

Original article

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Genotoxic effects of the carbamate insecticide Pirimor-50[®] in *Vicia faba* root tip meristems and human lymphocyte culture after direct application and treatment with its metabolic extracts

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The aim of the study was to evaluate genotoxic effects of Pirimor-50[®], a pirimicarb-based formulation (50 % active ingredient), in human lymphocyte cultures and *Vicia faba* root meristems. Furthermore, the objective was to examine a combined influence of insecticide treatment with mammalian microsomal S9 and vegetal S10 metabolic fractions or S10 mix metabolic transformation extracts (after *Vicia faba* primary roots treatment with Pirimor-50[®]). We used sister chromatid exchange assay-SCE and measured cell cycle progression and proliferation (proportion of M₁-M₂ metaphases and replication index ratio-RI). Two processes were used for plant promutagen activation: *in vivo* activation-Pirimor-50[®] was applied for 4 h to the plant and then S10 mix was added to lymphocytes; and, *in vitro* activation-lymphocytes were treated with Pirimor-50[®] and S10 or S9 for 2 h. Direct treatment induced significantly higher SCE frequencies in meristems at 0.01 mg mL⁻¹. In lymphocytes, significantly higher SCE was at 1 mg mL⁻¹ with decrease in RI and M₁-M₃ metaphase proportions at 0.5 mg mL⁻¹ and cell division stop at 2.5 mg mL⁻¹. S10 mix lymphocyte treatment showed significantly elevated SCE values at 2-2.5 mg mL⁻¹, with cell death at 3 mg mL⁻¹. Lymphocyte treatment with Pirimor-50[®] together with S9 or S10 showed slightly elevated SCE frequency but had a significant influence on RI decrease, with lowest values in S9 treatment. Since no data are available on the genotoxicity of Pirimor-50[®], this study is one of the first to evaluate and compare its direct effect in two bioassays, animal and vegetal, and also the effect of plant and animal metabolism on its genotoxic potential.

KEY WORDS: *cellular proliferation kinetics; plant and animal promutagen activation; replication index; sister chromatid exchange*

Industrial and agricultural workers worldwide unintentionally get exposed to pesticide poisoning every year (1, 2). Epidemiological studies on pesticides have demonstrated that these can cause cancer in non-target species, including humans. The risk assessment of cytotoxic/genotoxic effects due to direct or indirect exposure to pesticides has become a major concern to public health because of their widespread use in households and the industry. Carbamates are a large group of synthetic pesticides extensively applied in modern agriculture as insecticides, fungicides, herbicides, nematicides, and/or sprout inhibitors (3). Although various experimental data

have provided evidence that pesticides can possess genotoxic properties in animals and *in vitro* test systems after acute and chronic exposure, information about the genotoxic effects of some carbamates, like Pirimor-50[®], are still limited and inconsistent (4).

Pirimor-50[®] is a *N*-methyl carbamate insecticide in which pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yl dimethylcarbamate) is the active ingredient. Pirimor-50[®] represents a commercial formulation widely used in Mexico (5) and Croatia (6) for aphid control in a broad range of crops, including vegetable, cereal, and orchard crops (7). The WHO has classified it (8, 9) as "moderately hazardous" (class II) and USEPA as a compound with possible carcinogenic potential in humans (Classes II-III) (10).

Pirimicarb is transported systemically through the plant and acts by contact, ingestion, and inhalation (11). Its main mechanism of action is the inhibition of acetylcholinesterase activity (12). This mechanism has been reported in mice, rats, and dogs but also in pesticide workers exposed for a longer period (plasma and erythrocyte cholinesterase inhibition) (9). The main property of pirimicarb is its rapid and extensive absorption in the organism, after which its intensive biotransformation begins. It results in 24 metabolites, 17 of which have been identified, some with toxic effect. Besides possible metabolite toxicity, risk assessment is also difficult because pesticides are generally not used as a single active ingredient but rather as a complex commercial formulation. Formulated products, in addition to the active component, contain different solvents and adjuvants, some of which have been reported to induce damage (7, 13-16). Hence, additional toxic effects exerted by adjuvants must also be taken into consideration for risk assessment. Accordingly, workers and the environment are exposed to a simultaneous action of the active ingredient and a variety of other chemicals contained in the formulated product.

Although widely used, with 23 formulated products containing pirimicarb as an active ingredient registered worldwide, studies on the genotoxic effects of pirimicarb and some commercial formulations are scarce (11, 17-18) and there are few cytogenetic studies. Pirimicarb has been reported as non-genotoxic in bacteria, yeast, fungi, and mammalian cells (19). However, positive results were found in mouse lymphoma cells L5178Y in the presence of metabolic activation (11) and in *Drosophila* (20). Furthermore, in human lymphocyte cultures, elevated levels of DNA single-strand breaks were identified by the use of comet assay (4). An increase in the frequency of chromosomal aberrations and sister chromatid exchange (SCE) in Chinese hamster ovary (CHO-K1) cells was observed by Soloneski and Larramendy (7). In addition, an increase in the micronuclei frequency in erythrocytes in *Rana arenarum* and in the fish *Cnesterodon decemmaculatus* exposed to Aficida® (50 % pirimicarb) was observed by Vera-Candioti *et al.* (17-18). Patton Flow® (50 % pirimicarb) also induced DNA single-strand breaks and cytotoxicity on *Cnesterodon decemmaculatus* (21). In individuals occupationally exposed to Pirimor-50®, Pilinskaia (22) found a significant increase in chromosomal aberrations in peripheral lymphocytes.

