*Research Article*

# Evaluation of Genome Damage and Its Relation to Oxidative Stress Induced by Glyphosate in Human Lymphocytes in Vitro

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In the present study we evaluated the genotoxic and oxidative potential of glyphosate on human lymphocytes at concentrations likely to be encountered in residential and occupational exposure. Testing was done with and without metabolic activation (S9). Ferric-reducing ability of plasma (FRAP), thiobarbituric acid reactive substances (TBARS) and the hOGG1 modified comet assay were used to measure glyphosate's oxidative potential and its impact on DNA. Genotoxicity was evaluated by alkaline comet and analysis of micronuclei and other nuclear instabilities applying centromere probes. The alkaline comet assay showed significantly increased tail length (20.39 μm) and intensity (2.19%) for 580 μg/ml, and increased tail intensity (1.88%) at 92.8 μg/ml, compared to control values of 18.15 μm for tail length and 1.14% for tail intensity. With S9, tail length was significantly increased for all concentrations tested: 3.5, 92.8, and 580 μg/ml. Using the hOGG1 comet assay, a significant increase in tail intensity was observed at 2.91 μg/ml with S9 and 580 μg/ml without S9. Without S9, the frequency of micronuclei, nuclear buds and nucleoplasmic bridges slightly increased at concentrations 3.5 μg/ml and higher. The presence of S9 significantly elevated the frequency of nuclear instabilities only for 580 μg/ml. FRAP values slightly increased only at 580 μg/ml regardless of metabolic activation, while TBARS values increased significantly. Since for any of the assays applied, no clear dose-dependent effect was observed, it indicates that glyphosate in concentrations relevant to human exposure do not pose significant health risk. Environ. Mol. Mutagen. 00:000–000, 2009. 

**Key words: hOGG1 comet assay; centromere; glyphosate; genotoxicity; metabolic activation; thiobarbituric acid reactive substances (TBARS)**

## INTRODUCTION

Glyphosate (*N*-phosphomethyl glycine) is a nonselective, broad spectrum, postemergence organophosphorus herbicide used to control broad-leaf weeds in emerged grasses, pastures and rice, corn and soy [Smith and Oehme, 1992]. It was discovered in 1970 by scientists from Monsanto Company. Since then it has become one of the world's most widely used herbicides with estimated annual consumption of 51,000 tons in USA, 1,600 tons in Great Britain and 20,000 tons in Europe [Kiely et al., 2004]. In plants glyphosate inhibits 5-enolpyruvoylshikimate-3-phosphate synthase resulting in a retardation of the shikimate pathway in aromatic amino acid biosynthesis [Alibhai and Stallings, 2001]. Since the pathway operates only in plants and microorganisms, glyphosate is not considered to pose a risk for humans. Nevertheless, some recent data show that glyphosate is able to interfere with specific physiological pathways in eukaryotic cells. By the molecular switch with the CDK1/cyclin B complex, it leads to the dysfunction of the G2/M cell-cycle checkpoint [Marc et al., 2004]. Although there are no published data on glyphosate apoptotic activity, the fact that it arrests cell division might indicate its ability to induce apoptosis [Belle et al., 2007].

Benachour et al. [2007] confirmed that glyphosate based pesticide reduces estrogen production in human pla-

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cental and embryonic cells. In rats, the observed endocrine disruption resulted in reduced maternal weight gain, a significant decrease in the number of implantations, viable fetuses, and fetal body weight [US EPA, 1993a]. Data on glyphosate's teratogenicity and genotoxicity are ambiguous. Of all the observed teratogenic effects (alterations of skull, sternebra and limbs) only delayed ossification of skull showed a dose related response [Dallegrave et al., 2003]. Treatment of human lymphocytes with glyphosate in vitro induced an increase in sister chromatid exchange [Bolognesi et al., 1997], chromosomal aberrations and indicators of oxidative stress [Lioi et al., 1998]. Lack of any genotoxic effect has been reported [Vigfusson and Vyse, 1980; Dimitrov et al., 2006]. However, some epidemiological studies have demonstrated a correlation between glyphosate exposure and non-Hodgkin's lymphoma [Hardell et al., 2002; De Roos et al., 2003]. Currently, there are increasing numbers of genetically modified crops being sown that are resistant to pesticides. Most of these are glyphosate resistant which enables them to tolerate higher concentrations of the active ingredient needed for effective weed control. Hence, higher amounts of glyphosate are introduced into the environment. Due to the yearly increase of its consumption, it should be evaluated with new tests. In our study we decided to test five concentrations for the possibility of oxidative damage to DNA with and without exogenous metabolic activation system (S9); three of these concentrations are likely to be encountered in residential and occupational exposures to glyphosate. Since organophosphorous (OP) pesticides may induce oxidative stress leading to generation of free radicals and alterations in the antioxidant system, blood samples were also analyzed for total antioxidant capacity (TAC) and lipid peroxidation.

## MATERIALS AND METHODS

### Blood Sampling

Blood samples were taken from three healthy male nonsmoking donors. According to questionnaire, which the donors completed, they had not been exposed to any physical or chemical agent in the 12 months before blood sampling that might interfere with the results of the testing. Blood was drawn by antecubital venipuncture into heparinized vacutainers (Becton Dickenson, Plymouth, UK). All donors were acquainted with the study and they signed permission for their blood samples to be used for scientific purposes.

### Treatment in vitro

The treatment was performed in accordance with OECD chemical testing guidelines [OECD, 2006]. Half a milliliter of whole blood was introduced to 0.5 ml RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA) medium without the mitogen and newborn calf serum. For each donor duplicate cultures were treated with a technical grade glyphosate (98%, Supelco, Sigma, St. Louis, MO) as a pure active ingredient. Before treatment, glyphosate was diluted in PBS and pH was adjusted to 7.2. Glyphosate was tested in the final concentrations of 0.5, 2.91, 3.5, 92.8, and 580 μg/ml. Concentrations were chosen to correspond to values of acceptable daily intake (Annex I, EU directive 91/414/EEC), residential exposure level [US EPA, 2004], occupational exposure level (OEL) [US EPA, 2004], 1/100 $LD_{50}$ (Annex I, EU directive 91/414/EEC) and 1/16 of oral $LD_{50}$ in rats (Annex I, EU directive 91/414/EEC), respectively. Extrapolation was made according to Guyton and Hall [1996]. Each concentration was tested both with and without metabolic activation [10% (w/w) using human liver S9 mix; Sigma] in duplicate cultures. Cultures were incubated for 4 hat 37°C. Negative control was treated with standard physiological solution. For the vital staining, alkaline and hOGG1 modified comet assay blood samples were treated with 100 μl of 1 mM $H_2O_2$ at +4°C as a positive control. For the chromosome and nuclear instability assay cultures without S9 were treated with ethyl methanesulfonate (Sigma, St. Louis, MO) at a final concentration of 200 μg/ml as a positive control. Since cyclophosphamide (Sigma, St. Louis, MO) requires metabolic activation to exert its clastogenic effect, it was used at a final concentration of 30 μg/ml as the positive control for the S9 system mix. The positive control cultures were treated for 72 hr of cultivation.

### Vital Staining Using Ethidium Bromide and Acridine Orange

The indices of cell viability and necrosis were obtained from differential staining with acridine orange and ethidium bromide, using fluorescence microscopy [Duke and Cohen, 1992]. Fifty microlitres of treated blood was mixed with 50 μl of a solution of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml, 1:1; v/v). The suspension mixed with dye was covered with a cover slip and analyzed under the epifluorescence microscope AX 70 (Olympus, Tokyo, Japan) at 400× magnification. 400 lymphocytes were analyzed (200 per duplicate culture) for each lymphocyte culture (concentration), counting the unstained (viable) cells. The nuclei of vital cells emitted a green fluorescence; apoptotic lymphocytes emitted a green fluorescence surrounded by a red echo and necrotic red fluorescence.

### Alkaline and hOGG1 Modified Comet Assay

After treatment, cells were centrifuged, supernatant removed and a standard alkaline comet assay was performed on the whole-blood samples in accordance with the protocols of Singh et al. [1988] and Smith et al. [2006]. All the chemicals were obtained from Sigma Chemical Company and Trevigen. Fully frosted slides were coated with 1% and 0.6% normal melting point agarose. Blood samples (8 μl) were mixed with 0.5% low melting point agarose, placed on the slides, and were immersed in freshly prepared ice-cold lysis solution (Trevigen lysis solution, 10% DMSO, pH 10) and stored at 4°C overnight. For the alkaline comet assay the standard procedure was followed [Singh et al., 1988]. Comet assay analysis was done in duplicate.

For the hOGG1 modified comet assay, the slides were rinsed in three changes of 1× Flare buffer (Trevigen) for 5 min at room temperature. Afterwards, hOGG1 was added to the gel in 100 μl of Flare reaction buffer dilutions (1:4000), as described by Smith et al. [2006]. Simultaneously, for each concentration parallel slides were treated with 100 μl of Flare reaction buffer only and gels were incubated in a humidified chamber for 10 min at 37°C. Alkaline denaturation and electrophoresis were carried out at 4°C in electrophoretic solution (1.5 M NaCl, 1 mM $Na_2EDTA$, pH 12.1). After 20 min, the slides were placed in the horizontal gel-electrophoresis tank. Electrophoresis at 0.7 V/cm, 300 mA lasted another 15 min. The slides were neutralized in three changes of buffer (0.4 M Tris-HCl, pH 7.5) at 5 min. intervals. Slides were stained with ethidium bromide (20 g/ml). Each slide was examined using a 250× magnification fluorescence microscope (Zeiss, Oberkochen, Germany). A total of 100 comets per concentration tested were scored on each duplicate slide. The edges of the gel, occasional dead cells and superimposed comets were avoided. Tail length and tail intensity were

measured using the Comet Assay IV analysis system (Perceptive Instruments, Suffolk, UK). Oxidative DNA damage was given as a difference in mean values between gels treated with hOGG1 enzyme and gels treated with reaction buffer.

### Lymphocyte Cultures and FISH Analysis of Chromosomal and Nuclear Instability

After the treatment period, samples were centrifuged. Samples were washed in 0.5 ml of RPMI, centrifuged, supernatant removed and the pellet used to set up cultures by adding it to 6 ml of RPMI supplemented with 15% foetal calf serum (Sigma, St. Louis, MO), and 1% antibiotics (penicillin and streptomycin; Gibco, UK). Lymphocytes were stimulated by 1% phytohaemagglutinin (Remel, UK) and incubated for 72 hr at 37°C. Cultivation and slide preparation was done according to standard protocol [Fenech, 2006]. Cytokinesis was arrested using cytochalasin B (Sigma, St. Louis, MO), at a final concentration of 6 μg/ml and added to the culture after 44 hr of incubation. Cells were centrifuged, washed in saline solution (0.9% NaCl, Sigma) and fixed with 3:1 (v/v) methanol/acetic acid solution. Slides were stained with 5% Giemsa (Merck, Germany). One thousand binucleated cells with well-preserved cytoplasm were scored per subject, to determine the total number of micronuclei in binucleated lymphocytes (MN), nuclear buds (NBs), and nucleoplasmic bridges (NPBs). We applied scoring criteria described by Fenech [2006]. The cytokinesis-block proliferation index (CBPI) was evaluated by classifying 1000 cells per number of nuclei, as suggested by Surralles et al. [1995] according to the formula: CBPI = [M1+2M2+3(M3+M4)]/N, where M1-M4 indicate the number of cells with 1–4 nuclei respectively, and N the total number of cells scored. To minimize the variability, the same researcher carried out all the microscopic analysis. To detect the ratio of micronuclei (C+MN), nuclear buds (C+NB), and nucleoplasmic bridges (C+NPB) originating from whole chromosomes that contain centromeres, and the number of DAPI signal positive micronuclei (+MN), slides were kept in dark for a month. Slides were hybridized with All Human Centromere Satellite Probes (Q biogen, UK) directly labelled with a red fluorophore (Texas Red spectrum) following the supplier's instructions. Slides were counterstained with DAPI prepared in an antifade solution (Q biogen, UK). Probed slides were scored using an Olympus AX70 epifluorescence microscope. One thousand binucleated lymphocytes were analyzed for each concentration.

### Ferric-Reducing Ability of Plasma (FRAP) Assay

Plasma samples were separated by centrifugation and antioxidant power was determined by measuring their ability to reduce $Fe^{3+}$ to $Fe^{2+}$ as established by the Ferric-reducing ability of plasma (FRAP) test [Benzie and Strain, 1996]. The reagents included 300 mM acetate buffer (pH 3.6) with 16 ml acetic acid per 1 ml of buffer solution, 10 mM 2,4,6-tri[2-pyridyl]-s-triatine (TPTZ; Sigma, St. Louis, MO) in 40 mM HCl and 20 mM $FeCl_3$. Working FRAP reagent was prepared as required by mixing 20 ml acetate buffer, 2.0 ml TPTZ solution, 2.0 ml $FeCl_3$ solution and 2.4 ml distilled water. Thirty microliters of plasma sample diluted in saline (1:1) was then added to 1 ml of freshly prepared reagent warmed at 37°C. The complex between $Fe^{2+}$ and TPTZ gives a blue color with absorbance at 593 nm. Water solutions of known $FeSO_4$ concentration, in the range of 0.1–1.0 mM, were used for obtaining the calibration curve. For FRAP assay, as a positive control half a milliliter of whole blood was treated with vitamin C (Sigma, St. Louis, MO) at a final concentration of 100 μg/ml.

