

## Metabolic activation of herbicide products by *Vicia faba* detected in human peripheral lymphocytes using alkaline single cell gel electrophoresis

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### Abstract

Ametryn and metribuzin *S*-triazines derivatives and EPTC thiocarbamate are herbicides used extensively in Mexican agriculture, for example in crops such as corn, sugar cane, tomato, wheat, and beans. The present study evaluated the DNA damage and cytotoxic effects of three herbicides after metabolism by *Vicia faba* roots in human peripheral lymphocytes using alkaline single cell gel electrophoresis. Three parameters were scored as indicators of DNA damage: tail length, percentage of cells with DNA damage (with comet), and level DNA damage. The lymphocytes were treated for 2 h with 0.5–5.0 µg/ml ametryn or metribuzin and 1.5–10 µg/ml EPTC. Lymphocytes also were coincubated for 2 h with 20 µl *V. faba* roots extracts that had been treated for 4 h with 50–500 mg/l of the two triazines or with the thiocarbamate herbicide or with ethanol (3600 mg/l), as positive control. The lymphocytes treated with three pesticides without *in vivo* metabolic activation by *V. faba* root did not show significant differences in the mean values between genotoxic parameters compared with negative control. But when human cells were exposed to three herbicides after they had been metabolized the frequency of cell comet, tail length and level DNA damage all increased. At highest concentrations of the three herbicides produced severe DNA damage compared with S10 fraction and negative control. The linear regression analysis of the tail length values of three herbicides indicated that there was genotoxic effect concentration-response relationship with ametryn and ametryn but no EPTC. The ethanol induced major increase DNA damage compared with S10 fraction and the three pesticides. There were not effects in cell viability with treatment EPTC and metribuzin whether or not it had been metabolized. High concentrations of ametryn alone and after it had been metabolized decreased cell viability compared with the negative control. The results demonstrated that the three herbicides needed to be activated by the *V. faba* root metabolism to produce DNA damage in human peripheral lymphocyte. The alkaline comet technique is a rapid and sensitive assay, to quickly evaluate DNA damage the metabolic activation of herbicide products by *V. faba* root in human cells *in vitro*.

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**Keywords:** Ametryn; Metribuzin; EPTC herbicides; Human peripheral lymphocytes; *In vivo Vicia faba* metabolic activation; Comet tail length; DNA damaged cells

**Abbreviations:** EPTC (eptam), *S*-ethyl-*N,N*-diproylthiocarbamate; EDTA, ethylenediaminetetraacetic acid; PBS, Dulbecco's phosphate-buffered saline, pH 7.4; S10, 10,000g supernatant fraction *Vicia faba* root microsomal enzymes.

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## 1. Introduction

Many pesticides are not mutagens by themselves but become active by metabolic transformations. Thus, a promutagen may be converted into a mutagen through metabolic activation (Plewa, 1978; Plewa and Gentile, 1982). Plant activation is the process by which a promutagen is activated to a mutagen by plant enzymatic systems (Plewa, 1978; Plewa and Gentile, 1982). Many food plants are exposed to pesticides and other chemicals used in agriculture (Plewa, 1978; Plewa and Gentile, 1982).

It has been shown that the enzymatic system S10 of *Vicia faba* roots can activate pesticides *in vivo* and *in vitro*, and the genotoxic and cytotoxic action of the metabolites contained in the extracts, through sister chromatid exchanges, have been shown in human peripheral lymphocytes in culture (Calderón-Segura et al., 1999; Gómez-Arroyo et al., 1995, 2000; Flores-Maya et al., 2005) and in Chinese hamster ovary cell (Takehisa et al., 1988).

S-triazines herbicides are widely used in Mexican agricultural. Their principal mode of action is the inhibition of plant photosynthesis. S-triazines derivatives, including ametryn, cyanazine, metribuzin, prometryn, terbuthylazine, terbutryne, and some metabolites have been found in eggs, fruit and vegetables (Tadeo et al., 2000). Thiocarbamate herbicides belong to the group of S-thiocarbamate esters. They are widely used in Mexico for pre-emergent and post-emergent control of broadleaf weeds and annual grasses. Molinate, butylate and EPTC are applied to crops such as corn, rice, wheat, sugar cane, and beans (Bayer de México, 1994). The compounds in this family are considered to be meristematic inhibitors (Barret and Harwood, 1998).

Ametryn is used in México for the pre and post-emergence control of annual grasses and broad-leaved weeds in crops of pineapples, sugarcane, bananas, citrus, maize, and coffee. It is absorbed by leaves and roots, translocated acropetally in xylem and accumulates in the apical meristems (Edwards and Owen, 1989). It is metabolized in plants by hydroxylation and dealkylation reactions. The toxicity of Ametryn is Class III, which means that it is slightly toxic to humans (IARC, 2000). It is relatively nontoxic to mammals and fish (Davies et al., 1994), but highly toxic to crustaceans and mollusks (IARC, 2000), and it is embryotoxic in rats (Asongalem and Akintonwa, 1997). Ametryn inhibits bioluminescence of *Vibrio fischeri* after biodegradation (Farré et al., 2002).

Metribuzin is used as a photosynthesis inhibitor in corn, sugar soybeans, and carrot crops (Bayer de México, 1994; Frear et al., 1983, 1985). It is absorbed mainly by roots but also by leaves and is translocated in the xylem (Frear et al., 1983, 1985). The metabolism of metribuzin has been studied in several crop plants (Frear et al., 1983, 1985). The major primary metabolic reactions are deamination, sulfoxidation and demethylation (Frear et al., 1983, 1985). It is nonmutagenic in bacterial reversion assays (Moriya

et al., 1983); it is mutagenic in *Escherichia coli* (Pauli et al., 1990); and has moderate genotoxic activity using SOS microplate assays (Venkat et al., 1995).

EPTC is used to control of annual and perennial grasses, and is used, as a selective herbicide in corn, sorghum, and tomato crops (Bayer de México, 1994). EPTC has been shown to undergo metabolic bioactivation via oxidation to form the reactive metabolites EPTC-sulfoxide or EPTC-sulfone, which are excellent carbamoylating agents for tissue thiols. Both of these electrophilic species are capable to interact with proteins (Lamoureux and Rusness, 1987). EPTC-sulfoxide inhibits aldehyde dehydrogenase (ALDH), one of the key enzymes involved in ethanol metabolism in humans, and to increase their hepatotoxic potential in mammals and fish (Staub et al., 1999; Coleman et al., 2000). It is a potent neurotoxicant in rats (Smulders et al., 2003).

The alkaline comet assay is a rapid, simple, and sensitive procedure to quantify DNA lesions in individual cells, and is used in environmental genotoxic monitoring both *in vivo* and *in vitro* (Tice et al., 2000). The alkaline comet assay was specially developed to detect DNA single-strand breaks and alkali-labile sites (Singh et al., 1988). It is also used to evaluate *in vivo* genotoxicity induced by the exposure to carcinogens (Tice et al., 2000).