The metabolites of pesticides produced by plants and animals can act as promutagens, which is why it is necessary to evaluate the vegetal/animal metabolite after exposure to insecticides. When working with human lymphocyte culture, it is necessary to use in parallel the S9 rat liver enzymatic fraction in order to obtain data on the metabolites that would normally be found after the biotransformation of a chemical in the human body. In case of plant exposure, *Vicia faba* is considered a good plant system for treatment since it is metabolically active and contains the S10

enzymatic fraction. As such, it is a sensitive and effective plant to study the effects of pesticide metabolites on the genome stability using the SCE assay, in the plant itself, or on other cell lines with promutagen extracts from the treated *Vicia faba* roots (23, 24).

Although other commercial products as Aficida® and Patton Flow®, both pirimicarb-based pesticides (50 %) like Pirimor-50®, have been found capable of inducing genotoxic damage (17-18, 21, 25), no data/studies are available on the genotoxicity of Pirimor-50® as a pirimicarb-based formulation. Since major pesticides' commercial formulation showed more toxic effects than their active principles, it is important to examine the effect of the formulation on the metabolism in living organisms and to compare the results with the active compound (26). This is even more so since one of the formulations, Pirimor G, showed similar cell viability in three different cell line types, with no effect on the activation of different apoptotic or necrotic pathways, while the active ingredient showed significantly elevated levels of enzymes involved in these pathways (26).

Although nowadays most commonly used cytogenetic assays are micronucleus test and chromosomal aberration assay for *in vitro* and *in vivo* chemical tests, the SCE assay can serve not just as an indicator of genotoxic effects in cells but also as a biomarker of exposure and repair. It does so by reporting the changes in the frequency of reciprocal exchange of DNA segments between sister chromatids at identical loci, indicating a possible destabilisation of the cell genome, persistent damage, and the ability of cells to repair the possible (even persistent) damage. Although this assay has been removed from the OECD guideline list for mutagenicity testing assays on chemicals in 2013 (27), it is still widely used (more than 54 publications in 2016 already, *Web of Science*) due to its specificity and the information that other cytogenetic tests cannot offer. This is true when there is no chromosomal breakage or loss but only otherwise invisible exchanges that can make the genome more unstable and possibly result in a future loss or breakage of the chromosome.

The first aim of this study was to examine the influence of Pirimor-50® alone, as a formulated carbamate insecticide (50 % pirimicarb), on a possible genotoxic effect (measured as SCE frequency) caused in the *Vicia faba* root tip meristems and human lymphocyte cultures in a wide range of concentrations.

The second aim was to examine the *in vivo* and *in vitro* transformation of the Pirimor-50®. Plant and animal metabolites of the pesticide were applied to lymphocytes to estimate their influence on genotoxic effects, cell cycle progression, and proliferation measured as a proportion of the first (M₁), second (M₂), and third (M₃) metaphases, as well as the RI. This study is one of the first to evaluate and compare not only the direct effect of Pirimor-50® in two bioassays, animal and vegetal but also the effect of plant and animal drug metabolism on its genotoxic potential.

MATERIALS AND METHODS

Chemicals

RPMI medium 1640 with L-glutamine and phytohemagglutinin were purchased from Gibco of Mexico; Pirimor-50® (pirimicarb CAS number 23103-98-2) was provided by Zeneca of Mexico. The following chemicals were purchased from Sigma Chemical, St. Louis MO, USA: mitomycin C (MMC, CAS number 50-07-7), cyclophosphamide (CAS number 6055-19-2), dimethyl sulfoxide (DMSO, CAS No. 67-85-5), bromodeoxyuridine (BrdU, CAS number 59-14-3), uridine (Urd, CAS number 58-96-8), fluorodeoxyuridine (FdU, CAS number 3094-09-5), Giemsa (CAS number 51811-82), ethanol (purity 99.2 %, CAS number 6415-5), methanol (purity 99.8 %, CAS number 6756-1), acetic acid (purity 99.7 %, CAS number 5323-26-45), sodium phosphate monobasic (NaH_2PO_4 , CAS number 7558-80-7), sodium phosphate dibasic (Na_2HPO_4 , CAS number 7758-79-4), and sodium chloride (NaCl, CAS number 7647-14-5). Colchicine (CAS number 64-86-8) and potassium chloride (KCl, CAS number 222425) were purchased from Merck of Mexico.

