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SHORT COMMUNICATION

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In vivo genotoxicity of selected herbicides in the mouse bone-marrow micronucleus test

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Abstract The herbicides alachlor, atrazine, terbuthylazine, gluphosinate-ammonium, isoproturon, pendimethaline and trifluralin were tested for genotoxicity in the mouse bone-marrow micronucleus test (MNT). Both atrazine and trifluraline caused a significant increase in the number of micronuclei at doses of 1400 mg/kg body weight in female mice only. Alachlor, terbuthylazine, gluphosinate-ammonium, isoproturon and pendimethaline did not have any genotoxic effect in the mouse bone-marrow micronucleus test in either female or male animals.

Key words Mouse bone-marrow micronucleus test · Herbicides · Atrazine · Trifluraline

Introduction

Pesticides of worldwide application are used in agriculture in vast amounts each year, of which herbicides are the most prominent class. Putative detrimental effects on life caused by pesticides should be known and minimized, most especially because of the extensive environmental distribution of these xenobiotics. In the present study, selected herbicides of different chemical classes were tested for genotoxicity in the micronucleus test (MNT) in vivo. Alachlor is a chloroacetanilide and is chemically decomposed via aniline derivatives. Atrazine and terbuthylazine are triazines, which inhibit plant photosynthesis. Gluphosinate-ammonium is a phosphorous-containing amino acid derivative, which inhibits plant photosynthesis and the enzyme glutamine synthetase. Isoproturon is a derivative of urea and is decomposed via dealkylation and hydrolysis. Pendimethaline and trifluralin are 2,6-dinitroanilines, which inhibit cell mitosis by interaction with microtubuli (reviewed in Dunkelberg and Edenharder 1994). In Germany, a restriction was put on atrazine in March 1991 because this compound had been detected several times in ground and drinking water (Börner 1994; Mull et al. 1994). Furthermore, the use of alachlor is no longer legalized in Germany as it was not readmitted for use.

Micronucleus test systems play an important role in genetic toxicology in evaluating the genotoxic potentials of xenobiotics. The quantitative assessment of micronucleus-containing cells serves as an indicator for the induction of structural and/or numerical chromosomal aberrations. The mouse bone-marrow MNT is accepted and widely used as a rapid test in science and industry. The genotoxic potency of a chemical in this test is assessed by scoring micronuclei in polychromatic erythrocytes (Romagna 1993).

The present study was part of an interdisciplinary programme constituting ten research groups in geology, soil ecology, and drinking water hygiene. The main aim of this programme was to investigate the spread of pesticides in soil and ground water. Herbicides, insecticides and nematicides are the classes of compounds most likely to endanger ground water purity. The compounds included in the programme were selected because they had been detected in German drinking water or because they were presumed to be able potentially to contaminate ground water. Twenty-one pesticides in widespread use meeting these criteria were chosen, comprising mainly the chemical classes triazines, urea derivatives, carbamates, phenoxy acids and esters of phosphoric acid. Seven of these xenobiotics were herbicides and were included in this project to evaluate any in vivo genotoxic mode of action because of a possible

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deleterious effect on human health in the groundwater, i.e. drinking-water contamination.

Materials and methods

Chemicals

The herbicides tested were obtained in the highest purity grade available (alachlor 98.3%, atrazine 98.7%, gluphosinate-ammonium 99.9%, isoproturon 99.9%, pendimethaline 99.0%, trifluralin 99.5%, terbuthylazine 99.9%) from Promochem (Wesel, Germany).

Test protocol

The in vivo micronucleus test was performed as follows (OECD-Guideline 474, 1983): healthy young NMRI mice (7–12 weeks old, body weight 25–35 g) were randomized and assigned to the respective treatment and control groups. Each pesticide was tested in male mice in four doses approximating 50, 80, 100 and 115% of published LD_{50}-values (Pesticide Manual 1995; Industrieverband Agrar 1990; Perkow 1988) and subsequently in female mice in two doses corresponding to 80 and 100% of the LD_{50}-values. By so doing, the LD_{50} was used to define a maximum tolerated dose (MTD; Mavournin et al. 1990). The lower number of treatment schedules with female mice was chosen as a result of the findings obtained with male mice in order to save animals. The respective doses were applied to four female and four male NMRI mice each. Cyclophosphamide (male, 600 mg/kg body weight; female, 450 mg/kg body weight) was used as positive control. The test substances were dissolved in 200–300 µl corn oil and administered to the animals by oral gavage. As negative control, 300 µl of corn oil only was used. Most of the herbicides were given by one application. However, because of the high concentration tested, isoproturon and terbutylazine had to be applied in six portions (each 300 µl of corn oil as solvent) with an intermission of 1 h between each application.