The use of the comet assay to detect the potential genotoxic effects of pesticides is particularly relevant in the evaluation of potential health risks to humans and animals, since pesticides are applied to food crops, and can be present in the air, soil and aquatic systems. Original compounds and metabolites may pass through animals' digestive tracts and be activated and when the animals are used as food. Thus, these compounds could represent a health risk (Sandermann, 1992). When agrochemicals get into food plants, they may remain unaltered; they may undergo further transformations, or be reactivated by digestive enzymes and produce, perhaps, adverse physiological effects in organisms (Sandermann, 1992).

The present study was carried out to study the *in vivo* metabolic capability of *Vicia faba* roots to bioactivate three herbicides, using the alkaline comet assay to measure DNA damage plant promutagens effects on peripheral blood lymphocytes *in vitro*.

## 2. Material and methods

### 2.1. Chemicals and reagents

Fresh stock solutions of ametryn (Gesapax 49% CAS number 014-69-3 kindly donated by CIBA-GEIGY México), metribuzin (Sencor 48% provided by Bayer of México, CAS number 21087-64-9) and EPTC (eptam, 79% CAS number 759-94-4 kindly donated by Quimica Lucava México) were prepared in deionized water and immediately used to treat human peripheral lymphocytes and *V. faba* roots.

The chemicals used in the alkaline comet assay were: RPMI 1640 medium with L-glutamine (Gibco), LMA, low melting agarose (Gibco), NMA, normal melting agarose (Gibco), Tris (Sigma), sodium lauryl sarcosinate (Sigma), dimethylsulfoxide (DMSO) (Sigma), Triton X-100 (Sigma), EDTA (Sigma), NaOH, sodium hydroxide (Baker), ethidium bromide (Sigma); NaCl, sodium chloride (Baker); Trypan Blue 0.4% (Sigma), PBS (Gibco), ethanol (Sigma); HBSS, Ca<sup>2+</sup>, Mg<sup>2+</sup> free, Hanks balanced salt solution (Ca<sup>2+</sup>, Mg<sup>2+</sup> free) (Gibco); Tris Base (Sigma).

## 2.2. Human peripheral lymphocyte isolation

The experiments were carried out using human peripheral lymphocytes which were isolated from whole blood samples. Twenty milliliters of heparinized venous blood was collected from each of three healthy donors. Each sample was diluted 1:1 with Hanks solution (HBSS) (pH 7.4, Gibco) by centrifugation based on Ficoll–Hystopaque density gradient (Gibco, lymphoprep Oslo, Norway) at 400g for 20 min. The lymphocytes were washed twice using 5 ml Hank's solution's (pH 7.5) (Gibco, Grand Island, NY). The resulting pellet was resuspended with 5 ml RPMI 1640 medium (Gibco BRL Life Technologies) at 37 °C, supplemented with 1% penicillin-streptomycin antibiotic (Gibco), and 10 µl of the suspension were used for cell counting in a Neubauer chamber and viability evaluated by staining with trypan blue. The cells were immediately used for the experiments.

## 2.3. Direct treatment of human peripheral lymphocytes with ametryn, metribuzin and EPTC

Lymphocytes ( $25 \times 10^5$ ,  $\geq 94\%$  viability) were exposed to 500 µl of RPMI 1640 medium containing different concentrations of herbicide for 2 h at 37 °C. The concentrations used were: ametryn, 0.5, 1.0, 1.5, 2.5 and 5.0 µg/ml; metribuzin, 0.5, 1.0, 2.0, 3.5 and 5.0 µg/ml; and EPTC 1.5, 2.5, 5.0, 8.0 and 10 µg/ml. The negative control was 500 µl RPMI 1640 medium. The pH value was 7.5 and no changes were detected in the coincubate after the addition of *V. faba* extracts treated or untreated with ametryn, metribuzin and EPTC. After treatment, the human lymphocytes were washed twice with RPMI 1640 at 37 °C and immediately used in the cell viability test and alkaline comet assay.

## 2.4. In vivo metabolic activation of *V. faba*: exposure of *V. faba* primary roots to ametryn, metribuzin and EPTC herbicides and ethanol

Seven-day-old primary roots of *V. faba* 4–6 cm long were immersed in different concentrations of the three herbicides for 4 h in a dark at room temperature to extract the S10 fraction. The concentrations used for each herbicide were 50, 100, 200, 400, and 500 mg/l dissolved in deionized water. Root samples were also treated with ethanol

(3600 mg/ml) as a positive control. After treatment, the primary roots were washed three times with deionized water. The 2 cm tips of the main roots were homogenized at 4 °C in 0.1 M Na–phosphate buffer, pH 7.4. The ratio of the volume of buffer solution in ml (2.0–2.5) to fresh weight (2.0–2.5) of roots cutting in grams was 1:1 (Takehisa et al., 1988; Calderón-Segura et al., 1999; Gómez-Arroyo et al., 1995, 2000). The homogenate was centrifuged for 15 min at 10,000g at 4 °C. The supernatant was sterilized using Millipore filters (pore size 0.45 µm) and the protein concentration immediately measured using Beckman spectrophotometry (Bradford, 1976) before being used to treat human peripheral lymphocytes. The protein concentration in the three experiments was fairly constant, 0.4 and 0.8 µg/ml in each *V. faba* root extract treated with ametryn and metribuzin, respectively, and 0.6 µg/ml of EPTC (Bradford, 1976).

Lymphocytes ( $25 \times 10^5$ ) were treated with 20 µl of *V. faba* root extract that had been previously exposed to different concentrations of the three herbicides, S10 fraction and positive control (3600 mg/ml ethanol, *in vivo* metabolic activation) for 2 h at 37 °C. The mean value of pH was 7.5 and no changes were detected in the coincubate after the addition of *V. faba* extracts treated or untreated with ametryn, metribuzin, EPTC and ethanol. After treatment, the human lymphocytes were washed twice with RPMI 1640 at 37 °C and immediately used for the cell viability test and alkaline comet assay.

## 2.5. Cell viability test

Cell viability was routinely determined before and immediately after treatments with or without metabolic activation of the three herbicides, ethanol and S10 fraction using trypan blue dye-exclusion staining. Trypan blue penetrates dead cells through damaged membrane, staining the nucleus. Ten microliters of cell suspension were mixed with 10 µl trypan blue (0.4% in PBS). For all samples, cell viability was  $\geq 94\%$  before each concentration assayed. For each experiment 100 cells were counted and the results were expressed as percentages of viable cells among all cells.