Direct treatments without metabolic activation

Treatment of Vicia faba root tips

To verify the effect of Pirimor-50® on the root tip meristems of the *Vicia faba* (var. minor) chromosomes, SCEs were scored. Once the seeds germinated and reached 2-3 cm, root tips were put into a solution containing 100 $\mu\text{mol L}^{-1}$ bromodeoxyuridine (BrdU), 0.1 $\mu\text{mol L}^{-1}$ fluorodeoxyuridine (FdU), and 5 $\mu\text{mol L}^{-1}$ uridine (Urd) for one replicative 20-h cycle at 20 °C in the dark. Afterwards, the root tips were treated for 4 h with Pirimor-50® at following concentrations: 0.005, 0.01, 0.015, 0.02, 0.025, 0.05, 0.075, and 0.1 mg mL^{-1} , all concentrations dissolved in distilled water. A fresh solution containing BrdU, FdU, and Urd was applied for a second replicative 20-h cycle.

The treatments were performed at 20 °C in the dark. Two experiments were run for each concentration. Two millimetres of the root tips were cut and treated with colchicine (0.05 %) for 3 h in the dark, then stained using the Feulgen differential technique described by Tempelaar et al. (28) and modified by Gómez-Arroyo and Villalobos-Pietrini (29).

Treatment of human lymphocyte cultures

When DMSO was used as a solvent, in all cases its final concentration was ≤ 1 %. Two experiments were carried out for each treatment using 3 mL of RPMI medium 1640 with L-glutamine and 0.2 mL of phytohemagglutinin added to 0.5 mL of a healthy donor's blood in a culture flask. The cultures were incubated at 37 °C for 72 h. After 24 h, BrdU was added to the culture medium at a final concentration

of 5 $\mu\text{g mL}^{-1}$. In the experiments without metabolic activation, cultures were directly exposed to 0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, and 2.5 mg mL^{-1} of Pirimor-50®. Mitomycin C (400 ng mL^{-1}) was used as a positive control. Pirimor-50® was dissolved in 1 % DMSO, concentration that did not affect the SCE frequency according to our previous results (not shown here) and to the results of other studies (30-32). Preliminary experiments were performed in order to identify the optimal concentrations of Pirimor-50® that did not cause cell death. The cultures were incubated for additional 48 h at 37 °C, and 0.1 mL of colchicine (5 $\mu\text{mol L}^{-1}$) was added 2 h prior to the end of cell culture. Metaphase cells were harvested by centrifugation, treated with 0.075 mol L^{-1} of KCl for 20 min, and fixed in methanol-acetic acid (3:1). Slides were stained using the fluorescence-plus-Giemsa technique (33). In addition to the study of SCE, a BrdU differential staining technique was used to assay the effect of Pirimor-50® on cell replication. For evaluation of cytokinetics, the proportion of the first (M_1), second (M_2), and third (M_3) metaphases was obtained from 100 consecutive mitoses for each treatment, and the RI was calculated as follows: $\text{RI} = 1M_1 + 2M_2 + 3M_3 / 100$ (34). To avoid bias, the slides were scored blinded.

The blood from three donors was used separately in different experiments. Because the SCE baseline frequencies among the donors were not significantly different, the influence of the inter-individual variability was negligible in response to the mitogens, culture conditions, or blood samples, as suggested by Morgan and Crossen (35), and Speit et al. (36). Samples were taken from three healthy male donors, nonsmokers and nondrinkers, with an average age of 30 years. The donors did not use any medication in six months prior to sampling and did not undergo any radiation/diagnostic treatment in the same period. This study was performed in accordance with the principles stated in the Declaration of Helsinki. All participants were informed regarding the study, accepted the idea of taking part in it, and they subsequently signed a document in which they agreed.

Treatments with metabolic activation

In vivo activation. Treatment of human lymphocyte cultures with extracts of Vicia faba roots exposed to Pirimor-50®

Vicia faba (var. minor) seeds were germinated between two cotton layers soaked in tap water. When the primary roots reached a length of 4-6 cm, they were immersed in Pirimor-50® at 0.5, 1, 1.5, 2, 2.5, and 3 mg mL^{-1} in a water solution at 20 °C in the dark for 4 h. The concentrations were chosen based on preliminary experiments that examined the phytotoxicity of the insecticide. The positive control roots were exposed to 3.6 mg mL^{-1} of ethanol (0.1 mol L^{-1}) for 4 h at 20 °C. Ethanol is a promutagen in *Vicia faba*, which increases the SCE frequency (27, 37-39).

The negative control was handled under the same experimental conditions, but the roots of *Vicia faba* were immersed in distilled water to obtain the S10 fraction.