The animals were caged by sex in groups of four. Standard laboratory diet and drinking water were supplied ad libitum. Temperature (22 ± 2 °C), humidity (55 ± 5%) and day-night light cycles (12:12 h) were controlled as dictated by good procedures of animal husbandry. After 48 h the animals were killed by cervical dislocation and the femurs were excised. With the help of a syringe, the bone-marrow from both femurs was flushed into a tube using 5 ml fetal calf serum and centrifuged for 15 min at 4 °C at 200 *g*. The supernatant was discarded, and the pellet was carefully resuspended in the remaining ~100 µl. One drop of this suspension was applied to a slide, lying on a chilled glass top. Slides were prepared in quadruplicate for each animal. The slides were air-dried for 12 h, stained for 3 min in May-Grünwald solution, and for 2 min in a solution of May-Grünwald/demineralized water, 1:1 (v/v). The slides were washed with demineralized water, stained in Giemsa solution for 10 min and washed once again. After air-drying for 30 min the smears were finally fixed in xylene for 10 min, two drops of Entellan (Merck, Darmstadt, Germany) were added and the slides were dried again.

Counting and statistic evaluations

For MNT cell and micronuclei counting, the slides prepared as described above were coded for blind analysis. At least 1000 polychromatic erythrocytes per animal were scored for the presence of micronuclei. The ratio of polychromatic to normochromatic erythrocytes was determined by counting a total of 1000 erythrocytes. The chi-square test and the *U*-test according to Mann, Whitney and Wilcoxon were used to evaluate statistically significant differences between the collectives examined. Data given are of mean values and standard deviations of *n* number of individuals examined.

Results and discussion

The results of the herbicides tested in the present study are provided in Table 1. Only the highest tested dose that was tolerated by all animals is listed, thus resulting in a 100% survival rate. The fact should be taken into account that all herbicides had been used in the highest grade of purity available. The genotoxicity of the pesticide products in agricultural use was not tested. In some cases the genotoxicity may differ because of technical impurities, as is known for e.g. trifluralin (see below). Alachlor, gluphosinate-ammonium, isoproturon, pendimethaline, and terbuthylazine did not significantly induce the rate of micronucleated polychromatic erythrocytes either in male or female animals. In contrast, application of atrazine and trifluralin resulted in a significant increase in the frequencies of micronuclei in female mice in comparison to the untreated animals (Table 2). Surprisingly, nearly all the means of the PCE/NCE ratios were higher in female compared to male animals. We have no explanation for these observations and no reason to assume that there is a technical cause for the differences observed.

A statistically significant increase ($P < 0.05$) with a mean of 12.44 micronuclei per 10^3 polychromatic erythrocytes was caused in female mice by 1400 mg atrazine/kg body weight (Tables 1 and 2). The results of the application regime of 1750 mg/kg body weight were not utilized because only two animals survived (Table 1). In contrast, the frequency of micronuclei was not increased in male mice by atrazine at any concentration. Most investigations on cells in vitro did not show any genotoxic potential of atrazine (IARC 1991a). Another study concludes that a definitive conclusion concerning a putative clastogenicity of atrazine cannot be drawn (EPA 1988). Meisner et al. (1992) reported in vivo genotoxicity for atrazine only in combination with alachlor: a significant increase in the frequency of chromosome aberrations in mouse bone-marrow cells was found. In cultivated human peripheral human lymphocytes these authors found a single application of atrazine to increase the rate of chromosomal aberrations. Furthermore, atrazine genotoxicity was found using as plant metabolic activation assays either potato microsomes or extracts of atrazine-treated plants (reviewed in Dunkelberg and Edenharder 1994; IARC 1991a; Gentile et al. 1977; Plewa et al. 1984). Atrazine induced tumours in the lymphatic and the haematopoetic system (Pinter et al. 1990), and was therefore classified as a substance with limited evidence for carcinogenicity in experimental animals by the IARC (1991a).