## 2.6. Alkaline comet assay procedure

The comet assay was performed according with the methods of Singh et al. (1988) and Tice et al. (2000). Fully frosted slides (Fisher) were first immersed in normal agarose 1% (Gibco) prepared in PBS (pH 7.5) (Gibco) and melted by heating in a microwave oven and dried at room temperature. Then 90 µl of 0.5% low-melting point agarose (Gibco) in PBS (pH 7.5, Gibco) were mixed with a volume of  $5 \times 10^3$  cells per slide. After applying the coverslips, the slides were allowed to solidify at 4 °C for 5 min. The coverslips were gently removed and a third layer of 100 µl of 0.5% low-melting point agarose in PBS was applied and allowed to solidify at 4 °C for 5 min. The slides were immersed gently into a dark Koplín jar containing fresh

cold lysis solution (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 1% Triton X-100 and 10% DMSO, 10 mM Tris, 0.5% sodium lauroyl sarcosinate adjusted to pH 10) for 1 h. After lysis, the slides were transferred to a horizontal electrophoresis unit (Amersham Biosciences), filled with a freshly made cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13). The DNA in the samples was allowed to unwind for 20 min and then electrophoresed at 25 V, 300 mA current, and allowed to run for 20 min. After electrophoresis, the slides were washed three times with fresh neutralization buffer (0.4 M Tris at pH 7.5) every 5 min to remove any remaining alkali and detergent. Slides were drained with absolute methanol for 5 min, and stored in a black box until the images were scored. The cells were stained with 50  $\mu$ l of ethidium bromide solution (20  $\mu$ g/ml), then the slides were covered with coverslips, placed in a humidified black container to prevent gel from drying, and analyzed within 30 min. All the steps were conducted under yellow light (the unit was covered with a black cloth) to prevent additional DNA damage occurring. Two slides were prepared and coded for each herbicide concentration and examined with an Axiostar Plus Zeiss fluorescent microscope, equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. To visualize of DNA damage, slides were examined at 400 $\times$  magnification using an eyepiece micrometric objective (1 unit = 2.41  $\mu$ m

at 400 $\times$  magnification). Three parameters were scored to determine DNA damage: (a) frequency or percentage of damaged/undamaged cells (with or without comet) in 100 randomly selected cells (50 nuclei on each slide, two slides per herbicide concentration); (b) the comet tail length in the image was estimated in micrometers (from the nuclear region to the end of the tail) in a 100 consecutive cells; (c) damaged cells were visually allocated to five categories according to comet tail size and the proportions in each category were counted. The categories were: Level 0, no tail or undamaged nucleus; Level 1, tail  $\leq$ 50  $\mu$ m; Level 2, tail 51–100  $\mu$ m; Level 3, tail 101–150  $\mu$ m, and Level 4, tail 151–200  $\mu$ m (severely damaged nucleus; Fig. 1a–f). The values obtained were averaged from three experiments and expressed as percentages.

### 2.7. Statistical analysis

Results are expressed as mean  $\pm$  standard error (SE) of DNA migration ( $\mu$ m) of the mean ( $\pm$ SEM) or comet tail length for each herbicide and concentration, with or without *in vivo* metabolic activation by *V. faba* roots. Three experiments were carried out and the results obtained were compared using the Student's *t*-test. Comet tail length, percentages of damaged or undamaged cells were statistically analysed using analysis of variance (ANOVA) to determine

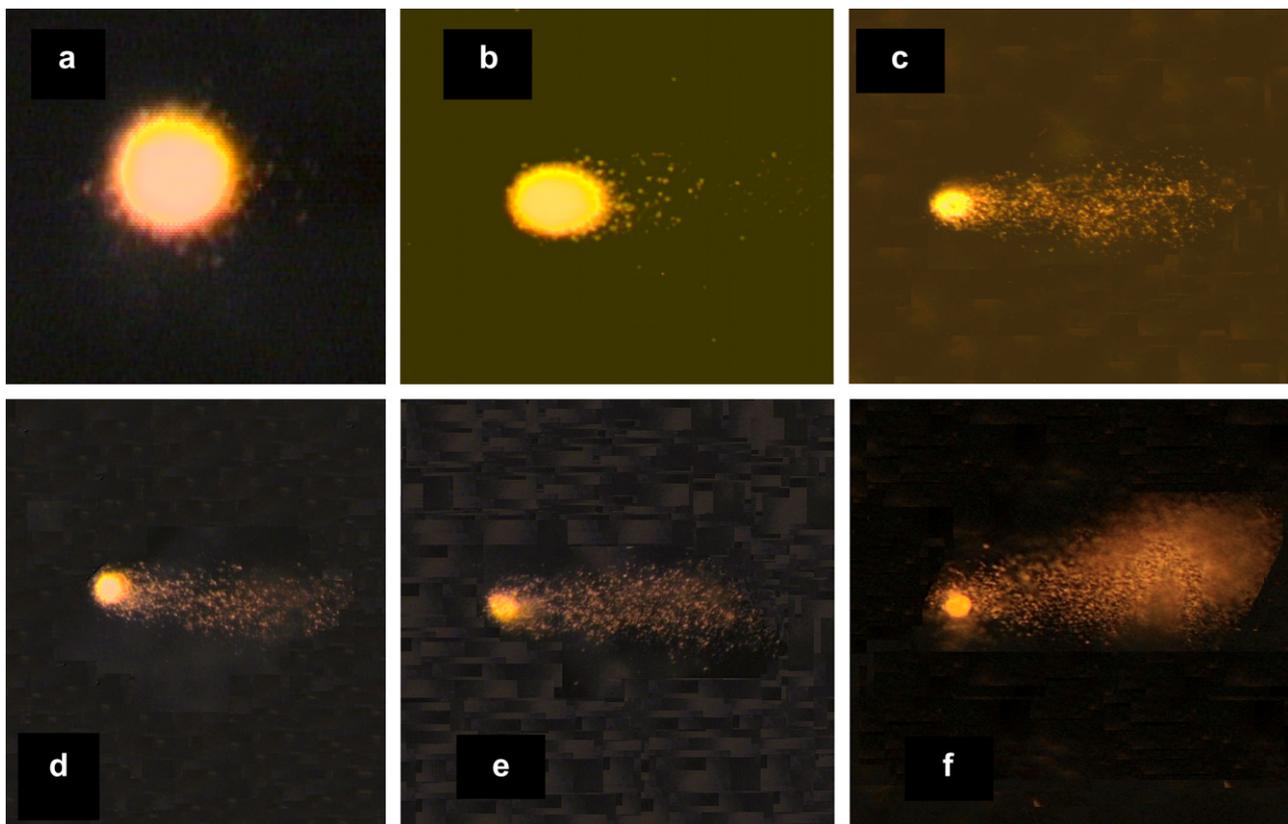


Fig. 1. Representative comet images (DNA damage) of nuclei from human lymphocytes induced by herbicides and ethanol after *in vivo* metabolic activation by *Vicia faba* root extracts, showing increase in tail length. Level 0 undamaged nuclei (a), Level 1, 4–50  $\mu$ m (b), Level 2, 51–100  $\mu$ m (c), Level 3, 101–150  $\mu$ m (d), Level 4, 151–200  $\mu$ m (e), and ethanol (positive control) (f).

whether differences among the treated groups, negative control and S10 were significant. When a relevant  $F$ -value was found ( $p < 0.05$ ), the Newman–Keuls multiple comparisons test was applied and the significant differences were established at  $p < 0.001$  when compared with the negative control and S10 alone. The relationship between comet tail length and each concentration used of the three herbicides were determined using linear regressions analysis (Fig. 4a–c).