### Lipid Peroxidation Thiobarbituric Acid Reactive Substances (TBARS)

Malondialdehyde (MDA), the secondary product of lipid peroxidation, was estimated in the plasma samples using the colorimetric reaction of thiobarbituric acid (TBA). It gives an index of the extent of progress of lipid peroxidation. Since the assay estimates the amount of TBA reactive substances e.g., MDA, it is also known as thiobarbituric acid reactive substances (TBARS) test.

The concentration of TBARS, as a measure of lipid peroxidation, was determined using a modification of the method of Drury et al. [1997]. Five microliter 0.2% (w/v) butylated hydroxytoluene (BHT; Sigma, St. Louis, MO) and 750 μl 1% (v/v) phosphoric acid was added to 50 μl plasma sample. After mixing, 250 μl 0.6% (w/w) TBA (Sigma, St. Louis, MO) and 445 μl $H_2O$ were added and the reaction mixture was incubated in a water bath at 90°C for 30 min. The mixture was cooled and absorbance was measured at 532 nm on spectrophotometer Cecil 9000 (Cecil Instruments Limited, Cambridge, UK). The concentration of TBARS was calculated using standard curves of increasing 1,1,3,3-tetramethoxypropane (Sigma, St. Louis, MO) concentrations, and expressed as μmol/l.

### Statistical Analysis

Differences in tail length and tail intensity (% DNA) were statistically analyzed between each concentration used and between concentrations and control in accordance with the presence of S9 using Mann-Whitney U-test. For the hOGG1 modified comet assay, for each replicate slide the mean tail length and tail intensity values were calculated. Means obtained with buffer were compared with the means for the corresponding enzyme treated slide. Additionally, after subtractions of the means obtained with the buffer from the means obtained with the enzyme, the resulting values that represent 8-hydroxy-2′-deoxyguanosine (8-OHdG) damage were compared between treated and control cultures [Smith et al., 2006]. Differences in the number of MN, C+MN, +MN, NB, C+NB, NPB, C+NPB, and CBPI between the treated and control cultures, with and without S9, were evaluated with Wilcoxon Rank Sum Test. An unpaired Student's *t*-test was used for statistical analysis of FRAP and TBARS values.

## RESULTS

The percentages of viable and nonviable lymphocytes in samples treated with glyphosate in vitro indicate a linear dose response (Table I). A significant increase in the number of early apoptotic and necrotic cells without S9 was detected only at 580 μg/ml. In the presence of S9 an increase in the number of apoptotic cells was already observed at 2.91 μg/ml, but necrotic cells were only observed at 580 μg/ml. As shown in Tables II and III, glyphosate induced a limited DNA damage in the treated lymphocytes evaluated using both the alkaline and hOGG1 comet assay. The alkaline comet assay (Table II) without S9 showed a significant increase in the mean tail intensity at the three highest concentrations (3.5, 92.8, and 580 μg/ml). The addition of S9 significantly elevated DNA migration already at 3.5 μg/ml. Tail intensity was significantly affected only at the highest concentration tested. Thus, the dynamics of the DNA damage intensity slightly differed between treatments with and without exogenous metabolic activation system (Table II and III).

In the hOGG1 comet assay without S9 a significant increase was observed only for the tail intensity at 3.5 μg/ml. Addition of S9 significantly elevated only the tail length at the highest concentration (580 μg/ml). Again,

**TABLE I. Results of Vital Staining of Peripheral Blood Lymphocytes Treated with Glyphosate With and Without Metabolic Activation System (S9) for 4 hr**

| | −S9 | | | +S9 | | |
|---|---|---|---|---|---|---|
| Concentration µg/ml | Early apoptosis % | Late apoptosis % | Necrosis % | Early apoptosis % | Late apoptosis % | Necrosis % |
| 0.50 | 9.0 | — | 0.5 | 10.5 | 1.0 | 2.5 |
| 2.91 | 11.0 | — | 2.0 | 13.5* | 0.5 | 3.0 |
| 3.50 | 13.0 | — | 3.0 | 20.0** | 3.0 | 2.5 |
| 92.8 | 14.2 | — | 3.4 | 23.1**# | 2.8 | 2.7 |
| 580 | 19.5** | — | 5.5** | 30.0**# | 2.0 | 6.5* |
| Positive control | 21.0** | 30.5** | 19.0** | 17.5** | 36.0** | 14.5** |
| Negative control | 6.5 | — | 0.5 | 5.5 | — | 2.0 |

For each treatment procedure 400 cells were analyzed. *$P < 0.05$; **$P < 0.01$ vs. control; #$P < 0.05$ vs. treatment without S9; positive control 100 µl of 1 mM $H_2O_2$ 15 min; negative control 100 µl of 0.9 % NaCl 4 hr.

**TABLE II. DNA Strand Breaks (Standard Comet Assay) in Lymphocytes Treated With Glyphosate With and Without Metabolic Activation System (S9)**

| | Alkaline comet assay | | | |
|---|---|---|---|---|
| | −S9 | | +S9 | |
| Concentration µg/ml | Tail length (µm) ± SD | Tail intensity (% DNA) ± SD | Tail length (µm) ± SD | Tail intensity (% DNA) ± SD |
| 0.50 | 18.3 ± 2.11 | 1.32 ± 1.96 | 20.03 ± 3.48 | 3.11 ± 3.93## |
| 2.91 | 18.2 ± 3.41 | 1.57 ± 2.34 | 20.32 ± 4.21 | 3.27 ± 4.07## |
| 3.50 | 17.11 ± 2.34 | 1.80 ± 2.85** | 22.08 ± 5.83*## | 3.46 ± 4.55## |
| 92.8 | 17.92 ± 2.56 | 1.88 ± 2.71** | 23.44 ± 5.97*## | 3.59 ± 4.62## |
| 580 | 20.39 ± 4.63**++ | 2.19 ± 3.88** | 26.12 ± 7.92**++## | 4.69 ± 5.36*++## |
| Positive control | 41.22 ± 25.73** | 29.80 ± 9.58** | 35.84 ± 17.32** | 31.15 ± 10.48** |
| Negative control | 18.15 ± 2.29 | 1.14 ± 2.40 | 19.84 ± 4.60 | 3.24 ± 4.51 |

Measured damage is presented as mean values of the tail length and tail intensity.
*$P < 0.05$; ***$P < 0.01$ vs. control; ***$P < 0.05$ and ++$P < 0.01$ vs. lower concentration; #$P < 0.05$ and ##$P < 0.01$ vs. treatment without S9; positive control 100 µl of 1 mM $H_2O_2$ 15 min; negative control 100 µl of 0.9% NaCl 4 hr.

**TABLE III. DNA Base Oxidation (Comet Assay + hOGG1 Enzyme) in Lymphocytes Treated with Glyphosate With and Without Metabolic Activation System (S9)**

| | hOGG1 Comet assay | | | |
|---|---|---|---|---|
| | −S9 | | +S9 | |
| Concentration µg/ml | Tail length (µm) ± SD | Tail intensity (% DNA) ± SD | Tail length (µm) ± SD | Tail intensity (% DNA) ± SD |
| 0.50 | 0.5 ± 0.23 | 0.26 ± 0.47 | 0.5 ± 0.18 | 0.41 ± 0.32 |
| 2.91 | 0.6 ± 0.37 | 0.35 ± 0.82 | 0.4 ± 0.55 | 0.58 ± 0.26# |
| 3.50 | 1.0 ± 0.83 | 1.15 ± 1.61*+ | 0.3 ± 0.95 | 0.36 ± 0.17 |
| 92.8 | 0.7 ± 0.46 | 0.44 ± 0.64 | 0.8 ± 0.77 | 0.55 ± 0.23+ |
| 580 | 0.6 ± 0.2 | 0.41 ± 0.40 | 1.9 ± 0.37**+# | 0.63 ± 0.94# |
| Positive control | 10.93 ± 4.38** | 12.57 ± 7.62** | 11.13 ± 3.78** | 15.22 ± 8.10** |
| Negative control | 0.6 ± 0.52 | 0.3 ± 0.36 | 0.2 ± 0.33 | 0.43 ± 0.1 |

Presented endpoint values are calculated as the difference between values obtained with the hOGG1 enzyme and those obtained with buffer only.
*$P < 0.05$; **$P < 0.01$ vs. control; +$P < 0.05$ and #$P < 0.01$ vs. lower concentration; ##$P < 0.05$ and *$P < 0.01$ vs. treatment without S9; positive control 100 µl of 1 mM $H_2O_2$ 15 min; negative control 100 µl of 0.9% NaCl 4 hr.

the detected DNA damage was not dose dependent. By comparing the results obtained with and without S9, we observed a significant increase of the tail intensity due to metabolic activation at 2.9 and 580 µg/ml (Table III). Without S9 the number of MN, NB, and NPB slightly increased at 3.5, 92.8, and 580 µg/ml (Table IV). A sig-

**TABLE IV. Frequency of micronuclei (MN), nuclear buds (NB), nucleoplasmic bridges (NPB), and cytokinesis-block proliferation index (CBPI) without S9**

| | MN | | | NB | | NPB | | |
|---|---|---|---|---|---|---|---|---|
| Concentration | Mean/1,000 | | | Mean/1,000 | | Mean/1,000 | | |
| μg/ml | BNC | % C+ | % DAPI + | BNC | % C+ | BNC | % C+ | CBPI |
| 0.50 | 7.3 | 37.0 | 37.0 | 1.7 | 17.6 | 1.3 | 0.0 | 1.91 |
| 2.91 | 8.3 | 24.1 | 36.1 | 2.3 | 0.0 | 1.3 | $23.1^{**}$ | 1.72 |
| 3.50 | 15.7 | 40.1 | $42.7^{**}$ | 5.7 | 12.3 | 3.3 | $9.1^{**}$ | 1.54 |
| 92.8 | 16.3 | 39.4 | 37.2 | 7.3 | 11.3 | 4.3 | $12.2^{**}$ | 1.59 |
| 580 | 17.7 | 41.2 | 32.2 | $12.0^{*}$ | 14.2 | 6.0 | $16.7^{**}$ | 1.68 |
| Positive control | $45.8^{**}$ | 44.4 | $29.0^{**}$ | $28.4^{**}$ | $18.3^{**}$ | $12.4^{**}$ | $20.6^{**}$ | $1.35^{*}$ |
| Negative control | 10.3 | 41.7 | 22.3 | 3.0 | 10.0 | 0.7 | 0.0 | 1.89 |

For each of three donors 1,000 lymphocytes per treatment procedure were analyzed.
$^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; C+ centromere containing chromatin structure; DAPI+ chromatin structure with intense DAPI signal; BNC, binuclear cell; positive control ethyl methanesulfonate 200 μg/ml; negative control 100 μl of 0.9 % NaCl 4 hr.

**TABLE V. Frequency of micronuclei (MN, C+MN, +MN), nuclear buds (NB, C+NB), nucleoplasmic bridges (NPB, C+NPB) and CBPI with S9**

| Concentration | MN | | | NB | | NPB | | |
|---|---|---|---|---|---|---|---|---|
| μg/ml | Σ | % C+ | % DAPI+ | Σ | % C+ | Σ | % C+ | CBPI |
| 0.50 | 11.3 | 35.4 | 23.9 | 4.7 | $21.3^{**}$ | 2.7 | 0.0 | 1.70 |
| 2.91 | 14.0 | 26.4 | 16.4 | 4.0 | $32.5^{**\#\#}$ | 3.3 | 0.0 | 1.65 |
| 3.50 | 19.3 | 43.0 | $39.9^{**++}$ | 8.7 | $42.5^{**\#\#}$ | 3.7 | 0.0 | 1.57 |
| 92.8 | 22.3 | 45.0 | $40.3^{**}$ | 11.0 | $36.3^{**}$ | 4.6 | $21.4^{**++}$ | 1.63 |
| 580 | $28.7^{**}$ | $65.2^{**+\#\#}$ | $46.3^{**}$ | $19.7^{**+}$ | $37.0^{**}$ | $9.7^{*}$ | $27.8^{**}$ | 1.77 |
| Positive control | $32.2^{**}$ | 48.1 | 20.5 | $17.9^{**}$ | 16.5 | $11.2^{**}$ | $32.1^{**}$ | $1.42^{*}$ |
| Negative control | 11.3 | 32.7 | 20.4 | 2.7 | 0.0 | 0.3 | 0.0 | 1.86 |

$^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{+}P < 0.05$ and $^{++}P < 0.01$ vs. lower concentration; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. treatment without S9; C+ centromere containing chromatin structure; DAPI+ chromatin structure with intense DAPI signal; BNC, binuclear cell; positive control cyclophosphamide 30 μg/ml; negative control 100 μl of 0.9% NaCl 4 hr.

nificant effect was detected only for NBs at the highest concentration. With addition of S9 an increase was observed for all concentrations within the tested range, but it was significant for MN, NB, and NPB at 580 μg/ml (Table V). For the treatment without S9 only the proportion of C+NPB increased significantly at 2.91 μg/ml (Table IV). For the treatment with S9 a proportion of chromatic formations containing centromeres, and MN with DAPI signal increased significantly compared at the highest concentration (580 μg/ml) as compared to the control. Moreover, the number of MN with DAPI signal was already significantly elevated at 3.5 μg/ml (Table V).

FRAP levels are shown in Figure 1: an significantly increased plasma antioxidant capacity was observed following glyphosate treatment at 580 μg/ml, both with and without S9. A significant increase in lipid peroxidation as compared to corresponding controls was observed at 580 μg/ml, with and without S9 (Fig. 2).