A weak, yet significant, induction of micronuclei frequencies ($P < 0.05$) was achieved with a dose of 1400 mg trifluralin/kg body weight again in female mice only (Tables 1 and 2). A further slight increase in the number of micronuclei (mean 5.88 micronuclei per 10^3 polychromatic erythrocytes) was caused by 2100 mg trifluralin/kg body weight. An increase in the rate of

Table 1 Frequencies (mean ± SD; $n = 4$) of micronucleated polychromatic erythrocytes in bone-marrow cells of NMRI mice after herbicide treatment (*MN* Micronuclei, *PCE* polychromatic erythrocytes, *NCE* normochromatic erythrocytes)

Treatment application	Highest tolerated dose (mg/kg body wt[a])	Sex	MN/10^3 PCE ± SD	PCE/NCE ± SD
Alachlor	531	m	0.55 ± 0.72	0.75 ± 0.14
	370	f	3.13 ± 0.25	1.39 ± 0.05
Atrazine	1750	m	1.45 ± 0.42	0.97 ± 0.29
	1400	f	12.44 ± 0.90*	1.31 ± 0.20
Gluphosinate-ammonium	474	m	3.38 ± 1.55	0.62 ± 0.23
	416	f	2.50 ± 0.54	1.15 ± 0.10
Isoproturon	3685	m	3.24 ± 0.38	0.56 ± 0.26
	3350	f	1.81 ± 0.31	1.38 ± 0.19
Pendimethaline	1280	m	3.00 ± 0.82	1.48 ± 0.38
	2899	f	1.75 ± 0.54	1.45 ± 0.11
Terbuthylazine	8250	m	2.25 ± 1.94	0.44 ± 0.13
	7700	f	1.44 ± 0.83	1.95 ± 0.07
Trifluralin	2300	m	2.30 ± 0.96	1.00 ± 0.10
	2100	f	5.88 ± 1.71*	1.55 ± 0.13
Cyclophosphamide ($n = 7$)	600	m	74.97 ± 8.79	0.63 ± 0.02
	450	f	78.43 ± 5.09	0.74 ± 0.07
Corn oil ($n = 7$)	–	m	0.63 ± 0.91	0.85 ± 0.46
	–	f	2.25 ± 1.05	1.61 ± 0.26

[a]Mice were tested in doses of 80 and 100% (50 and 115% additionally only for male mice) of the respective published LD_{50} values. The results are given for the highest tolerated dose, i.e. when all animals survived.
In each group a positive control was performed with cyclophosphamide (female mice, 450 mg/kg body wt.; male mice, 600 mg/kg body wt.)
*Statistically significant ($P < 0.05$) in the *U*-test according to Mann, Wilcoxon and Whitney in comparison to the female control

micronuclei in male mice was not detected at any trifluralin dose tested. The reason for the significant sex-specific genotoxicity in this study remains unclear. Other authors report trifluralin not to be genotoxic. It was neither mutagenic to *Salmonella* (Garriot et al. 1991; Shirasu et al. 1977; Waters et al. 1980) nor did it induce

Table 2 Induction (mean ± SD) of micronucleated polychromatic erythrocytes in bone-marrow cells of NMRI mice after treatment with atrazine and trifluralin

Herbicide	Dose in mg/kg body wt.	Number of animals	MN/10^3 PCE ± SD	PCE/NCE ± SD
Atrazine				
Male mice	900	4	0.63 ± 0.25	0.47 ± 0.10
	1200	4	0.38 ± 0.25	0.46 ± 0.16
	1500	4	2.00 ± 1.41	0.74 ± 0.27
	1750	4	1.45 ± 0.42	0.97 ± 0.29
	Corn oil	1	0.50	0.68
	Cyclophosphamide	1	78.0	0.61
Female mice	1400	4	12.44 ± 0.90*	1.31 ± 0.20
	1750	4/2[a]	6.50 ± 0.71	1.64 ± 0.08
	Corn oil	1	3.00	1.61
	Cyclophosphamide	1	71.5	0.84
Trifluralin				
Male mice	1200	4	0.68 ± 0.29	0.87 ± 0.22
	1600	4	1.10 ± 0.42	1.05 ± 0.33
	2000	4/3[a]	1.37 ± 1.09	0.69 ± 0.18
	2300	4	2.30 ± 0.96	1.00 ± 0.10
	Corn oil	1	2.0	1.81
	Cyclophosphamide	1	74.0	0.17
Female mice	1680	4	5.31 ± 0.69*	1.40 ± 0.04
	2100	4	5.88 ± 1.71*	1.55 ± 0.13
	Corn oil	1	3.00	1.44
	Cyclophosphamide	1	84.0	0.75