### 3. Results

#### 3.1. DNA damage to human lymphocytes exposed directly to ametryn, metribuzin, EPTC and ethanol

Fig. 2 and Tables 1–3, show the results obtained after direct treatments with different concentrations of ametryn (0.5, 1.0, 1.5, 2.5, 5.0  $\mu\text{g/ml}$ ), metribuzin (0.5, 1.0, 2.0, 3.5, 5.0), or EPTC (1.5, 2.5, 5.0, 8.0, 10.0  $\mu\text{g/ml}$ ). They did not induce DNA damage in human peripheral lymphocytes since no differences were found between the means of comet tail length, percentages of damaged and undamaged cells, and proportions of DNA damage cell level after the alkaline comet assay. Metribuzin and EPTC were not cytotoxic at any tested concentrations (Tables 2 and 3), Ametryn caused a decrease of between 13% and 19% in cell viability compared with the negative control (incubated in RPMI 1640 medium, free pesticides) for all concentrations (Table 1).

#### 3.2. *In vivo* promutagen activation by *Vicia faba*

Preliminary experiments were made with 10 and 20  $\mu\text{l}$  of *V. faba* root extract that had been treated for 4 h with each the three herbicides or ethanol (3600 mg/l) as a positive control, added to human cells and incubated for two differ-

ent periods (1 h and 2 h). In the 10  $\mu\text{l}$  tests, the pesticides produced a small amount of damage to DNA: the proportion of small comet tails was low; and the DNA damage compared with the negative control. But when human lymphocytes were exposed to 20  $\mu\text{l}$  of the same of root extracts, treated for 4 h with each of three herbicides, and coincubated for 2 h, there were significant increases in the genotoxic parameters in the human lymphocytes nuclei (Tables 1–3; Fig. 1b–e, Fig. 3).

When the S10 fraction of *V. faba* roots (without treatment with herbicides) was incubated with human peripheral lymphocytes for 2 h, we found short comet tail lengths in three healthy donors. In donor 1:  $9.0 \pm 1.4 \mu\text{m}$  (Fig. 3a and b); donor 2:  $24.5 \pm 1.8 \mu\text{m}$  (Fig. 3a and b), and donor 3:  $11.2 \pm 3.6 \mu\text{m}$  (Fig. 3a and b). The percentages of cells with DNA damage was 3% (level 1) compared with the negative control (Tables 1–3; Fig. 1b).

After *in vivo* metabolic activation and at a concentration of 50–500 mg/ml ametryn significantly increased the means of the comet tail length compared with the S10 fraction and the negative control: mean  $97.2 \pm 2.7$ – $111.8 \pm 2.0 \mu\text{m}$  (Fig. 3). DNA damage was also increased from 42% to 98% frequency (Table 1), and the cell damage was classified as level 2 or 3 (Fig. 1c and d). For metribuzin, the means of the comet tail length was  $44.4 \pm 3.9$ – $147.4 \pm 2.3 \mu\text{m}$  (Fig. 3), the frequency of DNA cell damage was 12–66% (Table 2) and the damage was classified at level 2 or 3 (Table 2; Fig. 1c and d). The means of the comet tail length for EPTC was  $32.7 \pm 2.2$ – $94.7 \pm 4.5 \mu\text{m}$  (Fig. 3) and the frequency of DNA cell damage increased to 21–82% (Table 3) and the damage was classified at level 2 or 3 (Fig. 1c and d). Lymphocytes exposed to extracts from *V. faba* roots and treated with 500 mg/ml ametryn and EPTC had 95% and 82% of DNA cell damage, respectively; most had comets (level 2 and 3) indicating severe DNA damage (Tables 1 and 3; Fig. 1c and d).

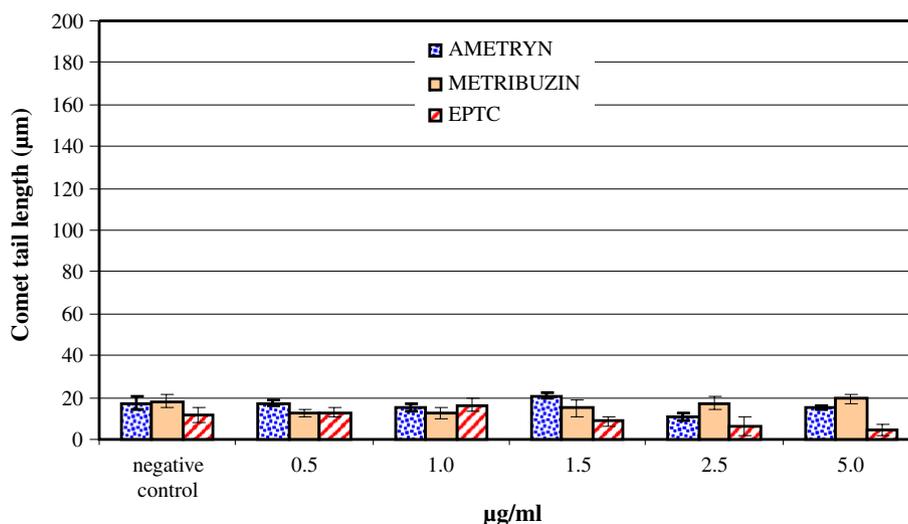


Fig. 2. DNA damage (comet tail length) in human lymphocytes induced by different concentrations of herbicides without *in vivo* metabolic activation by *Vicia faba* root extracts.

