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Evaluation of the Genotoxic Potential of Flumethrin in Mouse Bone Marrow by Chromosomal Analysis and Micronucleus Test

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The genotoxic potential of the pyrethroid flumethrin was evaluated by using the combined protocol of metaphase analysis and micronucleus test in vivo in mouse bone marrow. The dermal route was tested in a single treatment and the intraperitoneal (i.p.) route in a single and a multiple treatment. Flumethrin showed a cytotoxic effect on both myelopoiesis and erythropoiesis, as evidenced by a reduction in the mitotic index and in polychromatic erythrocyte values. An increase in the frequency of gaps after the dermal exposure and of breaks only at the highest dose tested in the i.p. treatment indicates a weak clastogenic potential of the compound. A significant increase in the frequency of micronucleated polychromatic erythrocytes was observed after single and multiple i.p. treatments. In the latter, the induction of micronuclei was highly significant but not accompanied by an increase in breaks. This may indicate that the clastogenic effect might not account by itself for the induction of micronuclei, which could also have arisen from an aneugenic potential of flumethrin.

Key words: **bayticol, synthetic pyrethroid, mutagenicity, micronuclei, chromosomal aberrations, mitotic index, in vivo test, combined protocol, single and multiple dosing**

INTRODUCTION

Natural pyrethrins and related synthetic compounds are known for their efficient insecticide properties, wide spectrum of activity, and low toxicity to mammals [1]. Restricted at first to closed environments, their use has been extended to agriculture, after the development of the first photostable compounds [2]. Today, pyrethroids

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represent 25% of all insecticides used per year in agriculture and public health [3]. The mutagenic potential of some of these compounds has been demonstrated [4–16].

The synthetic pyrethroid flumethrin is the main active element in Bayticol, an acaricide for veterinary use. There are no data regarding its mutagenic, teratogenic, or carcinogenic potential.

The use of several mutagenicity tests for a product performed in the same animal has been proposed by Connor et al. [17]. By combining chromosomal aberration analysis with the micronucleus test, information can be obtained both on the clastogenic and the aneugenic potential of a given agent, since micronuclei may also arise from effects on chromosomal segregation. This was the approach used in this paper for the genotoxic evaluation of flumethrin.

MATERIALS AND METHODS

Animals

Male albino Swiss Webster mice, 7–11 weeks old, were obtained from the colony maintained by the Instituto Butantan and acclimated to the laboratory animal-room environment for 1 week before use. The animals were kept in polypropylene cages, at temperatures ranging from 22 to 24°C and relative humidity 50–70%, and exposed to a 12 h light/12 hr dark cycle throughout the acclimation and experiment periods. Food (Purina Certified Rodent Chow) and water were given ad libitum.

Chemicals

Flumethrin (Technical Bayticol 60%, Bayer do Brasil) was administered as such for the dermal treatment and diluted in corn oil in the intraperitoneal (i.p.) exposure. Corn oil also served as the negative control for both types of exposure. Cyclophosphamide (Enduxan, injectable, Abbott Laboratories, Abbott Park, IL) was dissolved in bidistilled water and used as a positive control.

Treatment Schedule and Routes of Exposure

Dermal exposure was made according to Noakes and Sanderson [18]. Forty-eight hours prior to treatment, each animal had an area of approximately 3 × 4 cm shaved on the back. Animals with intact skin received either the product or corn oil; the area was then covered with aluminum paper and adhesive tape; both were removed 24 h afterward. Three groups of 6 animals received a single dose of technical Bayticol [0.05 ml/10 g body weight (b.w.), the maximum applicable dose (MAD)]. Three groups of 5 animals received the same volume of corn oil (controls with bandage). Fifteen animals received no treatment (controls without bandage). Animals were killed after 24, 48, and 72 hr. The MAD—5,325 mg/kg b.w.—was determined as the maximum applicable volume in the shaved area.

The i.p. route was tested in a single and a multiple treatment.

The choice of the doses used in the i.p. exposure was preceded by the determination of the acute LD50 and the maximum tolerated dose (MTD). The LD50 value (2,778 mg/kg b.w.) was determined according to Thompson [19]. A value of 128 mg/kg b.w. was determined according to Thompson and Gibson [20] as the MTD in terms of lethality (up to 20%).

For the single treatment, 3 groups of 10 animals were exposed to 0.25, 0.50,

and 0.75 LD50 (0.05 ml/10 g b.w.). The control group of 10 animals received the same volume of corn oil.

A positive control group of 6 animals receiving a single i.p. administration of cyclophosphamide (25 mg/kg b.w.—0.10 ml/10 g b.w.) and killed 24 hr afterward was used, merely to test for the sensitivity of the system.