After treatment, the roots were rinsed three times with distilled water and cut at approximately 2 cm from the primary root tips. The roots were macerated and homogenised at 4 °C in 0.1 mol L⁻¹ sodium-phosphate buffer, pH 7.4. The ratio of the buffer solution in millilitres (2.0-2.5) to the fresh weight of the root cuttings in grams (2.0-2.5) was 1:1 (23). The homogenised roots were centrifuged for 15 min at 10,000 x g and 4 °C. The supernatant was sterilised using Millipore filters (0.45 µm pore size) and immediately used to treat human lymphocytes in culture. The total protein concentration in these extracts was determined using the Bio-Rad method (40). Protein concentration was constant from one experiment to another, with values between 4.2-5.0 µg µL⁻¹.

In vitro activation. Treatment using in vitro promutagen activation by Vicia faba S10 mix

Lymphocytes cultured for 48 h were exposed to Pirimor-50® at 0.5, 1, 1.5, 2, and 2.5 mg mL⁻¹ (concentrations chosen in preliminary experiments) for 2 h in the dark at 37 °C with and without simultaneously adding the metabolic activation S10 mix (cultures were stationary for the first hour and gently shaken for the second hour). After treatment, cells were rinsed twice in 0.9 % sodium chloride and incubated for 24 h in an RPMI medium containing BrdU at a final concentration of 5 µg mL⁻¹. Colchicine (0.1 mL, 5 µmol L⁻¹) was added 70 h after starting the culture, and the same harvesting and staining procedures were performed as described above.

The metabolic activation system with the S10 mixture was prepared from the microsomal S10 fraction at a 1:9 ratio (v/v) with the following compounds: 8 mmol L⁻¹ MgCl₂, 3.3 mmol L⁻¹ KCl, 5 mmol L⁻¹ glucose-6 phosphate, 4 mmol L⁻¹ NADP and NAD, and 0.1 mol L⁻¹ Na₂HPO₄-NaH₂PO₄ at pH 7.4. The 48 h cultures were incubated for 2 h at 37 °C with 500 µL of activation system and several concentrations of the insecticide. As a positive control, 3.6 mg mL⁻¹ ethanol (0.1 mol L⁻¹) was used (23-24, 37, 39).

In vitro activation. Treatment using in vitro promutagen activation by mammalian S9 mix

The microsomal S9 fraction from rat liver in a commercial form (Mol Tox, from Molecular Toxicology Inc USA) was prepared according to Ames *et al.* (41) and Frantz and Malling (42).

The S9 mixture was prepared with 1 mL of S9 extract and NADPH generator system. It contained sterile cofactors: 0.2 mL (0.4 mol L⁻¹) MgCl₂, 5 mL of pH 7.4 phosphate buffer (0.2 mol L⁻¹ Na₂HPO₄ and NaH₂PO₄), 0.4 mL NADP (0.1 mol L⁻¹) and 0.05 mL of glucose-6-phosphate (1 mol L⁻¹), and 3.35 mL of distilled water; all of the reagents were freshly prepared. Similarly to direct

treatments, BrdU was added after 24 h, and 500 µL of the S9 mixture and Pirimor-50® at concentrations 0.5-2.5 mg L⁻¹ were added 48 h later and cultured for 2 h (cultures were stationary for the first hour and gently shaken for the second hour). After each treatment, cell cultures were washed twice with 0.9 % sodium chloride and incubated for 24 h with RPMI medium containing 100 µL of BrdU (0.4 mg mL⁻¹). The positive control was 50 µL (40 µmol L⁻¹) cyclophosphamide (CP) with and without the S9 mix, and the negative controls used BrdU (5 µg mL⁻¹) and DMSO (1 %).

Statistical analysis

To quantify the SCE frequencies, 25 well-spread metaphase cells at the second division were scored for each concentration (27). Two experiments were performed, and the results obtained were compared using Student's *t*-test. SCEs were statistically analysed using an ANOVA assay to determine significant differences among the treated groups. When a significant F value ($p < 0.0001$) was found, a Newman-Keuls multiple comparison test was applied to identify groups with significant differences at $p < 0.001$ when compared with controls. A chi squared (χ^2) test was used for RI. The parts of the decomposed χ^2 were used to compare the values of M₁, M₂, and M₃.

RESULTS

Direct treatments without metabolic activation

Treatment of Vicia faba root tips

Considering the SCE frequencies in *Vicia faba* root tip meristematic cells, a concentration-dependent response was observed between 0.005 mg mL⁻¹ and 0.025 mg mL⁻¹. Besides the first concentration of 0.005 mg mL⁻¹ of Pirimor-50®, all other were significantly higher ($p < 0.001$) than the control frequency. However, three of the highest concentrations showed a decrease in the SCE frequency when compared to other concentrations (Table 1).

Treatment of human lymphocyte cultures

Table 2 showed the averages of two experiments in which Pirimor-50® was added directly to the human lymphocyte culture. Although the SCE frequency in the treated samples was higher than in the negative control, only the concentrations 1-2 mg mL⁻¹ showed a significant difference from the control values ($p < 0.001$). At 2.5 mg mL⁻¹, the detected cells were only in the M₁ fraction and so the SCE frequency could not be determined.

