously treated with metolachlor to whole

Table I - Micronuclei (MN) mean in polychromatic erythrocytes of metolachlor-treated mice (6 animals per treatment group).

Treatment mg/kg	MN-PCE ($\mu \pm$ SD)	MN-NCE ($\mu \pm$ SD)	PCE %
24 h			
Control	1.8 \pm 0.08	2.4 \pm 0.12	49.1
20-CP	22.2 \pm 0.81	4.4 \pm 0.12	34.8
10	2.0 \pm 0.10	2.6 \pm 0.20	41.8
20	1.4 \pm 0.04	1.2 \pm 0.08	44.1
40	1.8 \pm 0.06	0.8 \pm 0.05	34.3
48 h			
Control	1.6 \pm 0.04	2.6 \pm 0.14	48.1
20-CP	18.2 \pm 1.00	4.6 \pm 0.90	33.7
10	2.8 \pm 0.06	3.4 \pm 0.86	39.7
20	2.4 \pm 0.03	2.0 \pm 0.12	34.8
40	1.6 \pm 0.05	1.2 \pm 0.06	26.7*

*0.01 < P < 0.05, *t*-test for paired samples. CP = Cyclophosphamide. PCE and NCE = Polychromatic and normochromatic erythrocytes, respectively.

$$\% \text{ PCE} = \frac{\text{PCE}}{\text{PCE} + \text{NCE}} \times 100$$

Table II - Chromosomal aberrations in human lymphocytes *in vitro* treated with metolachlor directly and indirectly or cyclophosphamide (CP).

Treatment	Cells analyzed	G'	B'	G''	B''	C.A. (%)	Polyploid cells	Aneuploid cells	MI (%)
Control	600	4	3	2	2	1.83	1	3	5.97
CP - 0.6 µg/ml	600	16	24	11	15	11.0*	3	1	2.18
0.5 µg/ml	400	2	1	0	0	0.75	1	3	3.25
1.0 µg/ml	382	7	9	1	1	4.71	-	2	2.15
2.0 µg/ml	365	5	0	4	4	3.56	2	5	1.50*
Non-treated rat plasma	320	6	9	1	-	5.00	1	2	1.90*
Treated rat plasma	323	7	9	1	2	5.88	-	1	1.11*
CP-treated rat plasma	560	8	13	11	15	8.39*	1	2	2.18

*0.01 < P < 0.05, *t*-test for paired samples. C.A. = Chromosomal aberrations; MI = mitotic index; G', G'', B' and B'' = chromatid and isochromatid-type gaps and breaks, respectively.

blood cultures to exploit the metabolic capability of rats highly contaminated with metolachlor. As we had insufficient information on the rate and pathway of metabolism of metolachlor under these conditions, a rat treatment schedule of 2 doses of 50 mg/kg body weight with a 24-h interval was chosen to avoid excessive cytotoxicity. Natarajan *et al.* (1983) used an *in vivo* activation system to assess possible genotoxic activity of chemical agents. This system demonstrates the mutagenicity of known indirectly acting chemical mutagens. Plasma of cyclophosphamide-pretreated rat significantly increased the frequencies of chromosomal aberrations in human lymphocytes (Table II). Darroudi and Natarajan (1985) detected increased sister chromatid exchange frequencies in Chinese hamster ovary cells, which were treated *in vitro* with rat plasma containing metabolites of indirectly acting chemicals, such as diethylnitrosamine, dimethylnitrosamine and benzo(a)pyrene.

Metolachlor was not found to be clastogenic in mouse polychromatic erythrocytes and in human lymphocytes, both directly and after rat metabolism (Table I and II). The mitotic indices in plasma-treated cultures were significantly lower than those found in the control group. This result reveals some anti-proliferative activity of plasma *per se*, while the effect of plasma from treated rats was not significantly different from that of control rats. Thus, the observed effect was probably due to rat plasma and not metabolites of metolachlor. In mice, cytotoxicity was demonstrated by a decrease in the percentage of PCE at 40 mg/kg for 48 h.

The present study demonstrates a non-genotoxic hazard by metolachlor. Considering the conflicting results reported here vs. previous studies, it is suggested that further assays using other short-term

mutagenicity test systems should be carried out for a better assessment of the genotoxic potential of metolachlor.

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RESUMO

O herbicida metolachlor foi avaliado quanto ao seu potencial genotóxico. Em camundongos não houve indução de micronúcleos nos eritrócitos policromáticos de medula óssea; entretanto, com a dose mais forte de 40 mg/kg peso por 48 h, observaram-se efeitos citotóxicos através da queda acentuada na porcentagem de eritrócitos policromáticos em relação aos normocromáticos. O metolachlor também não induziu aberrações cromossômicas em linfócitos humanos quando tratados *in vitro*, porém na concentração de 2,0 µg/ml de meio ocorreu queda no índice mitótico, demonstrando a toxidez do tratamento. Para se estudar os efeitos genotóxicos de possíveis metabolitos do metolachlor, ratos Wistar foram tratados com duas doses de 50 mg/kg com um intervalo de 24 h. Uma hora após o último tratamento, seus plasmas foram coletados através de punção cardíaca e adicionados às culturas de linfócitos humanos. Os resultados também demonstraram efeitos citotóxicos mas não de mutagenicidade.

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