The MTD replaced the LD50 in the multiple treatment, since the same fractions of the LD50 used in the single treatment were too toxic when used in multiple dosing.

Two experiments were performed using fractions of the MTD. In the first, 1 group of 12 animals received 4 daily administrations of 50 mg/kg b.w. (0.05 ml/10 g b.w.) of flumethrin solution; 12 animals received 4 daily administrations of 80 mg/kg b.w. (0.05 ml/10 g b.w.); and 10 animals received 4 daily administrations of equal volumes of corn oil. In the second, 1 group of 12 animals received 4 daily administrations of 128 mg/kg b.w. (0.05 ml/10 g b.w.). Ten animals served as negative controls.

All animals exposed through the i.p. route were killed 24 hr after the last administration. The choice of this sampling time was based on the evaluation of the mitotic index of 15 animals after a single administration of 0.75 LD50 of the product.

Bone Marrow Preparations

The animals received colchicine (100 mg/kg b.w.—0.10 ml/10 g b.w.) 2 hr prior to sacrifice by cervical dislocation. One femur from each was used for metaphase preparations [21] and the other for smears for the micronucleus test [22]. The slides were prepared, stained, and coded for analysis. Metaphase preparations were stained in buffered Giemsa, pH 6.8 (Merck, Darmstadt, Germany) and the smears in Wright stain solution (Fischer Scientific Company, Fair Lawn, NJ), according to Schmid [22].

One hundred metaphases per animal were scored for the frequency of gaps, breaks, and rearrangements. Gaps are defined as an unstained region in the chromatid with no dislocation of the fragment; the dislocation characterizes the break. Rearrangements result from chromatid or chromosomal breaks followed by exchange [23].

Mitotic Index (MI)

A total of 3,000 cells, 1,000 per slide, were scored for determining the frequency of dividing cells.

Micronuclei Frequency

A total of 2,000 erythrocytes were scored for determining the frequency of polychromatic erythrocytes (PCE); 2,000 PCE, 1,000 from each of 2 prepared slides, were scored to assess the frequency of micronuclei.

Statistical Analysis

The exact conditional test to compare Poisson means was used to evaluate the different groups with respect to gaps, breaks, rearrangements, and total number of cells with aberrations. This kind of analysis is appropriate for rare events.

An approximation of the exact test to chi-square was used in the case of the micronucleated PCE [24].

With respect to MI and the frequencies of PCE, the one-way analysis of variance (ANOVA) was used. These events, although having a low probability, are not rare, and a large number of cells was studied. Thus, an approximation of the binomial to normal is adequate.

Since most of the times $P > 0.10$, we state that there is no significant difference, but do not specify the values.

RESULTS

Dermal Treatment

Tables I and II show the data obtained from animals exposed through the dermal route.

The bandage technique per se did not affect the animals, since there were no statistically significant differences between the control groups with and without bandage in the MI, frequency of chromosomal aberrations, and micronucleated PCE.

MI. After finding no significant differences between control groups with or without bandage ($P = 0.1433$), nor among groups exposed to the MAD and killed 24, 48, and 72 hr afterward ($P = 0.3866$), these 2 homogeneous classes (controls × exposed) were compared and showed that flumethrin significantly reduced the MI ($P = 0.0001$).

Chromosomal aberrations. There was a significant increase in the frequency of gaps in the group exposed to the compound and killed after 24 hr ($P = 0.0018$). For breaks and rearrangements, no significant differences among groups were found. Thus, excluding gaps, there were no significant differences between exposed and control groups.

Micronucleus test. The frequencies of PCE and of micronucleated PCE were not significantly different either between exposed and non-exposed animals or among the different sampling times.

I.p. Treatment

Single dosing. Tables III and IV show the data obtained from single i.p. exposure.

MI. The single i.p. treatment caused a reduction in the MI of the animals exposed to 0.25, 0.50, and 0.75 LD50. Significant differences were found among all groups ($P = 0.004$). The highest dose had a stronger effect on cell division.

Chromosomal aberrations. No significant effect of flumethrin was observed on the frequencies of gaps and rearrangements for all doses tested. There were no statistically significant differences in the total number of cells with aberrations (including or excluding gaps) between controls and groups exposed to 0.25 and 0.50 LD50. When comparing these to the group exposed to 0.75 LD50, a significant increase was observed in the frequency of cells with aberrations (excluding gaps) ($P = 0.0128$); this difference was due to the higher frequency of breaks.