Cell kinetics and the RI are also listed in Table 2. Concentrations of Pirimor-50® starting at 0.5 mg mL⁻¹ increased the M₁ and M₂ cell fractions but significantly decreased the M₃ fraction. Treatments produced significant inhibition of the cell cycle progression compared with the corresponding negative control values ($p < 0.001$) starting

Table 1 Sister chromatid exchanges induced by Pirimor-50® in *Vicia faba* root tip meristems^a

(mg mL ⁻¹)	\bar{X}	±	S.E.
Control	26.20	±	0.89
0.005	32.84	±	1.21
0.010	37.10*	±	1.13
0.015	41.18*	±	1.21
0.020	42.06*	±	1.03
0.025	61.48*	±	1.33
0.050	59.75*	±	1.56
0.075	53.87*	±	1.27
0.100	40.26*	±	1.36

^an=50 metaphase cells in two experiments.

*Significant differences among controls and each treated group were found by analysis of variance $F=95.55$, p value is <0.0001 , and therefore the Newman-Keuls multiple comparisons test was applied, $p<0.001$

from 0.5 mg mL⁻¹. The RI diminished strongly as the concentration increased to 2.5 mg mL⁻¹, and only the M₁ fraction was observed at this concentration.

As expected, data revealed significant differences in the SCE frequency, cell kinetics, and RI between the negative and positive control (treated with MMC), ($p\leq 0.001$).

Treatments with metabolic activation

In vivo activation. Treatment of human lymphocyte cultures with extracts of Vicia faba roots exposed to Pirimor-50®

The *in vivo* activation by S10 *Vicia faba* mix is shown in Table 3. Extracts of the roots taken after 4 h insecticide treatment were applied to 24 h lymphocyte cultures. After

48 h of treatment, a concentration-dependent increase in the SCE frequency was observed for all concentrations, except for 3 mg mL⁻¹, where all of the examined cells were dead. Significant differences from the control values were detected for concentrations 2 and 2.5 mg mL⁻¹.

When ethanol was added directly to lymphocyte cultures, the SCE frequency was not significantly affected (negative control). Likewise, extracts from the untreated roots (also the negative control) did not increase the SCE frequency. However, significant differences were observed when lymphocytes were treated with extracts of the *Vicia faba* roots plus ethanol 3.6 mg mL⁻¹ (the positive control) (Table 3).

The metabolites of Pirimor-50® contained in the *Vicia faba* root extracts applied to lymphocyte cultures did not produce significant effects on cell kinetics or RI (Table 3), but at 3 mg mL⁻¹ we observed only dead cells.

In vitro activation

Pirimor-50® treatment with in vitro promutagen activation by mammalian S9 mix and Vicia faba S10 mix

As shown in Table 4, no significant increase in SCE frequencies was observed when the insecticide was applied directly for 2 h to lymphocyte cultures neither in the presence of animal (S9) or vegetal (S10) metabolic fractions, although the values were slightly higher than in the negative control. The M₃ cells and RI diminished significantly without and with S9 metabolic activation at all concentrations. Significant differences in the SCE frequency were observed with the positive control [we have added cyclophosphamide (CP) to the S9 mammalian metabolic fraction; and ethanol to the S10 *Vicia faba*

Table 2 Sister chromatid exchanges induction and effects on cell kinetics (M₁, M₂, and M₃ cells) and replication index (RI) by direct treatments of Pirimor-50® in human lymphocyte cultures^a

	$\bar{X}\pm$ S.E.	M ₁	M ₂	M ₃	% RI ^b	
Negative control	4.79 ± 0.35	26	38	36	2.10	
MMC (400 ng mL ⁻¹) (positive control)	15.36 ± 0.65*	51	31	18**	1.67**	
	0.05	26	33	41	2.15	
	0.10	26	35	39	2.13	
	0.25	25	39	36	2.11	
	0.50	49	40	11**	1.62**	
	0.75	50	40	10**	1.60**	
Lymphocyte cultures directly treated with Pirimor-50® (mg mL ⁻¹)	1.00	45	45	10**	1.65**	
	1.50	59	35	06**	1.47**	
	2.00	55	43	02**	1.47**	
	2.50	metaphases were not observed	100	0	0	1.00**

^an=50 metaphase cells in two experiments; ^b Replication index, n=200 consecutive metaphases. *Significant differences among controls and each treated group were obtained by analysis of variance $F=29.02$ the p value is <0.0001 , and therefore the Newman-Keuls multiple comparison test was applied $p<0.001$; **Significant with χ^2 , $p<0.05$.