[a]Number of surviving animals
Only one animal per sex, per negative and per positive control was chosen in order to save animals. In each group a positive control with cyclophosphamide (female mice, 450 mg/kg body wt.; male mice, 600 mg/kg body wt.) was performed.
*Statistically significant ($P < 0.05$) in the chi-square test in comparison to the control of the respective sex

chromosomal aberrations (Galloway et al. 1985; Garriot et al. 1991). Technical-grade trifluralin was often found to be contaminated with N-nitroso-*n*-propylamine, which was the reason for genotoxicity in some trifluralin studies (IARC 1991b). Thus, the maximum level of N-nitroso-*n*-propylamine occurring in technical trifluralin in Germany had been restricted to 0.5 mg/kg. The trifluralin used in the present study was of the highest available purity and thus is not contaminated with N-nitroso-*n*-propylamine. The US EPA (1987) classifies trifluralin as a substance with limited evidence for carcinogenicity in experimental animals.

Atrazine, pendimethaline and trifluralin were found to be negative in an analysis of sister chromatid exchange (SCE) either with or without metabolic activation (Dunkelberg et al. 1994). Alachlor has been shown to induce chromosomal aberrations, sister chromatid exchanges and micronuclei in mammalian cells in vitro (Georgian et al. 1983; Meisner et al. 1992; Lin et al. 1987). We found alachlor to be negative in provoking micronuclei (Table 1), but in a previous investigation we reported a significant induction of SCE without metabolic activation. In contrast, we could not find any SCE induction in the presence of metabolic activation (rat liver S9 mix, Dunkelberg et al. 1994).

Mutagenic effects were induced in prokaryotic and lower eukaryotic cells only after plant metabolic activation of alachlor (Gentile et al. 1977; Plewa et al. 1984). Although single treatment with either alachlor or atrazine did not show an effect on the rate of chromosomal aberrations, a combined treatment with 20 ppm alachlor and 20 ppm atrazine was shown to significantly increase the rate of chromosomal aberrations in bone-marrow cells of mice (Meisner et al. 1992). The US EPA (1986) classified alachlor as substance with a carcinogenic potential because of reported observations in mice, in which were induced nasal and thyroid gland adenomas as well as lung tumours.

Published data concerning the genotoxicity of terbuthylazine and gluphosinate-ammonium are sparse. Our own investigations did not show any evidence for genotoxicity in either the *Salmonella*-test or the SCE assay (Dunkelberg and Edenharder 1994). Gluphosinate-ammonium may be of increasing importance in the future because of economically important plants that are genetically engineered for resistance against this herbicide. To our knowledge, there are no data published concerning the carcinogenicity of isoproturon. In contrast to our findings for a lack of clastogenicity and aneugenicity of isoproturon in the in vivo micronucleus test (Table 1), Behera and Bhunya (1990) found significantly elevated rates for chromosomal aberrations and micronuclei in Swiss mice. Our own investigations in the *Salmonella*-test and in the SCE assay did not show any evidence for genotoxicity of isoproturon (Dunkelberg and Edenharder 1994). Few original data concerning the genotoxicity or carcinogenicity of pendimethaline are available. The data summarized by WHO (1987) did not give any indication for DNA reactivity of pendimethaline. The US EPA (1984) reported three studies concerning pendimethaline: mutagenic activity was not found in one dominant lethal study, a *Salmonella*-test and one mouse host-mediated assay.

Some authors proposed to use male mice exclusively for micronucleus testing of chemicals on account of an assumed higher sensitivity (Shelby 1987; The Collaborative Study Group for Micronucleus Test 1986). However, the analysis of available experimental data with positive results, presented separately for male and female animals, showed little or no sex differences for about two-thirds of the compounds tested but a greater micronucleus response of several substances in males or females with no preference for either sex (Mavournin et al. 1990). Our results showing a unique response of female mice support the concept of using male as well as female animals to check for micronuclei inducing pesticides, to ensure no positive results are missed.

The present study had been performed using only NMRI mice as test animals. The possibility of any interpretation of the results should take into account that species-specific differences in pesticide susceptibility as well as in metabolic capacity can influence genotoxicity. This is known e.g. for alachlor in mice, rats and monkeys (Feng et al. 1990; Li et al. 1992). In summary, of the herbicides tested in the present work, atrazine and trifluralin revealed significant aneugenic/clastogenic activities in the micronucleus test in vivo in female NMRI mice. However, these results only could be achieved in female animals at doses near to the maximum tolerated dose. Thus, an in vivo genotoxic potential for trifluralin and atrazine seems questionable.

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