## DISCUSSION

To enable more efficient weed control, there has been an increase in the planting of crops engineered to resist herbicides. The amounts of glyphosate introduced into the environment rise every year [Bradberry et al., 2004]. In this study we applied two cytogenetic techniques: (A) comet assay, alkaline and hOGG1 modified, to enable the evaluation of possible oxidative effects; and (B) chromosome and nuclear instability assay applying new scoring criteria [Fenech, 2006]. To consider the effect of glyphosate's metabolites we also used exogenous metabolic activation (S9). Results of previous genotoxicity studies are ambiguous, possibly due to differences in the purity of the evaluated active ingredient, the type of testing, and the experimental models used. A short overview of previous results is summarized in Table VI.

Our results of the hOGG1 comet assay did not demonstrate induction of oxidative damage 8-OHdG over the entire dose range tested. Significance was observed only at the highest concentration (580 μg/ml) with S9 for tail length, and without S9 at 3.5 μg/ml for tail intensity. Since we did not find a clear dose-response relation, the results do not indicate an unequivocal oxidative potential of glyphosate. Similarly, Bolognesi et al. [1997] reported an elevation in the values of 8-OHdG in liver and kidneys after treatment with glyphosate concentrations correspond-

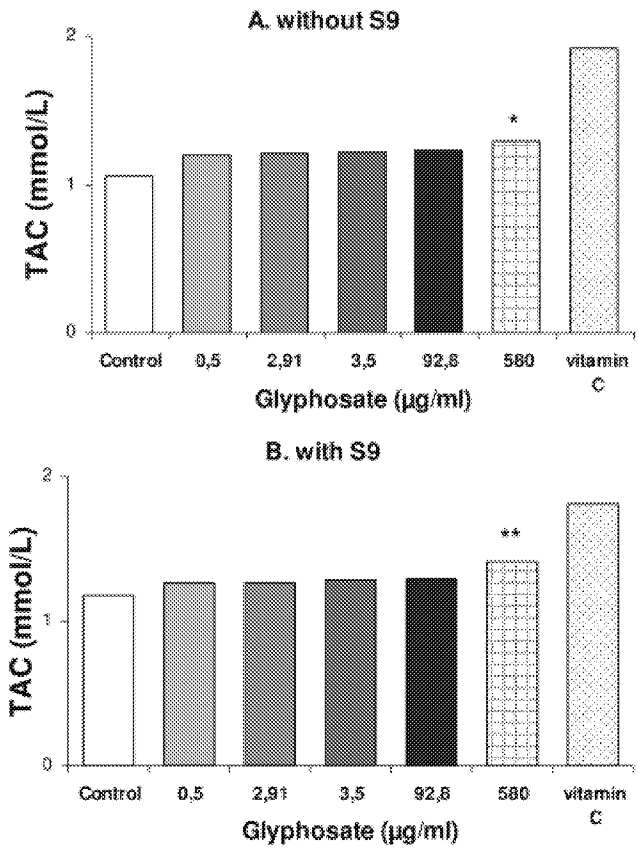


Fig. 1. Changes in plasma total antioxidant capacity (TAC) following treatment with glyphosate alone (A) or in combination with metabolic activation system S9 (B). Values are mean ± SD, $n = 3$. * Significantly different from control without S9 ($P < 0.05$). ** Significantly different from control with S9 ($P < 0.01$).

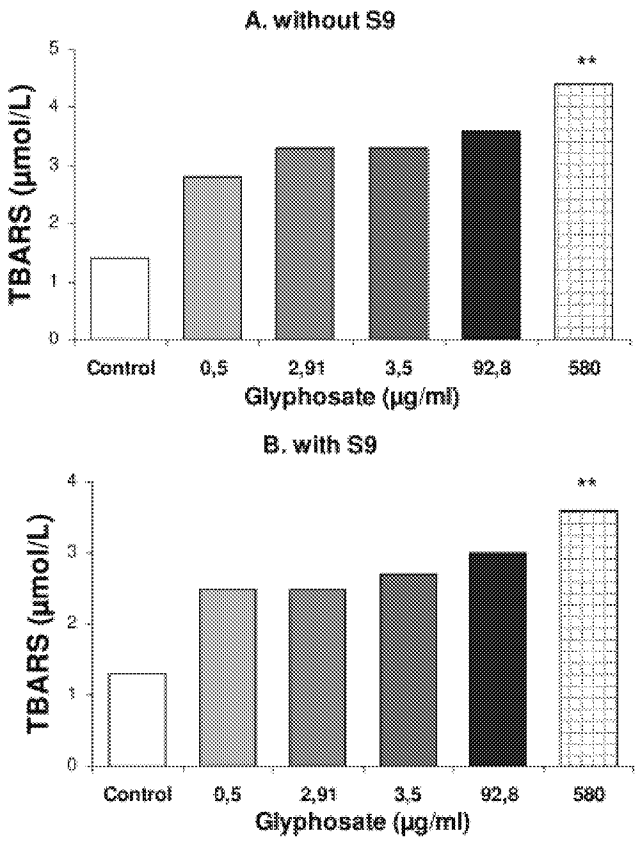


Fig. 2. Changes in plasma lipid peroxidation following treatment with glyphosate alone (A) or in combination with metabolic activation system S9 (B). Lipid peroxidation was determined by level of thiobarbituric reactive substances (TBARS). ** Significantly different from control with and without S9 ($P < 0.01$).

ing to ours. This may be explained by the study of Lueken et al. [2004]. The authors suggested that subtoxic concentrations of $H_2O_2$ that occur in the cell due to presence of xenobiotics may lead to the genotoxic effects. However, Heydens et al. [2008] showed no significant increase in the amount of 8-OHdG, despite kidney values being 143% higher than control values. Comparing the tail intensity values for both comet assay versions (Table II and III), at all tested concentrations, we obtained higher statistical differences between treated and untreated cells for the alkaline comet assay than for the hOGG1 modified comet assay. Thus, the proportion of oxidative damage is lower than the proportion of observed strand breakage. This could indicate an indirect and nonoxidative DNA damage induced by glyphosate. In addition, as suggested by Collins [2004], the results suggest that TI as a more valuable endpoint than TL for DNA damage.

In our study, the presence of metabolic activation mostly increased the parameters of alkaline comet assay. In humans, aminomethylphosphonic acid (AMPA) was identified as the only metabolite of glyphosate [WHO, 2003]. Still, it is not yet clear whether it originates from microbial digestion in the colon [Brewster et al., 1991] or from metabolic pathways in cells. A single study has reported a trial to evaluate the genotoxic potential of AMPA. The authors reported no increase in micronuclei formation in bone marrow cells of mice treated with a single dose of AMPA [Kier and Stegeman, 1993]. Since the assay applied is less sensitive than the comet assay, and its outcome depends on many other factors (differences in interspatial cell physiology), it is not possible to compare results from this report with our own results.

We observed a significant increase in the proportion of micronuclei that contained centromeres only at the highest concentration (580 µg/ml) in the presence of S9. This result could indicate aneugenic activity of glyphosate that is exhibited only above a threshold concentration.

The proportion of micronuclei containing a DAPI signal was significantly increased in lymphocytes treated with the highest concentrations of glyphosate in the presence of S9 (Table V). The result indicates more frequent involvement of chromosomal heteromorphic sites 1q, 9q, 15q, 16q, and Yq in micronucleus formation. Norppa and Falck [2003] showed that DAPI+MN contain mainly segments of chromosomes 1, 9, 16, and Y. Since our evaluation comprised lymphocytes from donors under the age of

**TABLE VI. Summary of the results of earlier studies of glyphosate genotoxicity and cytotoxicity**

| Cell type | Methodology | Active ingredient/formulation | Effect | Authors |
|---|---|---|---|---|
| *E. coli* | WP-2 reversion assays | Active ingredient | Negative | Li and Long [1988] |
| *Bacillus subtilis* | Recombination assay | Active ingredient | Negative | Li and Long [1988] |
| CHO cells | Gene mutation assay | Active ingredient | Negative | Li and Long [1988] |
| Rat hepatocytes | DNA repair assay | Active ingredient | Negative | Li and Long [1988] |
| Mouse bone marrow cells | Chromosome aberration analysis | Active ingredient formulation | Positive | Bolognesi et al. [1997] |
| Mouse bone marrow cells | Chromosome aberration analysis | Formulation | Negative | Dimitrov et al. [2006] |
| Mouse bone marrow cells | Micronucleus assay | Formulation | Negative | Dimitrov et al. [2006] |
| Human lymphocytes | Sister chromatid exchange | Formulation | Weak positive | Vigfusson and Vysse [1980] |
| Human GM38 cells | Comet assay | Formulation | positive | Monroy et al. [2005] |
| Fibrosarcoma HT1080 cells | Comet assay | Formulation | positive | Monroy et al. [2005] |
| Human JEG3 cells | MTT assay | Active ingredient | positive | Richard et al. [2005] |
| Human embryonic kidney cells | MTT assay | Formulation | positive | Benachour et al. [2007] |

30, in whom the exclusion of Y chromosome is rather unlikely to occur, it could be suggested that glyphosate-induced micronuclei originated mainly from autosomal chromosomes.

The significance of an increase in the proportion of C+NPB (Table IV) in treated lymphocytes is the consequence of certain limitations in statistical analysis due to complete absence of NPB in the controls. Thus, it could not be considered biologically relevant. The same observation could be made for glyphosate induced C+NB and C+NPB in the presence of S9 mix (Table V). A negative correlation of CBPI was observed with dose except for the two highest concentrations (92.8 and 580 μg/ml) at which an increase compared to the lower concentration treatment was observed. The recorded mitotic slippage might be explained as suggested by Marzin [1999]. He reported that chemical agents that do not cause severe DNA damage may exert certain effects on cytokinetics only above a threshold concentration. The effect of the threshold concentration was also observed by Sivikova and Dianovsky [2006]. Thus, it could be assumed that the endpoints exhibited only above a certain concentration are mediated by indirect mechanisms rather than by direct interaction with glyphosate. The same observation could be made regarding the hOGG1 comet assay results, as already discussed in preceding paragraphs.

With FRAP and TBARS we additionally tested glyphosate's ability to induce oxidative stress. The main system of defense against damage from free radicals is enzymatic, and if the oxidative stress is greater than the capacity of the system the second line of defense (vitamin C and E) may be invoked [Benzie and Strain, 1996]. Vitamins scavenge and quench free radicals and they often work synergistically to enhance the overall antioxidant capacity of the body [Halliwell, 1994]. Several studies have been performed to observe the balance between TAC and oxygen free radicals. For example, in a case control study of Ranjbar et al. [2005], toxicity was monitored in the blood samples of patients acutely poisoned with OP insecticides, by analysis of the TAC and lipid peroxidation, as well as by the determination of cholinesterase levels. FRAP values were statistically significant with and without S9 only for the highest tested concentration (Fig. 1). We can conclude that FRAP results are in agreement with comet assays parameters indicating that glyphosate can cause oxidative damage only at the highest tested concentration (580 μg/ml).

The role of lipid peroxidation and resulting oxidative stress has been reported for OP pesticides exposure in animals to result in increased levels of TBARS [Vandana and Poovolla, 1999; Dipanker and Tapas, 2000]. In humans that have been exposed to acute and subchronic concentrations of organophosphates, the levels of TBARS is also elevated. In our study, increased levels of TBARS in plasma after treatment with glyphosate (Fig. 2) could indicate increased peroxidation of cell membranes. Therefore our results, based on simultaneous measurements of total antioxidant power and lipid peroxidation, suggest that glyphosate exposure provoked some measure of oxidative stress only at the highest concentration.

In conclusion, only the highest concentration tested (580 μg/ml) of glyphosate showed statistical significance with various methods. However, the lack of statistical significance at lower concentrations could not unequivocally indicate an acceptable level of biocompatibility. The lack of the observed effect may be due to the low number of samples included in the study. Thus further studies applying even more sensitive techniques to detect physiological and metabolic changes at the cell level should be undertaken.

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*Research Article*

# Clastogenic Effects of Glyphosate in Bone Marrow Cells of Swiss Albino Mice

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Glyphosate (N-(phosphonomethyl) glycine, $C_3H_8NO_5P$), a herbicide, used to control unwanted annual and perennial plants all over the world. Nevertheless, occupational and environmental exposure to pesticides can pose a threat to nontarget species including human beings. Therefore, in the present study, genotoxic effects of the herbicide glyphosate were analyzed by measuring chromosomal aberrations (CAs) and micronuclei (MN) in bone marrow cells of Swiss albino mice. A single dose of glyphosate was given intraperitoneally (*i.p*) to the animals at a concentration of 25 and 50 mg/kg b.wt. Animals of positive control group were injected *i.p.* benzo(a)pyrene (100 mg/kg b.wt., once only), whereas, animals of control (vehicle) group were injected *i.p.* dimethyl sulfoxide (0.2 mL). Animals from all the groups were sacrificed at sampling times of 24, 48, and 72 hours and their bone marrow was analyzed for cytogenetic and chromosomal damage. Glyphosate treatment significantly increases CAs and MN induction at both treatments and time compared with the vehicle control ($P < .05$). The cytotoxic effects of glyphosate were also evident, as observed by significant decrease in mitotic index (MI). The present results indicate that glyphosate is clastogenic and cytotoxic to mouse bone marrow.



## 1. Introduction

Pesticides, including herbicides, insecticides, and fungicides are used extensively to improve crop yields and as a result, they accumulate in the environment and humans unavoidably exposed to them [1]. Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA [2–4]. Because of their biological activity, the indiscriminate use of pesticides may cause undesired effects to human health. For instance, the induction of DNA damage can potentially lead to adverse reproductive outcomes, the induction of cancer, and many other chronic diseases [5–8]. Epidemiological studies demonstrated that occupational exposure to some pesticides may be related to several kinds of cancer, including leukemia [9], bladder [10], and pancreatic cancers [11].