Table 3 Sister chromatid exchanges induction and effects on cell kinetics (M_1 , M_2 , and M_3 cells) and replication index (RI) by Pirimor-50[®] with in vivo metabolic activation by *Vicia faba* in human lymphocyte cultures^a

	$\bar{X} \pm S.E.$	M_1	M_2	M_3	% RI ^b	
Negative control	4.79±0.35	26	38	36	2.10	
Lymphocyte cultures + <i>V. faba</i> extracts, untreated (negative control)	4.57±0.35	20	37	43	2.23	
Lymphocyte cultures + ethanol (5 µg mL ⁻¹) (negative control)	4.35±0.21	28	39	38	2.20	
Lymphocyte cultures + ethanol 3600 mg L ⁻¹ + <i>V. faba</i> extracts (positive control)	8.96*±0.53	17	37	46	2.29	
	0.5	5.30±0.41	15	29	56	2.41
	1.0	5.42±0.58	12	33	55	2.43
Lymphocyte cultures + <i>V. faba</i> extracts	1.5	6.24±0.51	25	25	50	3.41
from treatment with Pirimor-50 [®] (mg mL ⁻¹)	2.0	7.56±0.52*	13	35	52	3.08
	2.5	9.54±0.53*	16	33	51	2.35
	3.0	Cellular death ^c				

^a $n=50$ metaphase cells in two experiments; ^bReplication index, $n=200$ consecutive metaphases; *Significant differences among controls and each treated group were obtained by analysis of variance; $F=17.90$ the p value is <0.0001 , and therefore the Newman-Keuls multiple comparison test was applied; $p<0.001$.

metabolic fraction]. Combined treatment did not change SCE significantly, influencing only proliferation kinetics and diminishing RI in the sequence: Pirimor-50[®]+S10mix> Pirimor-50[®]> Pirimor-50[®]+S9mix.

DISCUSSION

Pirimor-50[®] is the most widely used commercial formulation for aphid control. Aficida[®] and Patton Flow[®], have been found capable of inducing genotoxic damage (17-18, 21, 25) in water living organisms, but no data is available on the genotoxicity of Pirimor-50[®] as a pirimicarb-based formulation in human lymphocytes and *Vicia faba*. In the only one study with Pirimor G formulation, Mesnage et al (26) studied the cytotoxic effects on three different types of human cell lines, HepG2, HEK293, and JEG3. They discovered that the formulation does not produce the same cytotoxic effect as the active ingredient alone (almost no difference in cell viability and no activation of Adenylate kinase activity or Caspases 3/7 activity). The present study is one of the first to evaluate and compare not only the direct effect of Pirimor-50[®] in two bioassays, animal and vegetal, but also the effect of plant and animal drug metabolism on its genotoxic potential.

Although the evaluated concentrations of Pirimor-50[®] in this study are expected to be rare in the environment, perhaps only observed when specific events occur (e.g. direct application), we cannot rule out the possibility that organisms and occupationally exposed human workers could be exposed accidentally to such high concentrations. We also need to point out that plants are exposed to these concentrations, as pirimicarb is registered for use as a pesticide to control a large variety of chewing and sucking insects on a wide range of crops in many countries at

concentrations that go up to 0.2-2.5 mg mL⁻¹ (48), and this range was covered in our experiments.

Direct treatments with Pirimor-50[®] increased the SCE frequency efficiently in *Vicia faba* under laboratory conditions. Although the increase in SCE frequencies positively correlated with concentrations, these frequencies decreased at the highest concentrations (Table 1), with no alteration of the mitotic index (data not showed). Such an alteration could be explained by the presence of the cytotoxic potential exerted by the formulation and inhibitory effects of the concentrations tested due to the alterations in cell kinetics, as has been demonstrated in other assays (21). Although the mitotic index of *Vicia faba* was not altered, there is a possibility that SCE frequencies diminished through a damaged cell elimination pathway or by a damage repair process upon increasing pesticide exposure. On the other side, we cannot rule out the possibility that the metabolic system of *Vicia faba* can participate in the detoxification process and also have an impact on the DNA damage frequency and damaged cell elimination. Vera-Candiotti et al. (21) have found similar results but also no experimental evidence to explain this particular finding. Therefore, further experiments should be conducted to elucidate whether this observation is related to any of the above-mentioned possibilities or whether it is the result of several independent processes occurring simultaneously during metabolism and cell repair and elimination.

In direct treatments with Pirimor-50[®] in lymphocyte cultures for 48 h (Table 2), we have used the concentrations up to 3 mg mL⁻¹ in order to cover the entire range to which individuals can be exposed in direct contact. A positive significant SCE response was obtained for 1-2 mg mL⁻¹ concentrations but SCE frequencies were more or less similar for these concentrations. These results disagree with those reported by the USEPA (49) in which no induction of chromosomal aberrations on *in vitro* human lymphocytes

treated with a direct application of pirimicarb was observed. However, they are also in agreement with previous findings depicting the genotoxic cytotoxic potential of pirimicarb through the induction of SCE, chromosomal aberrations and cell cycle delay in CHO-K1 cells (7), and chromosomal damage in human lymphocytes *in vivo* (22), or *in vitro* (4). It should be highlighted that the behaviour found in human lymphocytes is similar to CHO-K1 cells treated with Aficida® and pirimicarb in the study by Soloneski and Larramendy (7).