Table 1  
DNA damage evaluated by alkaline comet assay and percentage cell viability in human peripheral lymphocytes induced by ametryn with or without activation by *Vicia faba* root metabolites (*in vivo* activation metabolic)<sup>a</sup>

	Means DNA damaged cells Mean ± SE	Level of DNA damage <sup>b</sup> Mean ± SE					% cell viability Mean ± SE
		0	1	2	3	4	
Negative control	3 ± 2.0	96	4	0	0	0	94 ± 2.3
Lymphocytes treated directly with ametryn (µg/mL)							
0.5	3 ± 2.0	97	3	0	0	0	87 ± 1.8
1.0	2 ± 1.6	98	2	0	0	0	81 ± 2.3
1.5	4 ± 1.9	96	4	0	0	0	78 <sup>c</sup> ± 0.7
2.5	3 ± 1.2	97	3	0	0	0	72 <sup>c</sup> ± 1.6
5.0	3 ± 2.0	97	3	0	0	0	72 <sup>c</sup> ± 1.6
Extract of untreated <i>V. faba</i> roots (S10 fraction)	3 ± 2.9	97	3	0	0	0	96 ± 0.2
Lymphocytes treated with 20 µl of <i>V. faba</i> root extracts after treatment with ametryn (mg/mL)							
50	42 <sup>c</sup> ± 2.0	58	10	23	9	0	80 ± 1.1
100	50 <sup>c</sup> ± 1.7	50	12	27	11	0	77 <sup>c</sup> ± 3.8
200	63 <sup>c</sup> ± 1.1	37	8	18	32	5	75 <sup>c</sup> ± 1.7
400	75 <sup>c</sup> ± 1.0	25	0	33	36	6	74 <sup>c</sup> ± 2.6
500	98 <sup>c</sup> ± 1.2	2	0	42	52	4	76 <sup>c</sup> ± 0.9
Positive control							
lymphocytes treated with 20 µl of <i>V. faba</i> root extracts after treatment with 3600 mg/l ethanol	75 <sup>c</sup> ± 1.1	25	5	26	41	3	96 ± 0.33

No significant differences among negative control and treated groups were found by analysis of variance  $F_{\text{ametryn}} = 2.46$ .

<sup>a</sup>  $n = 300$  cells in three experiments.

<sup>b</sup> DNA damaged cells were scored from level 0 (undamaged nucleus) to 4 (severely damaged nucleus): level 1, tail <50 µm; level 2, tail 51–100 µm; level 3, tail 101–150 µm and level 4, tail 151–200 µm.

<sup>c</sup> Significant differences among S10 and treated groups by analysis of variance  $F_{\text{ametryn}} = 95.73$ , the  $p$  value were found the <0.0001, and therefore the Newman–Keuls multiple comparison test was applied,  $p < 0.001$ .

Table 2  
DNA damage evaluated by alkaline comet assay and percentage cell viability in human peripheral lymphocytes induced by metribuzin with or without activation by *Vicia faba* root metabolites (*in vivo* metabolic activation)<sup>a</sup>

	Means DNA damaged cells Mean ± SE	Level of DNA damage <sup>b</sup> Mean ± SE					% cell viability Mean ± SE
		0	1	2	3	4	
Negative control	6 ± 2.0	94	6	0	0	0	97 ± 0.33
Lymphocyte treated directly with metribuzin (µg/ml)							
0.5	4 ± 3.0	96	4	0	0	0	96 ± 1.0
1.0	6 ± 2.5	94	6	0	0	0	94 ± 1.7
2.0	2 ± 5.0	98	2	0	0	0	96 ± 2.0
3.5	2 ± 3.5	98	2	0	0	0	94 ± 1.0
5.0	2 ± 5.0	98	2	0	0	0	90 ± 0.9
Extract of untreated <i>V. faba</i> roots (S10 fraction)	3 ± 2.5	97	3	0	0	0	98 ± 0.60
Lymphocytes treated with 20 µl of <i>V. faba</i> root extracts after treatment with metribuzin (mg/ml)							
50	12 <sup>c</sup> ± 3.5	88	9	3	0	0	94 ± 1.0
100	36 <sup>c</sup> ± 3.0	64	8	16	12	0	92 ± 0.8
200	46 <sup>c</sup> ± 2.9	54	2	26	18	0	96 ± 0.2
400	56 <sup>c</sup> ± 2.6	44	6	24	26	0	94 ± 0.8
500	66 <sup>c</sup> ± 3.7	34	5	22	32	7	92 ± 0.4
Positive control							
Lymphocytes treated with 20 µl of <i>V. faba</i> root extracts after treatment with 3600 mg/l ethanol	88 ± 2.7	12	0	34	52	2	98.3 ± 1.2

No significant differences among negative control and treated groups were found by analysis of variance  $F_{\text{metribuzin}} = 3.45$ .

<sup>a</sup>  $n = 300$  cells in three experiments.

<sup>b</sup> DNA damaged cells were scored from level 0 (undamaged nucleus) to 4 (severely damaged nucleus): level 1, tail <50 µm; level 2, tail 51–100 µm; level 3, tail 101–150 µm and level 4, tail 151–200 µm.

<sup>c</sup> Significant differences among S10 and treated groups by analysis of variance  $F_{\text{metribuzin}} = 55.88$ , were found the  $p$  value was <0.0001, and therefore the Newman–Keuls multiple comparison test was applied,  $p < 0.001$ .

Table 3

DNA damage evaluated by alkaline comet assay and percentage cell viability in human peripheral lymphocytes induced by EPTC with or without activation by *Vicia faba* root metabolites (*in vivo* metabolic activation)<sup>a</sup>

	Means DNA damaged cells Mean $\pm$ SE	Level of DNA damage <sup>b</sup> Mean $\pm$ SE					% cell viability Mean $\pm$ SE
		0	1	2	3	4	
Negative control	3 $\pm$ 2.7	97	3	0	0	0	97 $\pm$ 0.0
Lymphocytes treated directly with EPTC ( $\mu$ g/ml)							
1.5	3 $\pm$ 3.6	97	3	0	0	0	96 $\pm$ 0.0
2.5	8 $\pm$ 3.5	92	8	0	0	0	94 $\pm$ 0.0
5.0	2 $\pm$ 2.3	98	2	0	0	0	96 $\pm$ 0.9
8.0	3 $\pm$ 4.8	97	3	0	0	0	90 $\pm$ 1.0
10.0	2 $\pm$ 2.0	98	2	0	0	0	93 $\pm$ 0.8
Extract of untreated <i>V. faba</i> roots (S10 fraction)	3 $\pm$ 1.0	97	3	0	0	0	98 $\pm$ 0.0
Lymphocytes treated with 20 $\mu$ l of <i>V. faba</i> root extracts after treatment with EPTC (mg/l)							
50	21 <sup>c</sup> $\pm$ 4.3	79	12	9	0	0	96 $\pm$ 1.0
100	28 <sup>c</sup> $\pm$ 2.7	72	4	16	6	2	98 $\pm$ 0.0
200	35 <sup>c</sup> $\pm$ 2.2	65	6	14	13	2	96 $\pm$ 0.0
400	52 <sup>c</sup> $\pm$ 5.9	48	10	30	12	0	94 $\pm$ 0.5
500	82 <sup>c</sup> $\pm$ 6.2	18	4	43	35	0	92 $\pm$ 0.7
Positive control							
Lymphocytes treated with 20 $\mu$ l of <i>V. faba</i> root extracts after treatment with 3600 mg/l ethanol	78 <sup>c</sup> $\pm$ 2.4	22	6	13	22	37	98 $\pm$ 2.0

No significant differences among negative control and treated groups were found by analysis of variance  $F_{\text{EPTC}} = 1.73$ .