To assess the genetic damage induced by physical and chemical agents including pesticides, various test systems have been described in bacteria, in mammalian cells in vivo and in vitro and in plants [12–14]. Arguably, the most reliable genotoxicity evaluation for human health risk is conducted in mammals by the induction of chromosomal aberrations (CAs) and micronuclei (MN). In this regard, particular attention is focused on CAs because these are considered as early warning signals for neoplastic development [15, 16]. MN are defined as small, round, DNA containing cytoplasmic bodies formed during cell division by loss of acentric chromatin fragments and/or whole chromosomes and are used as a fast and reliable assay for detecting clastogenic or aneugenic action [17]. CAs qualitatively and quantitatively detect clastogenic activity, while the MN assay detects both clastogenic effects and damage to the mitotic apparatus, some of which might have aneugenic consequences[18].

Glyphosate [chemical name: N-(phosphonomethyl)-glycine-isopropylamine (IPA) salt; $C_3H_8NO_5P$; Figure 1], commonly sold in the commercial formulation named Roundup, Rodeo, Touchdown, and so forth, has been a frequently used herbicide on both cropland and noncropland areas of the world since its introduction in the 1970s [19]. Roundup (CAS # 1071-83-6) is a liquid water soluble

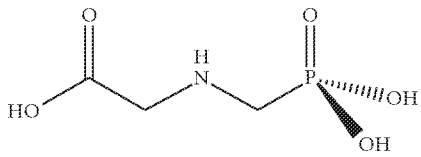


Figure 1: Chemical structure of glyphosate.

organophosphorus herbicide, containing glyphosate as its active ingredient and surfactant (polyoxyethyleneamine) that enhances the spreading of spray droplets when they contact foliage. As a herbicide Roundup works by being absorbed into the plant not only through its leaves but also through soft stalk tissue and applied at concentrations ranging from 0.26–1.152% of active ingredient, that is, glyphosate (20). Plants treated with glyphosate slowly die over a period of days or weeks [20]. Glyphosate is transported throughout the plant where it inhibits the shikimic acid pathway, which participates in the biosynthesis of phenylalanine and tyrosine and is also the major pathway in the biosynthesis of most plant phenolics [21]. Because this specific biologic pathway operates only in plants and microorganisms, the mechanism is not considered to be a risk for humans. Nevertheless, genotoxic, hormonal, and enzymatic effects of glyphosate in mammals have been reported [20, 22–25]. In rats, glyphosate was found to decrease the activity of some detoxifying enzymes, cytochrome P-450, and monooxygenase activities and the intestinal activity of aryl hydrocarbon hydroxylase when injected into the abdomen [26].

Li and Long [27] reported nonmutagenic effects from glyphosate in *Salmonella typhimurium*, *Escherichia coli*, *Bacillus subtilis*, Chinese hamster ovary cells gene mutation assay and chromosomal aberration in rat bone marrow cells. However, some other studies stated that glyphosate treatment on human lymphocytes in vitro resulted in increased sister chromatid exchanges [18, 22], CAs [22, 28], and oxidative stress measured by glucose 6-phosphate dehydrogenase (G6PD, marker of changes in the normal cell redox state) enzyme activity [22]. Roundup was associated with increased DNA adducts in mice [23] and DNA damage in *Rana catesbeiana* tadpolesas assessed by using Comet assay test [29]. Beside these, several assays also have demonstrated genotoxic activities of roundup, such as induction of reverse mutation in *S. typhimurium* (TA98 and TA100) and sex-linked recessive lethal mutation in *Drosophila melanogaster* [12, 28, 30] whereas glyphosate alone did not show these effects. In mammalian cells glyphosate was not also mutagenic [19]. Thus, so far there have been conflicting reports on the genotoxic hazards associated with the use of glyphosate.

On the basis of the information available, U.S. Environmental Protection Agency [31] and the World Health Organization [32] reviewed the toxicology data on glyphosate and concluded that glyphosate is not mutagenic or carcinogenic in humans. On the contrary, few recent studies have demonstrated cytotoxic effects of glyphosate [22, 33, 34]. Considering the widespread and frequent use of glyphosate throughout the world, ongoing risk assessment is of importance. In the present study we reported the genotoxic potential of glyphosate in mouse bone marrow cells.

## 2. Materials and Methods

*2.1. Chemicals.* Roundup containing active ingredient glyphosate >41% SL (IPA salt) was purchased from, Monsanto India Ltd. (Mumbai, India). Benzo(a)pyrene [B(a)P], colchicine and Giemsa were obtained from Sigma Chemical Company (St. Louis, USA). The rest of the chemicals used in the study were of analytical grade purity and obtained locally.

*2.2. Animals and Treatment.* Swiss albino mice (Male, 18 ± 2 g b.wt.; age: 10–12 weeks) were obtained from the Indian Institute of Toxicology Research (Lucknow, India) animal breeding colony. The ethical approval for the experiment was obtained from Institutional Ethical Committee. Animals were randomly selected and housed in polycarbonate boxes with steel wire tops and rice husk bedding. They were maintained in controlled atmosphere of 12 hours dark/light cycle, 25 ± 2°C temperature, and 57 ± 7% humidity with free access to pelleted feed (M/s. Ashirwad, Chandigarh, India) and fresh tap water.

The animals were divided into four groups of 15 animals each in two sets. The animals of group I were used as a control group and intraperitonialy (*i.p.*) treatment DMSO (0.2 mL, once only) was given. The animals of group II were served as positive control and only B(a)P was given at the single dose of 100 mg/kg b.wt. *i.p.* In groups III and IV single dose of glyphosate (diluted appropriately in DMSO) was given *i.p.* at the dose of 25 and 50 mg/kg b.wt., respectively.

*2.3. Chromosomal Aberration Assay.* After completion of the treatment period 5 animals from each group of set 1 were sacrificed at the sampling time of 24, 48, and 72 hours, respectively, by cervical dislocation (colchicine was given at a dose of 4 mg/kg of the b.wt. at 2 hours prior to sacrificing the animals to arrest cycling cells in metaphase). Cytogenetic analysis was performed as per the protocol of Preston et al. [35]. Briefly, the bone marrow was flushed out from both femurs using Hanks buffered salt solution (pH 7.2). The cells were centrifuged at 1000 rpm for 5 minutes and the pellet was redispersed in a hypotonic solution of 0.56% (w/v) KCl for 30 minutes at 37°C to permit osmotic swelling of cells. Swollen cells were fixed in ice-cold Carnoy's fluid, dropped onto slides, and stained with phosphate-buffered 5% Giemsa solution. A total of 75 well spread metaphase plates per animal in each group was analyzed for chromosomal aberrations at a magnification of 100x and the mitotic index (MI) was calculated from a scan of 2000 cells per animal. The chromosomal aberrations were classified as breaks, fragments, and exchanges. The incidence of aberrant cells was expressed as the percentage of damaged cells (aberrant metaphases).

*Mitotic Index (MI)* %:

$$\frac{\text{Number of dividing cells} \times 100}{\text{Total number of bone marrow cells counted}}, \quad (1)$$

*Incidence of aberrant cells* (%):

$$\frac{\text{Total number of aberrant metaphases} \times 100}{\text{Total number of metaphases counted}}. \quad (2)$$

*2.4. Micronuclei Induction Assay.* The rest of 5 animals from each group of set 2 were sacrificed after 24, 48, and 72 hours of treatment and the frequency of micronucleated polychromatic erythrocytes (MNPCEs) was evaluated using a modified protocol of Schmid [36]. The bone marrow was flushed from both femurs using Hanks' buffered salt solution, 1% (w/v) bovine serum albumin, and 0.15% (w/v) EDTA (pH 7.2). Evenly spread bone marrow smears were stained by using the May-Grunwald and Giemsa protocol. A minimum of 2000 erythrocytes was scored for each treated and control group. The stained slides were scored for number of MNPCE's/1000 PCE's.

*2.5. Statistical Analysis.* The data was analyzed for mean values and standard error (mean ± SE) for all groups. Statistical comparisons were made using Students $t$-test, and $P < .05$ was considered significant.

## 3. Results

In the results of chromosomal aberration assay, the percent incidence of aberrant cells in positive control B(a)P treated groups were found to be 12.76, 14.35, and 15.22 in 24, 48, and 72 hours of sampling time, respectively, in comparison to 1.88, 1.92, and 1.75 of untreated group I (Table 1, Figure 2). The frequency of percentage aberrant cells was also found to be significantly ($P < .05$) increased in glyphosate treated groups in dose- and time-dependent manner. The frequency of percent aberrant cells in glyphosate (25 mg/kg b.wt.) treated group III was found increased to 5.86, 7.24, and 7.76 in 24, 48, and 72 hours of sampling time, respectively, while in group IV (50 mg/kg b.wt.) it was 7.46, 8.85, and 9.24, respectively (Table 1, Figure 2).

Significant decrease in MI after B(a)P treatment was noticed and evaluated as percentage of dividing cells which was found to be 2.46, 2.12, and 1.94 in group II in comparison to 4.88, 4.90, and 4.84 of untreated control group I (Table 2, Figure 2). A significant ($P < .05$) decrease in MI was also observed in glyphosate treated groups III and IV in comparison to untreated controls (group I). Low-dose (25 mg/kg b.wt) glyphosate resulted in significant decrease in MI by 4.12, 3.84, and 3.75 in 24, 48, and 72 hours of treatment while high dose (50 mg/kg b.wt.) resulted in 3.54, 3.16, and 3.06, respectively (Table 2, Figure 3).

The frequency of MNPCEs/1000PCEs in the present study was 15.46, 17.50, and 18.25 in 24, 48, and 72 hours of B(a)P treatment (group II) and which was 1.24, 1.10, and 1.18 in control group I (Table 2, Figure 3). Glyphosate (25 mg/kg b.wt.) induced micronuclei induction in group III was 3.87, 5.76, and 6.12 whereas in group IV (50 mg/kg b.wt. glyphosate treated animals) it was 6.86, 8.25, and 8.48, in 24, 48, and 72 hours of sampling period, respectively, (Table 2, Figure 4), suggesting the genotoxic potential of glyphosate.

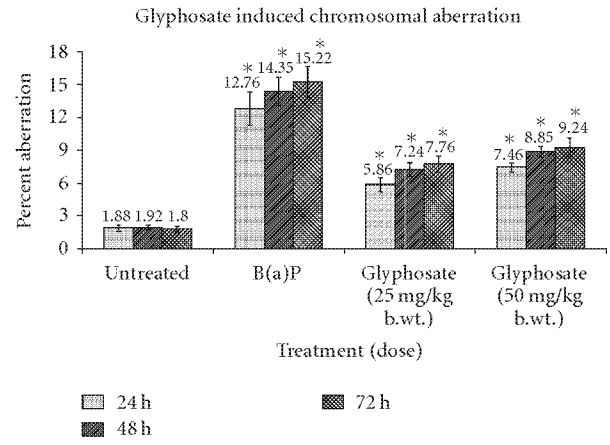


FIGURE 2: Mutagenic activity of glyphosate in Swiss albino mice showing incidence of aberrant cells at sampling time of 24, 48, and 72 hours. Values are expressed as mean ± SE of 5 animals. *Represent significant increase over untreated control group at their respective sampling time. Data are significant as $P < .05$.

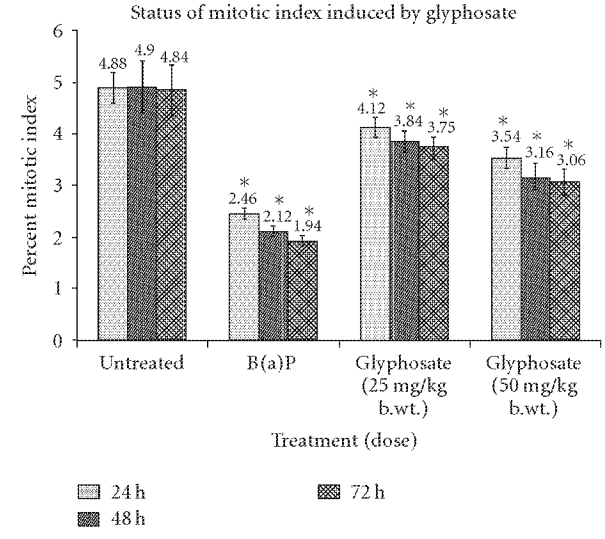


FIGURE 3: Cytotoxic effects glyphosate in Swiss albino mice indicated by decrease in mitotic index (MI) at 24, 48, and 72 hours of sampling time. Values are expressed as mean ± SE of 5 animals. *Represent significant decrease over untreated control group at their respective sampling time. Data are significant as $P < .05$.