The increase in the SCE frequency observed after direct exposure could suggest that high concentrations of Pirimor-50® can cause DNA strand breaks. Other authors have also noted that pirimicarb induces genotoxicity through chromosomal damage and DNA single-strand breaks both *in vivo* and *in vitro* (4, 7, 17-18, 22). It has been observed that pirimicarb can bind to DNA by intercalation (50). This mode of action could explain the results found in this study and also the decrease in RI that showed a significant effect on the concentration lower than the one where significantly higher SCE frequencies were found (starting from 0.5 mg mL⁻¹). Due to all these facts, a possible clastogenic effect of the insecticide could be suggested as already mentioned in the study by Soloneski et al. (51).

The results obtained in this study from the genotoxic and cytotoxic assays indicate that there are differences between the two bioassays employed concerning their sensitivity to Pirimor-50®. According to Soloneski et al. (51), this observation could be explained by the nature of each bioassay. Different assays can give different results depending on the test agent used and the bioassay employed, highlighting the importance of the use of more than one bioassay to determine the damage induced for a genotoxic agent.

On the other side, when *Vicia faba* extracts, obtained after treatment of *Vicia faba* roots for 4 h with different concentrations of the insecticide, were applied to lymphocyte cultures, concentrations of 2 and 2.5 mg mL⁻¹ of Pirimor-50® caused a concentration-dependent response in the SCE frequency, and cellular death was induced at 3 mg mL⁻¹. No significant differences were found in cell kinetics and RI. The products obtained from the *in vivo* promutagen activation of Pirimor-50® by *Vicia faba* applied to lymphocyte cultures were capable of increasing the SCE frequency, which means that this compound acted directly and indirectly. However, toxicity diminished with plant metabolism, possibly indicating that certain detoxification mechanisms are involved (52-53). The same detoxification was observed with the thiocarbamate herbicides molinate and butylate in the presence of the S10 fraction (38).

Comparing both plant activation systems, *in vivo* and *in vitro*, significant SCE frequencies were only observed *in vivo*, and these differences could be related to the exposure time of lymphocytes and/or to active metabolites. In the *in vivo* activation experiments, treatments lasted 48 h, while exposure lasted only 2 h for the *in vitro* activation. The latter

Table 4 Sister chromatid exchanges induction and effects on cell kinetics (M₁, M₂, and M₃ cells) and replication index (RI) by Pirimor-50® without and with *in vitro* animal and plant metabolic activation in human lymphocyte cultures^a

Concentration	Without metabolic activation			With S9 animal metabolic activation			With S10 plant metabolic activation		
	SCE / metaphase X̄±S.E.	Metaphases M ₁ M ₂ M ₃	RI ^b	SCE / metaphase X̄±S.E.	Metaphases M ₁ M ₂ M ₃	RI ^b	SCE / metaphase X̄±S.E.	Metaphases M ₁ M ₂ M ₃	RI ^b
Control	4.98±0.30	42 39 19	1.77	5.86±0.34	41 38 21	1.80	4.84±0.31	48 38 14	1.66
DMSO	6.10±0.51	42 37 21	1.79	5.95±0.42	47 33 20	1.73	6.56±0.52	43 34 23	1.80
CP	6.59±0.34	43 39 18	1.75	41.19±2.14*	40 40 20	1.80	7.40±0.34	50 33 17	1.67
Ethanol	6.01±0.35	47 39 14	1.67	7.96±0.47	48 37 15	1.67	9.10±0.25*	44 35 17	1.77
0.5	6.16±0.37	54 38 08**	1.54**	6.47±0.48	58 34 08**	1.50**	5.32±0.29	48 37 15	1.67
1.0	6.83±0.31	57 34 09**	1.52**	6.26±0.39	65 26 09**	1.44**	6.00±0.37	51 34 15	1.64
1.5	6.33±0.36	49 41 10**	1.61**	5.94±0.33	63 29 08**	1.45**	6.96±0.25	50 33 17	1.67
2.0	6.42±0.25	47 44 09**	1.62**	6.70±0.79	58 34 08**	1.50**	6.54±0.30	57 29 14	1.57
2.5	7.39±0.30	55 35 10**	1.55**	7.74±0.84	60 31 09**	1.49**	6.40±0.41	58 39 14	1.65

^an=50 metaphase cells in two experiments; ^bReplication index; *Significant differences among controls and each treated group were obtained by analysis of variance; F=29.02 the p value is < 0.0001, and therefore the Newman-Keuls multiple comparison test was applied p<0.001. **Significant with χ², p<0.05

time period may not have been sufficient for the cells to metabolise the insecticide. On the other hand, this may have been due to enzyme inactivation because direct contact of the insecticide with the S10 fraction could have inhibited the insecticide metabolism.

In plant metabolism, peroxidases are among the most important enzymes involved in the oxidative transformation of xenobiotics (54); peroxidases catalyse two categories of oxidative reactions in plant cells, the peroxidative reaction requiring H₂O₂ and the oxidative reaction using molecular oxygen (55). Calderón-Segura et al. (38) described the phytotoxic effect of the thiocarbamic herbicide butylate in *Vicia faba*. Cell death by this herbicide is caused by the absence of metabolism, and several other agents have been described to lower plant cell culture viability (56).