Micronucleus test. There were no statistically significant differences between controls and exposed animals in the frequencies of PCE.

A significant increase in the frequency of micronucleated PCE was observed only at the highest dose ($P = 0.0466$).

Multiple dosing. Tables V and VI show the data obtained from the multiple i.p. exposure.

MI and chromosomal aberrations. No significant effect of flumethrin was observed on the MI and on the frequencies of gaps, breaks, rearrangements, and total cells with aberrations (including or excluding gaps) for all doses tested.

TABLE I. Dermal Treatment: Chromosomal Aberrations and MI in Mouse Bone Marrow After Single Dosing of Flumethrin at the MAD

Group	No. of animals	Total no. of cells	Sampling time (hr)	Cells with abberations					MI	
							Total (%)		Individual data	
				Gaps	Breaks	Rearrangements	Without gaps	With gaps	(metaphases/3,000 cells)	Mean (SD)
Control without bandage	15	1,500	24	15	3	1	4 (0.27)	19 (1.27)	67; 78; 78; 78; 68; 68; 124 104; 91; 130; 55; 102; 98; 92; 72	87.00 (21.56)
Control	5	500	24	7	1	0	1 (0.20)	8 (1.60)	73; 79; 77; 85; 96	82.00 (8.94)
with	5	500	48	5	2	0	2 (0.40)	7 (1.50)	121; 96; 101; 106; 114	107.60 (10.01)
bandage	5	500	72	1	1	0	1 (0.20)	2 (0.40)	76; 59; 102; 107; 98	88.40 (20.26)
Exposed	6	600	24	13*	2	1	3 (0.50)	16 (2.67)*	59; 71; 71; 78; 56; 73	68.00 (8.58)*
	6	600	48	2	4	0	4 (0.67)	6 (1.00)	64; 81; 68; 56; 51; 78	66.33 (11.84)*
	6	600	72	4	3	0	3 (0.50)	7 (1.17)	58; 67; 64; 52; 44; 74	59.83 (10.81)*

*Significant difference.

TABLE II. Dermal Treatment: Frequencies of PCE and Micronucleated PCE in Mouse Bone Marrow After Single Dosing of Flumethrin at the MAD

Group	No. of animals	Sampling time (hr)	Micronucleated PCE: Individual data (in 2,000 PCE/animal)	Micronucleated PCE: Total (%)	PCE: Individual data (in 2,000 erythrocytes)
Control without bandage	15	24	4; 2; 0; 2; 3 5; 4; 4; 1; 4 1; 4; 1; 3; 5	43 (0.14)	441; 764; 428; 439; 460 520; 567; 736; 627; 948 431; 258; 442; 360; 514
Control with bandage	5	24	1; 5; 4; 6; 2	18 (0.18)	704; 411; 548; 516; 617
	5	48	2; 3; 6; 6; 3	20 (0.20)	721; 604; 535; 517; 508
	5	72	2; 1; 1; 1; 1	6 (0.06)	496; 533; 638; 783; 543
Exposed	6	24	1; 1; 1; 1; 4; 3	11 (0.09)	543; 494; 443; 349; 800; 763
	6	48	1; 1; 1; 2; 4; 3	12 (0.10)	604; 725; 447; 901; 641; 599
	6	72	1; 2; 2; 2; 1; 2	10 (0.08)	170; 670; 478; 432; 665; 537

Micronucleus test. The highest dose used (128 mg/kg b.w.) caused a reduction in the PCE values and an increase in the frequency of micronucleated PCE, both highly significant ($P = 0.0001$).

Positive controls. The animals exposed through the i.p. route to cyclophosphamide showed, as expected, increased frequencies of chromosomal aberrations and micronuclei, as well as a reduction in the MI and PCE values (Table VII).

DISCUSSION

The strategy in this work consisted in evaluating the genotoxicity of flumethrin using two exposure routes, two types of treatment, and the analysis of several cytogenetic endpoints in the same animal: MI, chromosomal aberrations, and frequency of PCE and of micronucleated PCE.

Dermal exposure was our first choice for administering the compound because it is mainly through the skin that absorption normally occurs in both man and animal. On the other hand, the bone marrow is not necessarily the most sensitive organ for the detection of mutagens topically applied, because of poor absorption or distribution to the bone marrow, or absence of metabolizing activity in this tissue [25,26]. The i.p. route eliminates the interindividual variability in absorption; it is also the route of choice for its simplicity and because it tends to maximize the exposure of the bone marrow [27].

Some experimental conditions are known to induce physiological alterations. Such is the case of stress caused by immobilization, to which an increase in chromosomal aberrations has been attributed [28]. There are no standard procedures for dermal exposure, but all of them restrict to some extent the animal's movements, causing some stress.