## 4. Discussion

Results of the present study reveals that single dose of glyphosate caused significant incidence of chromosomal aberration and induction of micronuclei in a dose- and time-dependent manner. Various cytogenetic results on commercial glyphosate are problematic. They may depend on purity of the active agent and on the nature of inert components. Surfactants and other inert compounds were previously suggested to increase the toxicity of the herbicide [37]. In a recent study, *Caiman latirostris* embryos were exposed at early embryonic stage to different sublethal concentrations of Roundup (range from 50–1750 μg/egg),

Table 1: Effect of glyphosate treatment on induction of chromosomal aberration in swiss albino mice.

| | | Groups | Untreated | B(a)P (100 mg/kg b.wt) | Glyphosate (25 mg/kg b.wt) | Glyphosate (50 mg/kg b.wt) |
|---|---|---|---|---|---|---|
| Number of aberrant cells (%) after | 24 hours of treatment | Breaks | 0.36 ± 0.1 | 5.65 ± 0.4 | 2.86 ± 0.2 | 3.79 ± 0.16 |
| | | Fragments | 0.17 ± 0.01 | 1.59 ± 0.03 | 0.39 ± 0.1 | 1.94 ± 0.02 |
| | | Exchange | 0.26 ± 0.02 | 0.69 ± 0.2 | 0.47 ± 0.3 | 0.41 ± 0.01 |
| | | Multiple damage | 1.02 ± 0.07 | 4.83 ± 0.3 | 2.14 ± 0.4 | 1.32 ± 0.07 |
| | | Total no. of aberrant cells | 1.81 ± 0.03 | 12.76 ± 0.17* | 5.86 ± 0.12* | 7.46 ± 0.14* |
| | 48 hours of treatment | Breaks | 0.33 ± 0.2 | 6.84 ± 0.5 | 3.37 ± 0.05 | 4.51 ± 0.07 |
| | | Fragments | 0.19 ± 0.01 | 2.63 ± 0.7 | 0.46 ± 0.03 | 0.89 ± 0.01 |
| | | Exchange | 0.23 ± 0.1 | 0.83 ± 0.03 | 0.59 ± 0.01 | 0.54 ± 0.02 |
| | | Multiple damage | 1.17 ± 0.04 | 4.05 ± 0.04 | 2.82 ± 0.06 | 2.91 ± 0.16 |
| | | Total no. of aberrant cells | 1.92 ± 0.03 | 14.35 ± 1.27* | 7.24 ± 0.15* | 8.85 ± 0.14* |
| | 72 hours of treatment | Breaks | 0.34 ± 0.02 | 6.91 ± 0.10 | 4.42 ± 0.07 | 4.49 ± 0.13 |
| | | Fragments | 0.15 ± 0.01 | 2.93 ± 0.04 | 0.53 ± 0.02 | 0.82 ± 0.02 |
| | | Exchange | 0.19 ± 0.01 | 1.65 ± 0.06 | 0.47 ± 0.03 | 0.63 ± 0.02 |
| | | Multiple damage | 1.12 ± 0.04 | 3.73 ± 0.1 | 2.34 ± 0.09 | 3.30 ± 0.15 |
| | | Total no. of aberrant cells | 1.80 ± .05 | 15.22* ± 1.19 | 7.76 ± 0.4* | 9.24 ± 0.18* |

Mean ± SE of animals $n = 5$.
* $P < .05$.

Table 2: Effects of glyphosate treatment on mitotic index and micronuclei induction in swiss albino mice.

| Groups (treatment) | Mitotic index (MI) after treatment | | | Micronuclei induction (MNPCEs/1000PCEs) after treatment | | |
|---|---|---|---|---|---|---|
| | 24 hours | 48 hours | 72 hours | 24 hours | 48 hours | 72 hours |
| Group I (untreated) | 4.88 ± 0.06 | 4.90 ± 0.02 | 4.84 ± 0.04 | 1.24 ± 0.01 | 1.10 ± 0.01 | 1.18 ± 0.03 |
| Group II B(a)P (100 mg/kg b.wt) | 2.46 ± 0.09# | 2.12 ± 0.01# | 1.94 ± 0.02# | 15.46 ± 0.03* | 17.50 ± 0.10* | 18.25 ± 0.12* |
| Group III (glyphosate dose 25 mg/kg b.wt) | 4.12 ± .05# | 3.84 ± 0.04# | 3.75 ± 0.03# | 3.87 ± 0.02* | 5.76 ± 0.08* | 6.12 ± 0.07* |
| Group IV (glyphosate dose 50 mg/kg b.wt) | 3.54 ± 0.01# | 3.16 ± 0.03# | 3.06 ± 0.01# | 6.86 ± 0.04* | 8.25 ± 0.04* | 8.48 ± 0.09* |

Data shows mean ± SE of 5 animals in each group.
#$P < .05$ represents significant decrease as compared to untreated control.
* $P < .05$ represents significant increase as compared to untreated control.
MNPCEs: Micronucleated polychromatic erythrocytes;
PCEs: Polychromatic erythrocytes.

results from both the comet assay and the MN test revealed a concentration dependent effect [4].

Glyphosate reported for positive clastogenic and genotoxic effects in vitro [22, 27] which are consistent with our results (Tables 1 and 2). Chromosomal damage is considered to detect early effects of xenobiotic insult and evaluation of the frequency of CAs is a sensitive cytogenetic assay for detecting exposure to mutagens and carcinogens [15]. In the present study, glyphosate induced CAs could be attributed to early changes either an increase in induced DNA lesions or interference with their repair (Table 1, Figure 1). Glyphosate has been reported to cause DNA damage in erythrocytes of bullfrog tadpoles (*R. catesbeiana*) [29]. However, few studies reported that glyphosate is weak or nonclastogenic in vivo [18, 28, 38].

The MN induction assay was used as an additional sensitive biological indicator of the damage to somatic cell genome of subjects exposed to pesticide mixtures occupationally. It is known that the appearance of MN is related to the loss of chromosome fragments due to chromosome breaks [39]. Our results revealed that there was elevation in the number of micronuclei in the glyphosate exposed

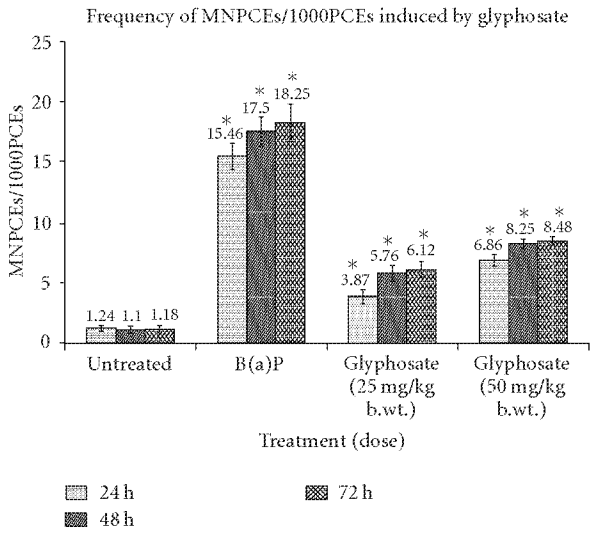


FIGURE 4: Mutagenic activity of glyphosate in Swiss albino mice showing increased micronuclei (MN) induction at sampling time of 24, 48, and 72 hours. Values are expressed as mean ± SE of five animals. *Represent significant increase over untreated control group at their respective sampling time. Data were significant as $P < .05$. MNPCEs: Micronucleated polychromatic erythrocytes and PCEs: polychromatic erythrocytes.

animals. Because MN could be the consequence of the mitotic spindle malfunction, it is possible that the glyphosate could also express an aneugenic mode of action as inhibiting cell division and mitotic spindle apparatus.

The molecular mechanisms responsible for the genotoxicity of glyphosate are not yet known clearly. However, the CAs and the micronucleus formation observed in animals clearly indicate that these compounds interact with chromatin DNA and induce damage there. Such interactions/DNA damage may be caused by an increased incidence of alkali labile sites in DNA as observed in kidney and liver with glyphosate treatment in CD-1 mice [23]. Alkali labile sites are generally produced at abasic sites in DNA and may be revealed under conditions that denature DNA secondary structure. Peluso et al. [23] also reported a dramatic increase in the number of oxidized guanine, 8-hydroxylguanine (8-OHdG), residues in DNA of liver cells from mice treated with glyphosate which also may be the reason of chromosomal damage in bone marrow cells of mice as observed in our study. It has also been shown in our study that CAs and MN induction increases in time as well as dose-dependent manner. It could be due to the glyphosate induced toxicity which produces reduced repair of spontaneous 8-OHdG and lead to an accumulation of oxidation products [23].

The sensitivities of two cytogenetic tests, chromosome analysis and the micronucleus test, were compared by using mice exposed to the substances glyphosate and B(a)P (Tables 1 and 2). Both test systems proved equally sensitive for genotoxicity assessment. Glyphosate at the tested doses significantly increased both the CAs rates and the MN induction in comparison to control. Thus, our results indicate that glyphosate is able to induce CAs and MN accompanied by inhibition of cell proliferation in Swiss albino mice following *i.p.* administration. In view of the earlier reports on mutagenic activity of glyphosate in laboratory experiments and from the present study, further studies are needed to assess the possible health hazard from glyphosate.

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# Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*

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ABSTRACT

Glyphosate-based herbicides, such as Roundup®, represent the most extensively used herbicides worldwide, including Brazil. Despite its extensive use, the genotoxic effects of this herbicide are not completely understood and studies with Roundup® show conflicting results with regard to the effects of this product on the genetic material. Thus, the aim of this study was to evaluate the genotoxic effects of acute exposures (6, 24 and 96 h) to 10 mg $L^{-1}$ of Roundup® on the neotropical fish *Prochilodus lineatus*. Accordingly, fish erythrocytes were used in the comet assay, micronucleus test and for the analysis of the occurrence of nuclear abnormalities and the comet assay was adjusted for branchial cells. The results showed that Roundup® produces genotoxic damage in erythrocytes and gill cells of *P. lineatus*. The comet scores obtained for *P. lineatus* erythrocytes after 6 and 96 h of exposure to Roundup® were significantly higher than respective negative controls. For branchial cells comet scores were significantly higher than negative controls after 6 and 24 h exposures. The frequencies of micronucleus and other erythrocyte nuclear abnormalities (ENAs) were not significantly different between Roundup® exposed fish and their respective negative controls, for all exposure periods. In conclusion, the results of this work showed that Roundup® produced genotoxic effects on the fish species *P. lineatus*. The comet assay with gill cells showed to be an important complementary tool for detecting genotoxicity, given that it revealed DNA damage in periods of exposure that erythrocytes did not. ENAs frequency was not a good indicator of genotoxicity, but further studies are needed to better understand the origin of these abnormalities.



## 1. Introduction

Herbicides constitute a heterogeneous category of chemical products, specifically made for weed control [1], which can reach aquatic ecosystems intentionally or indirectly, through soil surface run-off, from areas where they are applied [2]. Experimental findings have revealed that various pesticides possess genotoxic or mutagenic properties which constitute initial risk factors in the generation of carcinogenic and reproductive effects in the long term [1].

Currently, among the various existing pesticides in the market, glyphosate is the most extensively used, and its use in agriculture is continuously expanding on farms that grow genetically modified crops because they can tolerate treatments with this herbicide [3]. Glyphosate is a broad-spectrum nonselective herbicide used for inhibition of unwanted weeds and grasses in agricultural, industrial, urban, forest and aquatic landscapes [4].

Roundup® is the commercial name of an herbicide product in which glyphosate is formulated as isopropylamine salt (IPA) and a surfactant, polyethoxylene amine (POEA), is added to enhance the efficacy of the herbicide [5,6]. Due to its high water solubility and its extensive use, the exposure of non-target aquatic organisms to this herbicide is a concern especially in systems of shallow waters [7].

The acute toxicity of glyphosate is considered to be low by the World Health Organization [8]. However, glyphosate-based commercial formulations are generally more toxic than pure glyphosate [9,10] mainly because surfactants, such as the POEA used in Roundup® formulation, are toxic to aquatic organisms [7]. Giesy et al. [11] observed that POEA was more toxic to fish than pure glyphosate. Tests for acute toxicity, carried out on carps (*Cyprinus carpio*), revealed that the median lethal concentration for 96 h ($LC_{50}$96 h) of glyphosate is very high, that is, 620 mg $L^{-1}$ [12]. On the contrary, the $LC_{50}$96 h of the formulated product Roundup® was much lower, varying from 2 to 55 mg $L^{-1}$, depending on the species of fish, life stage and conditions of the test [13]. The $LC_{50}$96 h of Roundup® was determined as 13.7 mg $L^{-1}$ to juveniles of the Neotropical fish *Prochilodus lineatus* [14], a detrivorous fish species commonly found in rivers of the south and southeast regions of Brazil and considered as a potential bioindicator species [15,16].

Although studies regarding the biologic effects of pesticides have increased over the last years, the results on the genotoxicity of these products are often incomplete, and sometimes contradictory.

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The genotoxic potential of Roundup® has been studied extensively by the use of various methods, but conflicting results have been encountered [17].

The genotoxic effects of pollutants can be monitored using *in vitro* and *in vivo* tests and micronucleus test and comet assay are the most widely utilized tests in genotoxic evaluations, mainly because of the sensitivity of both in detecting DNA damage and their rapid performance [4]. In fish, the micronucleus test involving peripheral blood erythrocytes is most commonly used [18]. As a complement to the micronucleus test, many authors examine the occurrence of morphologic alterations in the erythrocyte nucleus of fish, also called erythrocytic nuclear abnormalities (ENAs), as possible indicators of genotoxicity [19].

There are very few studies that examined the genotoxic effects of glyphosate-based herbicides on fish. In a study performed by Grisolia [20] it was reported that intra-abdominal injection of Roundup significantly increased the micronuclei frequencies on erythrocytes of fish *Tilapia rendalli*. In an other study [4] treatment with Roundup induced significant increases in frequencies of micronuclei as well as DNA damage, as revealed by comet assay, in peripheral erythrocytes of *Carassius auratus*.