In the *in vitro* animal (S9)/vegetal (S10) metabolism assay, the SCE frequency was not significantly increased, but cell kinetics were affected with 2 h of treatment with Pirimor-50® both without and with the presence of the S9 fraction (Table 4). Both methods significantly decreased the number of M₃ cells, and the insecticide induced a cell cycle delay and significant differences in RI. This cell cycle arrest may be an adaptive process in which a surveillance mechanism delays the cell cycle when DNA lesions occur. The ability of cells to delay their cell cycle in order for the repair to take place is well known (57-58).

Several investigations have proven that commercial formulations have the ability to induce DNA damage (15, 59-62). For pirimicarb and its formulations, the results revealed, depending upon the endpoint employed, that the damage induced by the latter is, in general, greater than that produced by the pure pesticide. Unfortunately, manufacturers have not made the identity of the components present in excipient formulations available. However, the deleterious effect/s of the adjuvants/s present within the commercial formulation should neither be discarded nor underestimated.

It has been suggested that the damage induced by pirimicarb could be mostly related to the effect of desmethyl pirimicarb and/or desmethylformamido pirimicarb metabolites (11), two toxicologically relevant metabolites. So far, the mechanism/s by which N,N-dimethylcarbamate and their carbamate metabolites exert genotoxicity has not been fully established (7). The main metabolic pathway involves the loss of carbamate moiety to produce a range of substituted hydroxypyrimidines, some of which are glucuronide conjugates. Unfortunately, in our study we did not have the possibility to examine the content of the plant extracts and evaluate the metabolites found inside, as well as the level of other oxidative and antioxidative substances.

Taken together, our findings suggest the importance of further studies on this type of pesticide in order to achieve a complete knowledge on its genetic toxicology. Future studies either *in vivo* or *in vitro* are required in order to clarify its mechanism of action.

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Genotoksični učinci karbamatnog insekticida Pirimor-50® na vršni meristem korijena biljke *Vicia faba* i kulturu ljudskih limfocita nakon izravne primjene i tretiranja njegovim metaboličkim ekstraktima

Cilj ovog ispitivanja bio je procijeniti genotoksične učinke insekticida Pirimor-50®, formulacije koja se temelji na pirimikarbu (50 %-tni aktivni sastojak), u kulturama ljudskih limfocita i meristemu korijena biljke *Vicia faba*. Nadalje, cilj je bio ispitati objedinjeni utjecaj tretiranja insekticidom i metaboličkom mješavinom S9 za kultivirane stanice sisavaca i mješavinom S10 za stanice biljaka ili mješavinom S10 transformacijskih ekstrakata (nakon tretiranja primarnih korijena biljke *Vicia faba* insekticidom Pirimor-50®). Korišten je test izmjena sestrinskih kromatida (*Sister Chromatid Exchange* – SCE) i mjerena je progresija i proliferacija staničnog ciklusa (kroz omjer M_1 - M_3 metafaza i vrijednost replikacijskog indeksa – RI). Dva su procesa korištena za aktivaciju biljnog promutagena: *in vivo* aktivacija – Pirimor-50® primjenjivan je tijekom 4 h na biljci, a potom je mješavina S10 dodana limfocitima, i *in vitro* aktivacija – limfociti su 2 h tretirani insekticidom Pirimor-50® i mješavinom S10 ili S9. Izravno tretiranje proizvelo je značajno veću učestalost SCE-a u meristemu pri 0,01 mg mL⁻¹. U limfocitima je razina SCE-a značajno povećana pri 1 mg mL⁻¹, uz smanjenje RI-ja i omjera M_1 - M_3 metafaza pri 0,5 mg mL⁻¹, uz zastoj stanične diobe pri 2,5 mg mL⁻¹. Tretiranje limfocita mješavinom S10 značajno je povisilo vrijednosti SCE-a pri 2-2,5 mg mL⁻¹, a stanična smrt nastupila je pri 3 mg mL⁻¹. Tretiranje limfocita insekticidom Pirimor-50®, zajedno sa S9 ili S10, pokazalo je nešto veću učestalost SCE-a, ali i značajniji utjecaj na povećanje RI-ja, pri čemu su najniže vrijednosti utvrđene nakon tretiranja mješavinom S9. S obzirom na to da nema podataka o genotoksičnosti insekticida Pirimor-50®, ovo je istraživanje među prvima koje pomoću dvaju testova, životinjskoga i biljnoga, ispituje i uspoređuje njegov izravan učinak, ali i učinak biljnog i životinjskog metabolizma na njegov genotoksični potencijal.

KLJUČNE RIJEČI: aktivacija biljnog i životinjskog promutagena; izmjene sestrinskih kromatida; kinetika stanične proliferacije; replikacijski indeks