<sup>a</sup>  $n = 300$  cells in three separated experiments.

<sup>b</sup> DNA damaged cells were scored from level 0 (undamaged nucleus) to 4 (severely damaged nucleus): level 1, tail  $<50 \mu\text{m}$ ; level 2, tail  $51\text{--}100 \mu\text{m}$ ; level 3, tail  $101\text{--}150 \mu\text{m}$  and level 4, tail  $151\text{--}200 \mu\text{m}$ .

<sup>c</sup> Significant differences among S10 and treated groups were found by analysis of variance  $F_{\text{EPTC}} = 65.38$ , the  $p$ -value was  $<0.0001$ , and therefore the Newman–Keuls multiple comparisons test was applied,  $p < 0.001$ .

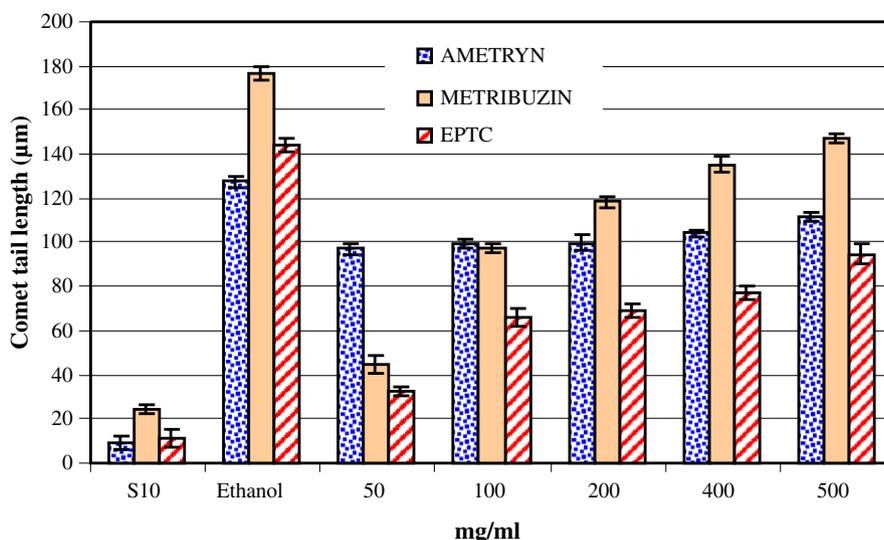


Fig. 3. DNA damage (comet tail length) in human lymphocytes induced by different concentrations of herbicides with *in vivo* metabolic activation by *Vicia faba* root extracts.

DNA damage (comet tail length) plotted as a function of the concentration (mg/ml) of herbicide applied is shown in Fig. 4a–c. Linear regression analysis showed that the slopes for the effects of two triazines with *in vivo* metabolic activation by *V. faba* root extract were significant (Fig. 4a–c). Comet tail length means ( $97 \pm 2.6\text{--}111 \pm 2.6 \mu\text{m}$ ) shows a

linear concentration-response with ametryn and metribuzin, but not EPTC compared with the S10 fraction value (Fig. 4a–c).

The positive control, ethanol, is a powerful promutagen activated by plant metabolism (Takehisa et al., 1988; Calderón-Segura et al., 1999; Gómez-Arroyo et al., 1995,

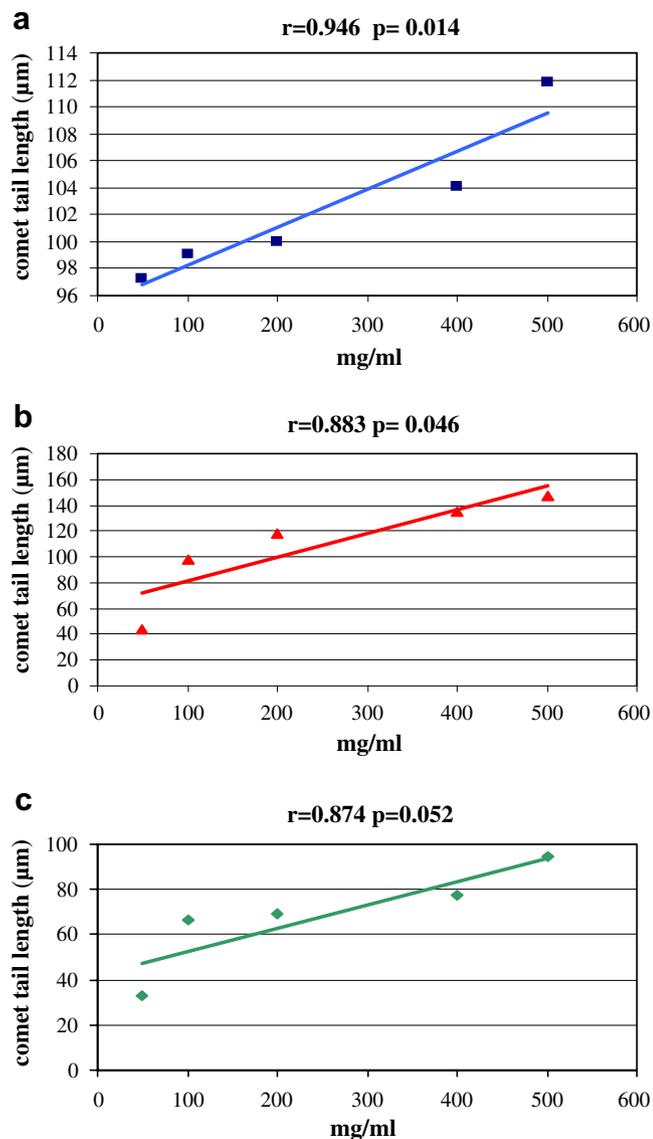


Fig. 4. Regression lines of comet tail length of human lymphocytes induced by different concentrations of herbicides with *in vivo* metabolic activation by *Vicia faba* root extracts. Statistics and some of the data are presented in Tables 1–3. Ametryn (a), metribuzin (b) and EPTC (c).

2000). Lymphocytes exposed for 2 h to 20 µl of the extract from *V. faba* roots treated with ethanol (3600 mg/l) for 4 h induced significant DNA damage as showed in the values of three parameters scored: comet tail length, mean DNA damaged cells and the classification of DNA damaged cell levels. Mean comet tail lengths for donor 1, donor 2 and donor 3 were  $127.6 \pm 2.3$  µm,  $176.5 \pm 3.0$  µm  $144.2 \pm 3.3$  µm, respectively (Fig. 3); for DNA cell damage frequency: 75%, 88% and 78% (Tables 1–3); and for comet classification: levels 1–4 (Table 1; Fig. 1b–f), level 2 and 4 (Table 2; Fig. 1c–f) and level 1 and 4 (Table 3; Fig. 1b–f).