In Brazil, glyphosate-based herbicides are most often utilized and their consumption increased 95% in the period of 2000–2004. Just in the state of Paraná (southern Brazil) alone, 4562 tons of glyphosate were used on soybean and corn crops, between 2000 and 2002 [21], and high concentrations of glyphosate have already been detected in water near to intense cultivation areas in southern Brazil [22]. Despite its extensive use, little is known about the genotoxic effects of this herbicide to Neotropical fish species.

In studies with fish, comet and micronucleus assays have been generally performed on peripheral blood erythrocytes due to their easy sampling and use [19,20,23–26]. Besides erythrocytes, other cell types such as gill cells have also been used, these cells have some advantages over erythrocytes because gill cells of fish exposed to a pollutant can demonstrate more frequent DNA damage than erythrocytes [23]. This can be explained by the fact that gill cells are continuously dividing and are also directly exposed to water contaminants [24].

The aim of this work was to evaluate the genotoxic effects of Roundup® in *P. lineatus* acutely exposed to the herbicide for different periods, using the comet assay, micronucleus test and the occurrence of erythrocytic nuclear abnormalities (ENAs).

## 2. Material and methods

### 2.1. Animals

Juveniles of *Prochilodus lineatus* (Valenciennes, 1847), with 9.6 ± 5.4 g and 9.7 ± 1.81 cm (mean ± S.D., $N = 50$), were supplied by the Hatchery Station of Londrina State University. Prior to the toxicity tests, fish were acclimated to laboratory conditions for a minimum of seven days in a 300-L tank with aerated dechlorinated water ($T \cong 25$ °C; pH ≅ 7.0) and a 14/10 h light/dark photoperiod. During this period, fish were fed every 48 h with commercial pellet food containing 36% of protein (Guabi®, BR). Animals were not fed during the toxicity tests.

### 2.2. Toxicity tests

Short-term (6, 24 and 96 h) static toxicity tests were performed to evaluate the genotoxic and mutagenic effects of 10 mg $L^{-1}$ of Roundup® (360 g glyphosate $L^{-1}$ or 41% of glyphosate, Monsanto Brazil LTDA) to *P. lineatus*. This Roundup® concentration corresponds to 75% of the $LC_{50}$ of this herbicide to *P. lineatus* [14]. Experiments were performed in 100 L glass aquaria containing 6 fish each, with continuously aerated dechlorinated water. One negative control group (NC), exposed only to clean water was terminally sampled at each experimental interval along with the experimental groups exposed to Roundup®. Replicates were carried out for each acute experimental interval. During the tests water was continuously monitored for temperature, dissolved oxygen, pH and conductivity. The mean values (±S.D.) for NC and experimental groups were, respectively, temperature: 25.7 ± 0.5 and 25.0 ± 0.0 °C; pH: 7.4 ± 0.2 and 7.4 ± 0.1; dissolved oxygen: 6.9 ± 0.8 and 7.0 ± 0.8 mg $O_2$. $L^{-1}$; conductivity: 53.7 ± 10.4 and 62.8 ± 4.4 μS $cm^{-1}$. Positive control groups (PC), consisting of fish injected with the clastogenic agent cyclophosphamide (40 mg $Kg^{-1}$, Sigma–CAS no. 64-86-8) were terminally sampled 6, 24 and 96 h after treatment.

Immediately after removal from the aquaria fish were anesthetized with benzocaine (0.1 g $L^{-1}$), and blood samples were taken from the caudal vein into heparinized plastic syringes. Subsequently animals were killed by cervical section and the gills were immediately removed. A small amount of each blood sample (10 μL) was diluted in 700 μL of phosphate-buffered saline (PBS: 126.6 mM NaCl, 4.8 mM KCL, 1.5 mM $CaCl_2$; 3.7 mM $NaHCO_3$; 8.9 mM $Na_2HPO_4$; 2.9 mM $NaH_2PO_4$) and kept in ice until the start of the comet assay.

Upon dissection, gills were immediately washed with PBS and filaments were gently cleaned using tiny brushes and then cut in small pieces. Gill filaments were stored in 700 μL of PBS and kept in ice until the moment of cell suspension preparation. All handling during gill dissection, dissociation, and preparations were performed on ice. The method for gill cellular suspensions preparation was based on Kilemade et al. [27]. Briefly, gill filaments were gently sectioned using disposable blades and sections were transferred to small plastic tubes, incubated for 15 min in 200 μL 0.25% trypsin – EDTA and homogenized by periodic manual inversion at room temperature for tissue dissociation. To halt the enzymatic digestion 200 μL of fetal calf serum was added to each tube. After 15 min the solution was filtered, leaving the larger undigested tissue pieces behind, the resulting cell suspensions were used in the comet assay.

### 2.3. Cell viability assay

Before running the comet assay, cell viability for erythrocytes and gill cells was determined using the trypan blue exclusion method. For each animal a total of 100 cells were scored per cell type, and the viability was expressed as the percentage of viable cells in the total number of cells counted. At least 80% of cells should be viable to run the comet assay [28].

### 2.4. Comet assay

Alkaline comet assay was performed according to Singh et al. [29] and Speit and Hartmann [30] with some modifications as described by Vanzella et al. [25]. Basic steps of the assay for both erythrocytes and gill cells were executed as follows: (a) lysis: one hour, at 4 °C, protected from light, in a lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1 mL Triton X-100, pH 10.0); (b) DNA unwinding: 30 min, in the dark, in an electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH > 13); (c) electrophoresis: 20 min, 300 mA, 25 V, 1 V $cm^{-1}$; d) neutralization: three washes for 5 min each in buffer (0.4 M Tris, pH 7.5). Slides were then fixed with absolute ethanol for 10 min and kept under refrigeration until cytological analyses.

Slides stained with ethidium bromide (20 μg $mL^{-1}$) were analyzed under a Nikon fluorescence microscope (1000× magnification). All slides were independently coded and scored without knowledge of the code [28]. The extent of DNA damage was quantified by the length of DNA migration which was visually determined in 100 randomly select and non-overlapping cells per fish. DNA damage was classified in four classes (0: undamaged; 1: minimum damage; 2: medium damage; 3: maximum damage) and each comet assigned a value of 0–3 according to its class, the total score will be between 0 and 300 "arbitrary units" [31]. Results for DNA damage in erythrocytes and gill cells were expressed as the mean number of damaged nucleoids (sum of classes 1, 2 and 3) and the mean comet score for each treatment group (CN, Roundup® and CP), for each exposure period.

### 2.5. Micronucleus test and the occurrence of erythrocytic nuclear abnormalities (ENAs)

The micronucleus test was performed with fish erythrocytes according to the methodology of Hooftman and Raat [32] and the analysis of erythrocytic nuclear abnormalities according to Carrasco et al. [33]. Immediately after sampling blood was smeared on clean glass slides, dried overnight, fixed with methanol for 10 min and stained with Giemsa (5%). A total of 3000 erythrocytes per fish were examined under an Olympus optical microscope (1000× magnification). The mean frequencies of micronucleus (MN) and erythrocytic nuclear abnormalities (ENA) found in each experimental group were calculated and expressed per 1000 cells (‰). ENAs were classified, following Pacheco and Santos [34], into three categories: segmented nuclei (SN), lobed nuclei (LN) and kidney-shaped nuclei (KSN).

### 2.6. Statistical analysis

Results are presented as the mean ± standard error. All the data were first tested for normality and homogeneity of variance to meet statistical demands. The results obtained for both controls (NC and PC) and for Roundup® group and negative controls, for each experimental period, were compared with each other using two-tailed Student $t$ test. Differences between means were considered significant when $p < 0.05$. Erythrocytic nuclear abnormalities (ENA) other than micronuclei were considered together for statistical analysis and micronuclei were always considered separately from the other nuclear abnormalities.

**Table 1**
Frequency of nucleoids observed in each comet class (0, 1, 2 and 3) and the number of damaged nucleoids (mean ± S.E.) in erythrocytes and branchial cells of *Prochilodus lineatus* exposed to Roundup (RDP) and the respective negative controls (NC) and positive controls (PC), taking into account the total number of fish (*N*) analyzed for each experimental period (6, 24 and 96 h)

| | Time | Groups | *N* | Comet Classes | | | | Damaged nucleoids (mean ± S.E.) |
|---|---|---|---|---|---|---|---|---|
| | | | | 0 (%) | 1 (%) | 2 (%) | 3 (%) | |
| Erythrocytes | 6 h | NC | 6 | 70.5 | 23.0 | 3.2 | 3.3 | 29.5 ± 2.4 |
| | | RDP | 6 | 54.5 | 33.3 | 8.8 | 3.3 | 45.5 ± 6.1* |
| | | PC | 6 | 25.7 | 63.1 | 10.2 | 1.0 | 74.3 ± 1.5* |
| | 24 h | NC | 5 | 77.8 | 17.6 | 2.8 | 1.7 | 22.2 ± 1.4 |
| | | RDP | 6 | 78.5 | 18.7 | 2.2 | 0.7 | 21.5 ± 2.4 |
| | | PC | 6 | 9.3 | 53.0 | 34.3 | 2.7 | 90.7 ± 1.6* |
| | 96 h | NC | 9 | 78.4 | 20.9 | 0.7 | 0.0 | 20.3 ± 1.6 |
| | | RDP | 12 | 76.7 | 21.3 | 1.6 | 0.3 | 23.3 ± 1.1 |
| | | PC | 8 | 25.1 | 69.0 | 4.5 | 0.3 | 74.9 ± 0.9* |
| Branchial cells | 6 h | NC | 6 | 71.5 | 24.5 | 2.2 | 1.8 | 28.5 ± 1.5 |
| | | RDP | 5 | 66.6 | 28.0 | 3.2 | 2.6 | 33.8 ± 1.1* |
| | | PC | 7 | 63.9 | 24.7 | 7.3 | 4.1 | 36.1 ± 2.7* |
| | 24 h | NC | 6 | 71.2 | 23.4 | 3.0 | 2.4 | 28.8 ± 1.0 |
| | | RDP | 6 | 61.2 | 29.2 | 4.8 | 4.5 | 38.5 ± 1.3* |
| | | PC | 4 | 65.0 | 23.3 | 6.8 | 5.0 | 35.0 ± 3.2 |
| | 96 h | NC | 4 | 76.7 | 13.2 | 5.8 | 4.3 | 23.5 ± 2.3 |
| | | RDP | 3 | 74.3 | 11.7 | 9.3 | 4.7 | 25.7 ± 1.7 |
| | | PC | 4 | 65.0 | 23.3 | 6.8 | 5.0 | 35.0 ± 3.2* |

One hundred nucleoids were analyzed per fish.
* Different from respective negative controls ($p < 0.05$).

## 3. Results

The cell viability assays which were run before the comet assays showed above 90% of viable erythrocytes and gill cells. The results obtained using the comet assay in erythrocytes of *P. lineatus* revealed that fish injected with cyclophosphamide (PC) showed a significant increase both in the number of damaged nucleoids and in the comet scores, in relation to their respective negative controls, in all experimental periods (Table 1 and Fig. 1). When branchial cells were used in the comet assay the results revealed that only after 6 h PC fish showed significant increase in the number of damaged cells and in the comet score, in relation to respective NC. After 96 h of cyclophosphamide injection a significant increase was observed in the number of damaged nucleoids, but the comet score remained similar to the one obtained with gill cells from NC (Table 1 and Fig. 1).

In terms of MN induction erythrocytes from PC fish showed a significant increase in MN frequency after 24 and 96 h of the treatment with the clastogenic agent in relation to respective NC (Table 2). On the other hand, analysis of the frequency of other nuclear abnormalities (SN + LN + KSN) in erythrocytes of *P. lineatus* injected with cyclophosphamide did not show any significant increase with respect to NC in any experimental period (Table 2). The frequencies of ENAs verified for both negative and positive controls showed to be low, varying, respectively, from 2.11 to 4.11 and from 2.33 to 4.00 (‰). The type of nuclear abnormality more commonly detected was a kidney-shaped nucleus, which was observed more frequently after 96 h, both for NC and PC.

**Table 2**
Frequencies of micronuclei (MN) and other nuclear abnormalities (ENA) in erythrocytes of *Prochilodus lineatus* exposed to Roundup (RDP) and the respective negative controls (NC) and positive controls (PC), taking into account the total number of fish (*N*) analyzed for each experimental period (6, 24 and 96 h)

| Time | Group | *N* | MN frequency (‰) | ENA frequency (‰) |
|---|---|---|---|---|
| 6 h | NC | 8 | 0 | 3.00 ± 0.34 |
| | RDP | 10 | 0 | 2.37 ± 0.16 |
| | PC | 7 | 0.05 ± 0.05 | 2.33 ± 0.38 |
| 24 h | NC | 9 | 0.07 ± 0.05 | 2.11 ± 0.30 |
| | RDP | 12 | 0.05 ± 0.05 | 2.33 ± 0.46 |
| | PC | 8 | 0.71 ± 0.22* | 2.33 ± 0.35 |
| 96 h | NC | 9 | 0.18 ± 0.11 | 4.11 ± 0.38 |
| | RDP | 12 | 0.11 ± 0.08 | 3.67 ± 0.36 |
| | PC | 8 | 0.54 ± 0.09* | 4.00 ± 0.30 |

Three thousand erythrocytes were analyzed per fish and results are shown as mean ± S.E.
* Different from respective negative controls ($p < 0.05$).