The results in this paper show that the bandage technique used did not alter any of the parameters analyzed, as seen by the comparison of the groups with and without bandage.

Flumethrin induced a reduction of the MI after the dermal treatment and the single i.p. exposure at all doses tested. This cytotoxic effect is shared by other

TABLE III. Single I.p. Treatment: Chromosomal Aberrations and MI in Mouse Bone Marrow After Single Dosing of Flumethrin

			Cells with aberrations					MI	
						Total (%)			
Dose (mg/kg)	No. of animals	Total no. of cells	Gaps	Breaks	Rearrangements	Without gaps	With gaps	Individual data (metaphases/3,000 cells)	Mean (SD)
0	10	1,000	2	5	1	6 (0.60)	8 (0.80)	71; 86; 104; 57; 76 78; 82; 86; 99; 90	82.90 (13.57)
694 (0.25 LD50)	10	1,000	5	3	0	3 (0.10)	8 (0.80)	82; 75; 73; 71; 78 71; 74; 73; 64; 60	72.10 (6.33)*
1,389 (0.50 LD50)	10	1,000	5	5	0	5 (0.50)	10 (1.00)	91; 82; 80; 76; 73 71; 87; 66; 83; 92	80.10 (8.62)*
2,083 (0.75 LD50)	10	1,000	4	12*	0	12 (1.20)*	16 (1.60)*	78; 80; 49; 75; 87 46; 64; 66; 39; 53	63.70 (16.33)*

*Significant difference.

TABLE IV. Single I.p. Treatment: Frequencies of PCE and Micronucleated PCE in Mouse Bone Marrow After Single Dosing of Flumethrin

		Micronucleated PCE		PCE
Dose (mg/kg)	No. of animals	Individual data (in 2,000 PCE/animal)	Total (%)	Individual data (in 2,000 erythrocytes)
0	10	5; 2; 1; 1; 2; 4; 4; 5; 3; 1	28 (0.14)	852; 363; 973; 783; 434 274; 613; 281; 292; 249
694 (0.25 LD50)	10	3; 3; 4; 4; 4; 5; 0; 2; 0; 2	27 (0.13)	793; 686; 473; 342; 931 953; 900; 700; 897; 555
1,389 (0.50 LD50)	10	2; 4; 0; 1; 1; 3; 0; 1; 4; 7	23 (0.11)	762; 733; 544; 416; 666 582; 644; 737; 368; 440
2,083 (0.75 LD50)	10	2; 5; 10; 5; 4; 10; 8; 4; 9; 1	58 (0.29)*	904; 575; 777; 992; 652 880; 593; 807; 346; 1289

*Significant difference.

pyrethroids such as fenvalerate and cypermethrin in rat bone marrow and human lymphocytes [11,12,13,29].

The analysis of the frequency of cells with chromosomal aberrations showed a weak clastogenic effect of flumethrin, as evidenced by the increase in the frequency of gaps in the dermal treatment and of breaks only at the highest dose in the single i.p. treatment.

The results obtained in the micronucleus test show that flumethrin had a weak toxic effect on erythropoiesis as indicated by the reduction in PCE values only in the group exposed to the highest dose in the multiple i.p. treatment. The compound proved to be an inducer of micronuclei in mice exposed intraperitoneally, both in single and multiple dosing.

Several studies have proposed the combination of chromosomal aberration analysis and micronucleus test in a single protocol for the evaluation of the clastogenic potential of a substance [30–34]. The two tests have different targets: erythro- and myelopoietic cells in metaphase and erythropoietic cells in interphase, possibly having different susceptibilities to a same agent [35,36]. Apparently, myelopoiesis and erythropoiesis are reacting differently to flumethrin: the MI was reduced after dermal and single i.p. treatments, but reduction in PCE values, also indicative of disturbances in the normal maturation of these cells, occurred only after the multiple i.p. treatment. Hayashi et al. [37] obtained different time-response curves for chromosomal aberrations and micronuclei in mice exposed to a same clastogen. The authors state that, when the two curves do not overlap, the use of several sampling times after a single treatment or a multiple treatment is necessary for the detection of an effect in both endpoints.

Although an increase in both the frequency of breaks and micronuclei was observed after the single i.p. treatment, it is noteworthy that in the multiple i.p. treatment the induction of micronuclei, which was highly significant, was not accompanied by an increase in breaks. The use of the multiple treatment discarded the possibility of a lack of detection of an effect on chromosomal aberrations. Our data suggest that the weak clastogenic effect of flumethrin might not account per se for the induction of micronuclei. These could result also from an aneugenic potential of the compound. Additional procedures would be necessary to identify the origin of these structures.