The S10 fraction produced small comet tails ( $9.0 \pm 1.4$  µm;  $24.5 \pm 1.8$  µm,  $11.2 \pm 3.6$  µm; Fig. 3b), low frequency of DNA damage (3%) and damaged cells were classified at level 1 (Fig. 1b) as compared with the negative control, in the three healthy donors (Tables 1–3).

A significant decrease of about 13–19% in cell viability, compared with negative control data, was observed at concentrations of 1.5–5.0 µg/ml of ametryn alone and a decrease of 20–22% with root extracts treated with 50.0–500 mg/l ametryn compared with S10 fraction value (Table 1). No differences in cell viability (percent) were found for metribuzin and EPTC with or without metabolic activation in relation to S10 fraction value (Tables 2 and 3).

#### 4. Discussion

In the last two decades, Mexico, as well as other Latin American countries, has used more agrochemicals in order to increase agricultural productivity. Pesticides are a group of chemical agents used as fungicides, nematicides, acaricides, herbicides, and insecticides. The most frequently used pesticides are organophosphorous, organochlorides, triazines, and carbamates. However, many of these chemicals released in the soil, water, and air affect nontarget organisms, including humans. The present study evaluated the *in vivo* metabolic capability of *V. faba* roots to bioactivate three promutagen herbicides in plant and the induction of DNA damage and cytotoxic effects in human peripheral lymphocytes using alkaline single cell gel electrophoresis. This assay offers considerable advantages over other cytogenetics methods such as chromosomes aberrations, sister chromatid exchanges (SCE) and micronuclei tests because cells do not need to be mitotically active, it requires low concentrations from genotoxicants, and small number cells for the analysis. The comet assay is used to detect DNA damage in individual cells (<10,000), tissues, and organs *in vivo* and *in vitro*. It is also applied to detect DNA repair, apoptosis and in the human and animal reproduction (Tice et al., 2000).

Low concentrations of ametryn and metribuzin (Flores-Maya et al., 2005) without *in vivo* metabolic activation by *V. faba* root extract did not produce sister chromatid exchanges in human peripheral lymphocytes culture. But after plant metabolic activation, two triazines were very effective inducers of SCE in human peripheral lymphocytes culture (Flores-Maya et al., 2005). Our goal was to test if the same extracts from *V. faba* roots treated with concentrations of ametryn, metribuzin produced DNA damage in the lymphocytes detectable by alkaline comet assay. In addition EPTC was tested. This is a thiocarbamate few known genotoxic effects.

When ametryn, metribuzin, and EPTC herbicides were directly applied to human peripheral lymphocytes, it was shown that they were not directly genotoxics as they did not produce DNA damage. These results are in agreement with our other studies with carbamate pesticides (Calderón-Segura et al., 1999; Gómez-Arroyo et al., 1995, 2000; Flores-Maya et al., 2005), and in agreement with the negative results obtained in *Escherichia coli* with metribuzin (Pauli et al., 1990; Xu and Scurr, 1990). Terbutryn s-triazine produced DNA damage in human leukocyte with S9 mix (Villarini et al., 2000), and lymphocyte cultures to higher

concentrations by alkaline comet assay (Moretti et al., 2002). Atrazine, cyanazine, and simazine Cl-triazines did not induce SCE, or chromosome aberrations in human peripheral lymphocytes exposed *in vitro* (Kligerman et al., 2000a; Tennant et al., 2001), neither produced micronuclei in mice bone marrow cells (Kligerman et al., 2000b). Metribuzin and other triazines did not affect *Drosophila melanogaster* (Kaya et al., 2000, 2004). Terbutryne did not induce SCE or micronuclei in human peripheral lymphocytes *in vitro* with or without the S9 metabolic mammalian fraction (Moretti et al., 2002). However, there are contradictory reports on the genotoxicity of triazines. It has been reported that low doses of simazine induced SCE in Chinese hamster V79 cells (Kuroda et al., 1992). High concentrations of atrazine significantly increase DNA migration (comet assay) in human lymphocyte cultures (Ribas et al., 1998). Atrazine, metalochlor, amsol, glifosat, and metribuzin produced DNA damage in tadpole erythrocytes of *Rana catesbeiana* detected using the comet assay (Clements et al., 1997). Atrazine, simazine, and cyanazine produced only marginal damage in mice leucocytes *in vivo* (Tennant et al., 2001).

When human lymphocytes were exposed to 20 µl of the same of root extract, treated for 4 h with ametryn and metribuzin triazines and EPTC thiocarbamate, and coincubated for 2 h, the human lymphocytes nuclei showed significant increase in the genotoxic indicators. Our results are in agreement with Calderón-Segura et al. (1999), Gómez-Arroyo et al. (1995, 2000) and Flores-Maya et al. (2005), who demonstrated that both triazines and molinate and butylate thiocarbamates and propoxur carbamate required plant metabolites to induce genotoxicity in human peripheral lymphocytes culture. Possibly, the root enzymatic system transformed the ametryn and metribuzin triazines and EPTC thiocarbamate to genotoxic metabolites or produced reactive intermediate metabolites that may produce DNA breaks.

The biotransformation of metribuzin and ametryn has been studied in microorganisms, plants (Frear et al., 1983, 1985) and animals (Coleman et al., 2000). The *in vitro* metabolic pathways of ametryn in liver microsomes from rats, pigs, and humans have been described. The dominant phase I-reactions in these studies were *N*-mono-dealkylations, and alkylhydroxylations and sulfoxidations of the thiomethyl ethers ametryn (Lang et al., 1996, 1997; Coleman et al., 2000). Ametryn sulfoxide (sulfoxidation) and deisopropylametryn (*N*-dealkylation) were catalyzed by cytochrome P-450 in liver human microsomes (Coleman et al., 2000; Hanioka et al., 1999). The ametryn sulfoxide metabolite has been shown to contain a putative active site where the rat ALDH-adduct is formed (Lang et al., 1997). In plants, this ametryn herbicide is quickly absorbed by roots and leaves, and transported to the whole plant (Frear et al., 1983, 1985). This herbicide is biotransformed by oxidation and hydroxylation reactions to produce 2-hydroxyametryn compounds (Edwards and Owen, 1989; Lamoureux and Rusness, 1987) which could be the inducers of DNA damage.