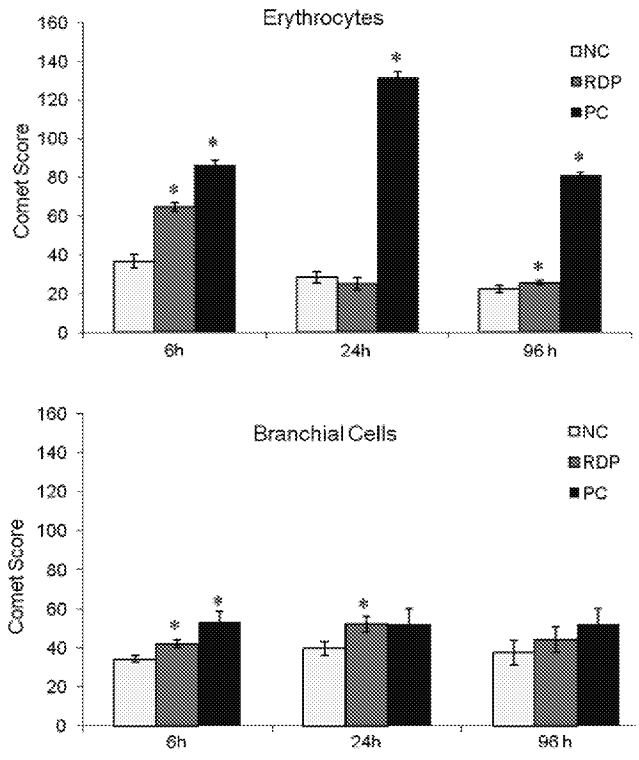


**Fig. 1.** Comet scores in erythrocytes and branchial cells of *Prochilodus lineatus* exposed to Roundup (RDP) and the respective negative (NC) and positive controls (PC) for each experimental period (6, 24 and 96 h). One hundred nucleoids were analyzed per fish. Bars represent means and vertical lines the S.E. *Significantly different from respective negative control ($p < 0.05$).

Concerning Roundup® effects, fish erythrocytes exhibited significantly higher DNA damage after 6 and 96 h of herbicide exposure, as demonstrated by the significant increases in the comet scores in relation to respective NC (Fig. 1). The number of damaged nucleoids was significantly different from respective controls only in fish erythrocytes after 6 h exposure to Roundup® (Table 1). For branchial cells both the number of damaged nucleoids and the comet scores were significantly higher in fish exposed to the herbicide during 6 and 24 h in relation to respective NC (Table 1 and Fig. 1).

Frequencies of MN and nuclear abnormalities in peripheral fish erythrocytes from groups of fish exposed to Roundup® and their respective negative controls groups are shown in Table 2. In contrast to comet results, both MN and ENAs frequencies registered in fish erythrocytes after herbicide exposure were not significantly different from the respective negative controls. As it was verified for negative control groups, the frequency of ENAs in erythrocytes of fish exposed to Roundup® showed to be low, varying from 2.33 to 3.67 (‰), and the type of nuclear abnormality more commonly detected was also a kidney-shaped nucleus, followed by segmented nuclei and lobed nuclei.

## 4. Discussion

Substantial progress has been made in the last decades to evaluate the impact of physical and chemical genotoxins in aquatic organisms [35]. The development of new methods and the application of assays that are more sensitive in the detection of genotoxicity for various xenobiotics in aquatic biota have been the main determinants for attaining these advances [36,37]. In the present work, the genotoxicity of the herbicide Roundup® was evaluated based on the comet assay applied to the analysis of peripheral blood erythrocytes and gill cells of *P. lineatus*, and based on the micronucleus test (in erythrocytes) and the test for erythrocytic nuclear abnormalities (ENAs).

Although the comet assay is suitable for genotoxicity studies in any nucleated eukaryotic cell [38], there may be various practical limitations to the application of this assay including the first stage of cell isolation [39]. For the comet assay to be applied in a reliable manner in cells from tissues such as gills and liver, it is necessary for the cells to be insolated using techniques that themselves do not cause DNA damage [28]. In fish, a tissue frequently chosen to perform the comet assay is blood because it is easy to collect and there is no need for a cell isolation step [27]. Besides erythrocytes, other cell types are used for monitoring the genotoxic effects of pollutants, thereby exploiting tissue-specific responses [38]. Thus, different tissues such as intestine, liver, gills, gonads, kidney, spleen and muscle are chosen for the determination of DNA damage by the comet assay [40]. However, regardless of the cell type to be studied, the results obtained in genotoxicity tests must be first checked in relation to the sensitivity of the test-organism and the overall credibility of the test system. In this context, the utilization of negative and positive control groups is part of the recommended guidelines [41].

In the present study, the results of the comet assay indicated that blood cells were more sensitive than the gill cells to DNA damage caused by cyclophosphamide. This stronger effect of cyclophosphamide in blood cells might be partially attributed to the route of administration of the genotoxic agent, which was by intraperitoneal injection, possibly resulting in a greater exposure of the erythrocytes than the gills cells [42].

The micronucleus test detects chromosomal fragments or acentric chromosomes that are not incorporated into the main nucleus after mitosis. Thus, for the detection of MN it is necessary that actively dividing cell populations undergo at least one cell cycle [18]. However, there is little information on the extent of the cell cycle in teleosts, considering that this cycle varies with temperature in poikilotherm animals, and the rate of erythropoiesis may vary in different fish species [43,44]. From the literature, it appears that a peak in micronucleated erythrocytes occurs 1–5 days after exposure, but in most fish species it takes place after 2 or 3 days [18]. Grisolia and Cordeiro [45] studied the effect of cyclophosphamide in peripheral blood erythrocytes of three fish species and observed an increase in MN frequency after 2–7 days of treatment. In the present study, cyclophosphamide induced an increased MN frequency in fish erythrocytes after 24 and 96 h of treatment. The absence of a significant MN increase after 6 h of cyclophosphamide injection is probably related to the short time interval of treatment, which was insufficient for the occurrence of a complete cell cycle and, consequently, for the detection of micronuclei in the erythrocytes examined.

In fish, besides the presence of micronuclei, there are various types of nuclear lesions in the erythrocytes, whose origin has not yet been very well elucidated [46]. Such abnormalities have been used by various authors as indicators of genotoxicity in fish [27,47–49]. Although the use of this method has indicated that cyclophosphamide induces a greater incidence of erythrocytic nuclear abnormalities (ENA) in other species of fish [19,23,50], this did not occur in the present work in relation to *P. lineatus* (Table 2). Pacheco and Santos [34] showed that at least 6 days exposure to cyclophosphamide was necessary to induce a significant increase in ENA frequency in *Anguilla anguilla*, and they suggested that a rapid catabolism of DNA-damaged erythrocytes and its slow replacement by the organism might be the cause of a delayed appearance.

Studies on the genotoxic potential of glyphosate and formulations based on this product, such as Roundup®, exhibit great variation due to the different formulations tested, doses applied, methods employed and organisms studied [4]. Such facts could explain, in part, the conflicting results that have been published with regard to the effects of these products. According to some of these studies, glyphosate and glyphosate-based herbicides can result in both the absence [51–54] and the incidence [53–59] of DNA damage.

In the present study, the comet assay revealed a significant increase in DNA damage in erythrocytes and gill cells in animals exposed to Roundup® for 6 h. However, after 24 h exposure, the erythrocytes and gill cells exhibited different behaviors (Table 1). At this time, the DNA damage in erythrocytes of *P. lineatus* exposed to Roundup® diminished returning to the mean score found in the respective control group (Fig. 1). It is possible that the repair system of fish had acted on the DNA of the erythrocytes or that the damaged cells had been removed by the spleen [18]. However, in the gill cells, DNA damage in fish exposed to Roundup® for 24 h remained increased, in relation to the respective negative control (Fig. 1). A possible explanation for this difference between erythrocytes and gill cells would be that the repair system in gill cells is slower and consequently damaged cells could have remained longer in the gill tissue, resulting in an increased comet score after 24 h.

The biotransformation of xenobiotics often results in the production of reactive intermediates such as reactive oxygen species (ROS), which are highly toxic and can cause oxidative damage to DNA. Although organisms are equipped with an antioxidant defense system to protect tissues against oxidative lesions, if the rate of ROS production exceeds the capacity of defense mechanisms, cellular and DNA lesions can occur [44,60]. Thus, it is possible that the increased DNA damage in erythrocytes of *P. lineatus* after 96 h of exposure to Roundup® could be due to ROS generated by the metabolism of the herbicide, which could have interacted with DNA of exposed fish, resulting in the lesions detected by the comet assay. In fact, *P. lineatus* exposed to 10 mg $L^{-1}$ of Roundup® for up

to 96 h showed a significant increase in hepatic catalase activity, indicating the activation of antioxidant defenses, probably due to the increased production of ROS [14].

While the comet assay showed a positive response following Roundup exposure, the MN test using *P. lineatus* erythrocytes did not indicate any genotoxic effect of the sub-lethal concentration of Roundup® (10 mg $L^{-1}$) here employed, which corresponds to 4.1 mg $L^{-1}$ of glyphosate. This result agrees with Çavas and Konen [4] who investigated the effects of glyphosate in *Carassius auratus* and observed that the lowest glyphosate concentration capable of inducing a significant increase in the number of micronucleated erythrocytes was 5 mg $L^{-1}$, after 96 h exposure. The sensitivity of the MN assay in fish erythrocytes has been always debatable due to its low level induction and it is not surprising that a correlation between MN induction and comet response under *in vivo* conditions in *P. lineatus* is not apparent [44].

Among the three methods employed in this study, the frequency of ENAs was the least efficacious in the identification of damage to the genetic material caused by the herbicide Roundup®. Considering that not even cyclophosphamide was capable of inducing an increase in ENAs frequency, it is recommended that for *P. lineatus* the comet assay and MN test be adopted as tools in studies of genotoxicity.

In conclusion, the results of this work showed that Roundup® produced genotoxic effects on the fish species *P. lineatus*. The comet assay with gill cells showed to be an important complementary tool for detecting genotocixity, given that it revealed DNA damage in periods of exposure that erythrocytes did not. ENAs frequency was not a good indicator of genotoxicity, but further studies are needed to better understand the origin of these abnormalities. Finally, the use of the comet assay represents an efficient tool for monitoring genotoxic agents in aquatic ecosystem.

## Conflict of interest statement

None.

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# Evaluation of Lethality and Genotoxicity in the Freshwater Mussel *Utterbackia imbecillis* (Bivalvia: Unionidae) Exposed Singly and in Combination to Chemicals Used in Lawn Care

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**Abstract.** Many chemicals, including fertilizers, herbicides, and insecticides, are routinely applied to turf in the care and maintenance of lawns. These chemicals have the potential to leach into nearby surface waters and adversely affect aquatic biota. In this study, we evaluated the lethal and genotoxic effects of chemicals used in lawn care on an early life stage of freshwater mussels (*Utterbackia imbecillis*). The chemicals tested were copper and commercial formulations of atrazine, glyphosate, carbaryl, and diazinon. Mussel glochidia were exposed to chemicals singly or in combination (equitoxic and environmentally realistic mixtures) for 24 h and toxic interactions were evaluated with Marking's additive index. Genotoxicity was quantified with the alkaline single-cell gel electrophoresis assay (Comet assay). In acute tests, copper was the most toxic of all chemicals evaluated (LC50 = 37.4 μg/L) and carbaryl was the most toxic of all pesticides evaluated (LC50 = 7.9 mg/L). In comparison to other aquatic organisms commonly used in toxicity tests (e.g., amphipods, cladocerans, and chironomids), mussel glochidia were as or more sensitive to the chemicals evaluated with the exception of diazinon, where mussels were observed to be less sensitive. The combined toxicity of equitoxic and environmentally realistic mixtures to mussels was additive. Genotoxic responses were observed in mussels exposed to copper, atrazine and diazinon at levels below their respective no-observed-effect concentrations. Together, these data indicate that freshwater mussels are among the most sensitive aquatic organisms tested for some chemicals commonly used in lawn care and that DNA damage may be useful as a screening tool to evaluate potential sublethal effects of lawn care products on non-target aquatic organisms.

Many fertilizers, herbicides, and insecticides used in residential areas and golf courses for the care and maintenance of lawns have been detected in adjacent surface waters (Larson *et al.* 1999; Nowell *et al.* 1999), yet little is known of their toxicity to non-target aquatic organisms during realistic exposure scenarios. In the environment, organisms are exposed to mixtures of pesticides in commercial formulations with additives that may modify toxicity. Furthermore, environmental exposures to pesticides occur at low concentrations so that sublethal effects on organisms may be more pronounced than overt toxic effects such as lethality. The purpose of this study was to quantify the lethal effects of chemicals commonly used in lawn care, singly and in combination, and to evaluate the genotoxicity of their respective no-observed-effect concentrations (NOECs) on an early life stage of a freshwater mussel (*Utterbackia imbecillis*).

Freshwater mussels (Bivalvia: Unionidae) are among the most imperiled aquatic fauna in the United States, and pollution by chemical contaminants has been cited as a major factor contributing to their decline (Williams *et al.* 1993; Fleming *et al.* 1995; Richter *et al.* 1997). *U. imbecillis* is fairly abundant and widespread in many warm, low-flow habitats, and may represent a reasonable surrogate species for other Unionid mussels. Toxicity tests that have been performed on freshwater mussels suggest that early life stages (glochidia and juveniles) are among aquatic invertebrates most sensitive to contaminants such as metals (Keller and Zam 1991; Jacobson *et al.* 1997), chlorine (Goudreau *et al.* 1993), ammonia (Goudreau *et al.* 1993; Augspurger *et al.* 2003), photoactivated fluoranthene (Weinstein 2001), and pulp and paper mill effluents (McKinney and Wade 1996). In contrast, juvenile *U. imbecillis* were observed to be less sensitive to many insecticides (organochlorine cyclodienes, organophosphates and carbamates) and herbicides (triazines and pyrethoids) than other aquatic invertebrates commonly used in aquatic toxicity tests (*e.g.*, cladocerans and amphipods) (Johnson *et al.* 1993; Keller 1993; Keller and Ruessler 1997). Information on the toxicity of pesticides that are currently in widespread use to freshwater mussels is lacking.