TABLE V. Multiple I.p. Treatment: Chromosomal Aberrations and MI in Mouse Bone Marrow After 4 Daily Administration of 50, 80, and 128 mg/kg b.w. of Flumethrin

			Cells with aberrations					MI	
						Total (%)			
Dose (mg/k b.w.)	No. of animals	Total no. of cells	Gaps	Breaks	Rearrangements	Without gaps	With gaps	Individual data (metaphases/3,000 cells)	Mean (SD)
0	22	2,200	14	4	0	4 (0.18)	18 (0.82)	77; 78; 78; 93; 96; 93; 87; 65; 79; 85; 106 75; 108; 102; 45; 77; 82; 82; 85; 85; 101; 92	85.04 (14.14)
50	12	1,200	5	4	1	5 (0.42)	10 (0.83)	93; 71; 73; 67; 89; 47; 52; 70; 73; 74; 75; 75	71.58 (12.82)
80	12	1,200	4	3	0	3 (0.25)	7 (0.58)	95; 134; 79; 84; 65; 105; 64; 52; 70; 84; 77; 85	82.83 (21.54)
128	11[a]	1,100	3	3	0	3 (0.27)	6 (0.54)	77; 60; 50; 77; 95; 79; 78; 104; 109; 74; 79	80.18 (17.32)

[a]One animal died before sacrifice.

TABLE VI. Multiple I.p. Treatment: Frequencies of PCE and Micronucleated PCE in Mouse Bone Marrow After 4 Daily Administrations of 50, 80, and 128 mg/kg b.w. of Flumethrin

		Micronucleated PCE		PCE
Dose (mg/kg b.w.)	No. of animals	Individual data (in 2,000 PCE/animal)	Total (%)	Individual data (in 2,000 erythrocytes)
0	22	4; 6; 1; 4; 4; 2; 4; 1; 1; 3 4; 4; 4; 3; 3; 5; 4; 8; 4; 4; 0; 5	78 (0.18)	496; 686; 664; 748; 816; 840; 694 693; 749; 667; 328; 398; 444; 192 267; 502; 417; 245; 294; 419; 486; 368
50	12	4; 3; 1; 2; 1; 7; 6; 4; 4; 7; 2; 4	45 (0.19)	985; 729; 710; 697; 829; 595 746; 643; 449; 546; 315; 658
80	12	4; 3; 2; 2; 4; 3; 2; 2; 2; 1; 1; 3	29 (0.12)	573; 859; 277; 282; 657; 538 496; 621; 599; 624; 656; 563
128	10[a]	11; 9; 6; 6; 7; 12; 5; 3; 20; 9	88 (0.44)*	151; 222; 237; 270; 117 110; 582; 566; 215; 238*

[a]One animal did not yield a sufficient number of PCE.
*Significant difference.

Other pyrethroids have been shown to induce micronuclei, such as cypermethrin in mice bone marrow [8] and human lymphocytes [16], fenvalerate and fenpropathrin in human peripheral lymphocytes [11,16], deltamethrin in mice [10,15], rat [14], human lymphocytes [16], and in *Allium cepa* [38].

Micronuclei can originate both from chromosomal breaks or malsegregation caused by disturbance in the spindle formation. In the case of fenvalerate and of deltamethrin the induction of chromosomal aberrations [10,13,14] and an effect on the spindle were demonstrated [12,14,29,38]. Cypermethrin was not found to be clastogenic [13].

The induction of chromosomal malsegregation and aneuploidy seem then to be characteristics shared by some pyrethroids. Aneuploidy is a significant cause of abortion, perinatal death, malformation, and mental retardation in humans.

Pyrethroids have been presented to the general public as harmless and prevail over the market of the domestic insecticides. The results of this and other papers regarding their genotoxic potential warn against the indiscriminate use of these compounds.

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TABLE VII. Positive Control: Chromosomal Aberrations, MI, PCE, and Micronucleated PCE in Mouse Bone Marrow After a Single I.p. Exposure of 6 Animals to 25 mg/kg b.w. of Cyclophosphamide*

Cells with aberrations (%)						
Total	N < 10	N > 10	P	MI	PCE (%)	MPCE (%)
42.7	20.7	20.5	1.5	1.3	16.8	1.2

*N < 10 and N > 10 = number of cells with less or more than 10 aberrations; P = cells with pulverized chromosomes; MPCE = micronucleated PCE.

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