The metabolism of metribuzin has been studied in several crop plants such as soybean, sugarcane, tomato, potato, wheat, lentil, and weed (Bardalaye and Wheeler, 1984; Frear et al., 1983, 1985). The major primary metabolic reactions are: (i) deamination, (ii) sulfoxidation, (iii) demethylation, and (iv) *S*-oxidation to two sulfoxide metabolites. In the case of high concentrations, the conjugation of metribuzin sulfoxide and homoglutathione seemed to be the predominant reaction to disable metribuzin, and it has been proposed, that ametryn is biotransformed into an *N*-dealkylation reaction oxidizer (Bardalaye and Wheeler, 1984; Frear et al., 1983, 1985; Tchounwoul et al., 2000). Therefore, the 2-hydroxy-sulfoxide-, sulfone compounds for ametryn could be the inducers of DNA single strand breaks, indicating that these compounds are promutagens.

The genotoxic activity of ametryn, and metribuzin, on human peripheral lymphocytes had a concentration–response relationship between the comet tail length and the concentration applied, compared with S10 fraction. These results were similar to those found Flores-Maya et al. (2005), who demonstrated that both triazines applied to *V. faba* root tip meristems increase SCE frequency with a concentration–response relationship.

EPTC thiocarbamate exerts its major herbicidal effects after metabolic transformation to its sulfoxide or sulfone derivatives. It may be through cytochrome P-450 monooxygenase or, more likely, peroxidase in *V. faba* root (Barret and Harwood, 1998; Casida et al., 1975; Lamoureux and Rusness, 1987). The sulfoxidation of EPTC represents a metabolic pathway where reactive electrophilic intermediates are generated and sulfoxides of thiocarbamates are the most potent carbamylating agents (Casida et al., 1975). It has been shown that EPTC sulfoxide inhibits fatty acid elongation in plants (Baldwin et al., 2003). It can covalently bind and inhibit the activity of mouse aldehyde dehydrogenase, (Staub et al., 1999), as well as covalently bind to DNA, and produce adducts in rat hepatocytes (Zimmerman et al., 2004). Probably, *V. faba* roots are able to transform EPTC into a sulfoxide or sulfone which may induce DNA strand breaks in human lymphocytes. Such results in DNA damage are consistent with our previously obtained results in the induction of SCE with butylate and molinate thiocarbamates after *V. faba* metabolic activation (Calderón-Segura et al., 1999; Gómez-Arroyo et al., 2000).

In our study, the three herbicides induced DNA damage in lymphocytes of human healthy volunteers at all concentrations. Ametryn and metribuzin were more genotoxic than EPTC because they induced greater comet tail lengths than the DNA damage caused by of the three herbicides depended on the concentration. The S10 fraction did not modified cell viability and basal DNA damage compared with the negative control value (free pesticides).

We used ethanol as a positive control because it is genotoxic in Chinese hamster ovary cells (Takehisa et al., 1988) and in human lymphocytes culture with the same plant

activation system (Calderón-Segura et al., 1999; Gómez-Arroyo et al., 2000; Flores-Maya et al., 2005). It has been demonstrated that DNA damage is induced by acetaldehyde, since ethanol is metabolized into acetaldehyde and free radicals which are mutagenic and carcinogenic. Ethanol is also a good inductor of sister chromatid exchange, chromosomal aberrations and adducts *in vivo* and *in vitro* in mammalian cells (Obe et al., 1986; Fang and Vaca, 1997; Pöschl and Seitz, 2004). In our research ethanol had longer mean comet tail lengths, a greater frequency of cells with damage to DNA, and a higher level of the DNA damage than the three herbicides and the S10 fraction. In addition, these results corroborated that alcohol is a good positive control and it can be used as a vegetal promutagen for alkaline comet assay. We have evaluated cytogenetic effects of different pesticides with or without vegetal metabolic activation by *V. faba* root in human peripheral lymphocytes in culture through frequency of SCE, in all our experiments we have used large enough pesticide concentrations, large enough volumes of vegetal extracts and long enough exposure times to ensure genotoxic response if they exist (Calderón-Segura et al., 1999; Gómez-Arroyo et al., 1995, 2000; Flores-Maya et al., 2005). The results support that the alkaline comet assay is more efficient, sensitive and rapid to detect DNA damage caused by promutagen herbicides by *V. faba* root than SCE method.

Cell viability was not statistically affected at all the concentrations of the herbicides with or without *in vivo* plant metabolic activation, except for ametryn alone which reduced cell viability by 13–19% (86–76%) compared to the negative control (94%) at concentrations of 1.5–5 µg/ml. Cytotoxic effects in human lymphocytes cultures were observed at similar concentrations of ametryn (Flores-Maya et al., 2005) and effects were also found in leukocyte cultures exposed to terbutryn with or without S9 metabolic activation (Moretti et al., 2002). This response could be due to secondary effects for there was not evidence of a relationship between cytotoxic effects on the three parameters of DNA damage (comet tail length, percentages, and proportions of damaged cells) with the parent compound. It is possible that lymphocytes were particularly more sensitive to the maximum concentrations of ametryn and levels of other intermediate metabolite-ametryn included in vegetal extract, perhaps they interfered with intracellular proteins that lead cell death. Furthermore, the reduction of cell viability produced by ametryn with and without metabolic activation was ≤22% (83–79%) compared with negative control (96%) and the S10 fraction (94%). This reduction is within acceptable limits for not interfering with genotoxic effects (Hartmann and Speit, 1997; Hartmann et al., 2001; Henderson et al., 1998). To avoid false positive responses in our genotoxic analysis with the three herbicides and ethanol, the analysis did not include cells with comets but without nuclei (clouds) (Hartmann and Speit, 1997). We measured the following biochemical parameters: the pH (mean 7.5) in the coincubated medium of human

lymphocytes with direct ametryn, the pH (mean 7.5) in the root extracts of *V. faba* treated with ametryn, and the protein concentrations (0.6 µg/ml) in the root extracts of *V. faba* treated with ametryn also incubated with human lymphocytes *in vitro*. Both parameters were within normal physiological conditions in order not to increase the basal DNA damage. It is unclear whether the cytotoxicity and genotoxicity produced by ametryn and their intermediate metabolites are induced via different mechanisms, and further research is needed.

The data generated in the present study provide further evidence that *in vivo* *V. faba* metabolism is important in the genotoxicity of ametryn, metribuzin triazines, and EPTC thiocarbamate herbicide, since they produce DNA single strand breaks, probably mainly by forming active metabolites. Comparing the genotoxic effect of the three herbicides, we found that ametryn and metribuzin *S*-triazines are more genotoxic than the EPTC thiocarbamate. Furthermore, our results suggest that the differences in DNA damage are due to the interindividual variability between donors.

In conclusion our results also show that comet assay seems to be a reliable, rapid and highly sensitive method for detecting DNA damage in human cells by plant promutagens and it showed that DNA damage occurs at low concentrations of these herbicides in conjunction with of *V. faba* metabolites. These pesticides are extensively used in agricultural at higher concentrations than those tested in this study. Thus they are could represent a risk factor to human and animal health.

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