The chemicals evaluated in this study were selected on the basis of their extensive use in nonagricultural settings (Nowell *et al.* 1999), and their frequency of detection in surface waters (Larson *et al.* 1999). Specifically, these chemicals were copper, atrazine [2-chloro-4-ethylamine-6-isopropylamino-*S*-triazine], glyphosate [*N*-(phosphonomethyl) glycine], carbaryl [1-napthyl methylcarbamate] and diazinon [*O, O*-diethyl *O*-2-isopropyl-

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6-methyl (pyrimidine-4-yl) phosphorothioate]. Copper is an essential metal that is a component of many fertilizers and fungicides. Atrazine, a triazine herbicide, and glyphosate, a phosphanoglycine herbicide, are widely used to control broad-leaved weeds in lawn care maintenance. Carbaryl, a carbamate insecticide, and diazinon, an organophosphate insecticide, are frequently used to control arthropod pests such as ants, ticks and leaf eaters.

Single-strand breaks in DNA have been used to evaluate genotoxicity in aquatic organisms (Mitchelmore and Chipman 1998). The potential of chemicals to cause DNA strand breakage, either directly or indirectly via alkali-labile sites and through the action of excision repair enzymes, has been correlated with their mutagenic and carcinogenic potential in mammals (Sina *et al.* 1983). Techniques commonly used to measure DNA strand breakage in aquatic organisms include the alkaline unwinding assay (Shugart 1988) and the single-cell gel electrophoresis or Comet assay (Singh *et al.* 1988). The Comet assay has the advantage of allowing for the quantification of intercellular heterogeneity in DNA damage. Recently, the Comet assay has been employed with aquatic invertebrates to (1) study mechanisms of chemically induced DNA damage (Mitchelmore *et al.* 1998), (2) evaluate the significance of chemically induced DNA strand breaks to embryonic development (Lee *et al.* 1999), and (3) monitor environmental quality (Steinert *et al.* 1998; Shaw *et al.* 2000; Frenzilli *et al.* 2001). In aquatic vertebrates, Clements *et al.* (1997) found the Comet assay to be a sensitive measure of DNA damage in tadpoles exposed to commercial formulations of pesticides.

## Materials and Methods

### *Toxicants*

All pesticides were purchased as commercial formulations from local retail suppliers to mimic products that organisms are exposed to in the environment. Herbicides used were atrazine (Atrazine 4L Herbicide, SA-50; 40.8% active ingredient; Southern Agricultural Insecticides, Inc.) and glyphosate isopropylamine salt (Roundup; 18.0% active ingredient; Monsanto Company). Insecticides used were carbaryl (Sevin, Garden Tech; 22.5% active ingredient; TechPac, LLC) and diazinon (Diazinon Ultra, Ortho; 22.4% active ingredient; The Solaris Group of Monsanto Company). Copper was purchased as technical-grade cupric sulfate from Fisher Scientific.

### *Test Organisms*

Gravid adult *U. imbecillis* mussels (average length = 54.7 mm, average height = 26.9 mm) were hand-collected by snorkeling or scuba from Lake Chapman, Sandy Creek Park, Athens, GA, during the spring and summer of 2000 and 2001. Mussels were transported to the laboratory at ambient temperature in aerated site water within 20 min. Prior to tests, mussels were held for 2 to 10 days in flow-through aquaria containing aerated, dechlorinated, soft tap water, and were fed 0.03 g Microfeast/mussel/day. Mature glochidia were obtained by excising the outer marsupial gill of at least two gravid adults, and shaking contents into reconstituted moderately hard water. Glochidia were rinsed five times to remove mucous, debris and immature or dead glochidia. Viable glochidia were used immediately in toxicity tests.

### *Lethality Tests*

Acute toxicity tests were carried out by methods similar to those described by Johnson *et al.* (1993). Glochidia were exposed to lawn care chemicals in 12-well polystyrene plates. Each well contained 5 mL of a randomly assigned test solution diluted with moderately hard water (measured water parameters: alkalinity = 63 mg/L, hardness = 85 mg/L, and pH 8.33) and approximately 100 glochidia (n = 3 wells per concentration). Plates were incubated at 25°C for 24 h with an 18 h light/6 h dark cycle. After exposures, mortality was assessed by adding 3 to 4 drops of a supersaturated NaCl solution to wells, which initiates shell closure in viable glochidia. Fifty glochidia were then immediately scored as being alive or dead (*i.e.*, unable to close shell during salt insult). Concentrations lethal to 50% of the exposed organisms (LC50s) were calculated by the Trimmed Spearman–Karber method (Hamilton *et al.* 1977) with statistical software provided by the U.S. Environmental Protection Agency (http://www.epa.gov/ nerleerd/ stat2.htm#tsk), and NOECs were computed as the highest concentration not significantly different from controls ($p < 0.05$) with SYSTAT statistical software (version 9; SPSS Inc.). Data are expressed as nominal concentrations (mg/L or μg/L) of chemicals calculated from their percentage active ingredients (for copper this was the free ion concentration). Test results were deemed acceptable if (1) the control mortality was <10% and (2) the LC50 of the positive copper control was within two standard deviations of the mean computed from seven separate tests (Fig. 1B). Three or four tests were run for each pesticide tested. LC50s of individual chemicals were compared to literature values reported for other sensitive aquatic invertebrates as recommended by the U.S. Environmental Protection Agency (1994). Marking's additive index was used to assess toxic interactions of the chemicals present in an equitoxic mixture (chemical concentrations in proportion to their individual LC50s) and an environmental mixture (chemical concentrations in proportion to those that typically occur in the environment, *i.e.*, 10 μg/L Cu and 0.5 μg/L pesticides) (Marking 1977).

### *Genotoxicity Tests*

Glochidia were exposed to sublethal concentrations of lawn care chemicals equivalent to ¼ and ½ NOECs, in 12-well polystyrene plates. Each well contained 5 ml of a randomly assigned test solution diluted with moderately hard water (measured water parameters: alkalinity = 66 mg/L, hardness = 88 mg/L, and pH 8.23) and approximately 150 glochidia ($n$ = 4 wells per concentration). Plates were incubated at 25°C for 24 h with an 18 h light/6 h dark cycle. After exposures, approximately 100 glochidia were collected to assess genotoxicity. The higher number of glochidia used in genotoxicity tests was necessary to obtain sufficient cells for analyses.

Genotoxicity was measured with the Comet assay described by Steinert (1996), modified to account for reduced osmolality of hemolymph in freshwater bivalves. Glochidia were transferred to 1.5-ml centrifuge tubes and allowed to settle. Test solutions were removed and glochidia were resuspended in 490 μl of an osmotically modified $Ca^{2+}/Mg^{2+}$ free Hanks balanced salt solution (HBSS) containing 4.2 m*M* $NaHCO_3$, 26.2 m*M* NaCl, 1.3 m*M* KCl, 0.44 m*M* $KH_2PO_4$, 0.34 m*M* $Na_2HPO_4$, and 5.55 m*M* D-glucose (adjusted pH 7.3). Proteinase K was added (10 μl at 10 mg/ml) and glochidia were crushed gently with a handheld, ground-glass tissue homogenizer (*i.e.*, three light pressured turns with a homogenizer having a large clearance between pestle and tube of 0.09 to 0.16 mm). Shell debris was allowed to settle for 30 s and cell suspensions were collected, pelleted (2000 rpm, 5 min, 10°C) and resuspended in 125 μL 0.65% low-melting agarose (made with HBSS and melted at a constant temperature of 37°C). Samples (50 μl) were transferred to microscope slides precoated with 1% normal melting agarose (made with 40 m*M* Tris–acetate EDTA), solidified (5 min at 4°C) and top-coated with 50 μl 0.65% low-melting

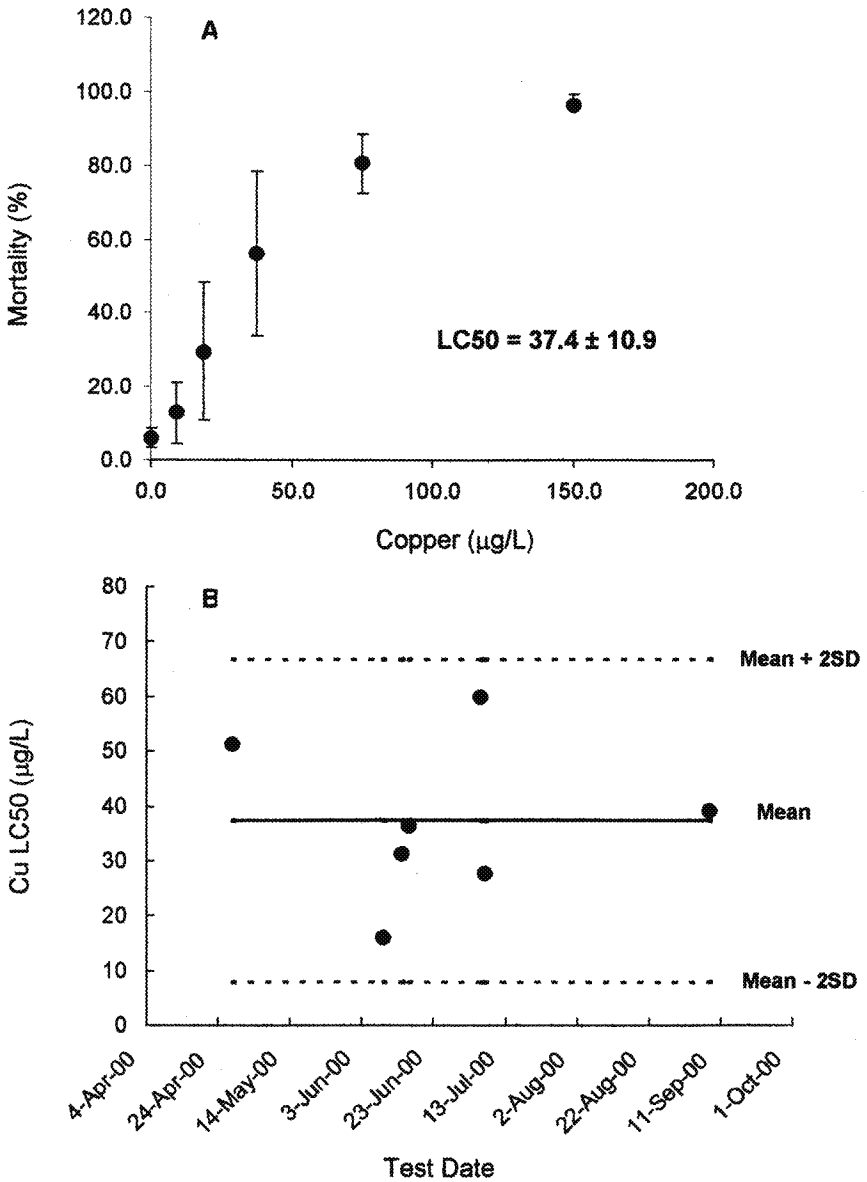


**Fig. 1.** Mortality of *U. imbecillis* glochidia exposed to copper (**A**). Data are means (SD) and LC50s are means ± 95% confidence intervals from seven replicate tests. Positive copper control chart (**B**)

agarose. Once solidified, slides were transferred to light-protected coplin jars containing cold lysis buffer (10 m*M* Tris–HCl, 2.5 *M* NaCl, 100 m*M* EDTA, 10% DMSO, and 1% Triton X-100, pH 10.0), and incubated at 4°C overnight. To prevent confounding DNA damage from ultraviolet light, samples were light-protected during all following steps. Slides were removed from lysis buffer, rinsed three times in cold water, and transferred to a submarine gel electrophoresis chamber containing cold DNA unwinding buffer (300 m*M* NaOH, 1 m*M* EDTA, pH 13.1). DNA was allowed to unwind for 15 min and then samples were electrophoresed at 25 V, ~300 mA for 10 min. After electrophoresis, samples were transferred to coplin jars and neutralized by rinsing in 400 m*M* Tris, three times for 2 min. Samples were then immersed in cold 100% ethanol for 5 min, air-dried, and stored in a desicicator until analyzed. For analyses, slides were stained with 50 µl ethidium bromide (20 µg/ml) and viewed under epifluorescent microscopy (200× magnification, 510- to 560-nm excitation filter, 590-nm barrier filter). DNA damage results in increased DNA migration away from individual cells and produces a characteristic comet shape. DNA damage was quantified by measuring tail moment (product of % DNA in comet tail and length of tail) with a Loats Image Analysis System. Twenty cells were scored per slide and geometric means were used to describe the damage because distributions of tail moments among cells on a slide were skewed. DNA damage among treatments were normalized by a square root transformation and evaluated for significant differences with a one way ANOVA and Fisher's post hoc comparison test ($p < 0.05$) (SYSTAT version 9; SPSS Inc.). Glochidia exposed to 4-nitroquinoline dissolved in dimethyl sulfoxide (DMSO) were used as a positive control.

## Results

### *Lethality*

Acute toxicity of lawn care chemicals to *U. imbecillis* glochidia decreased in the order of copper (Fig. 1A) > carbaryl (Fig. 3A) > glyphosate (Fig. 2B) > diazinon (Fig. 3B) > atrazine (Fig. 2A). In comparison to other aquatic invertebrates commonly

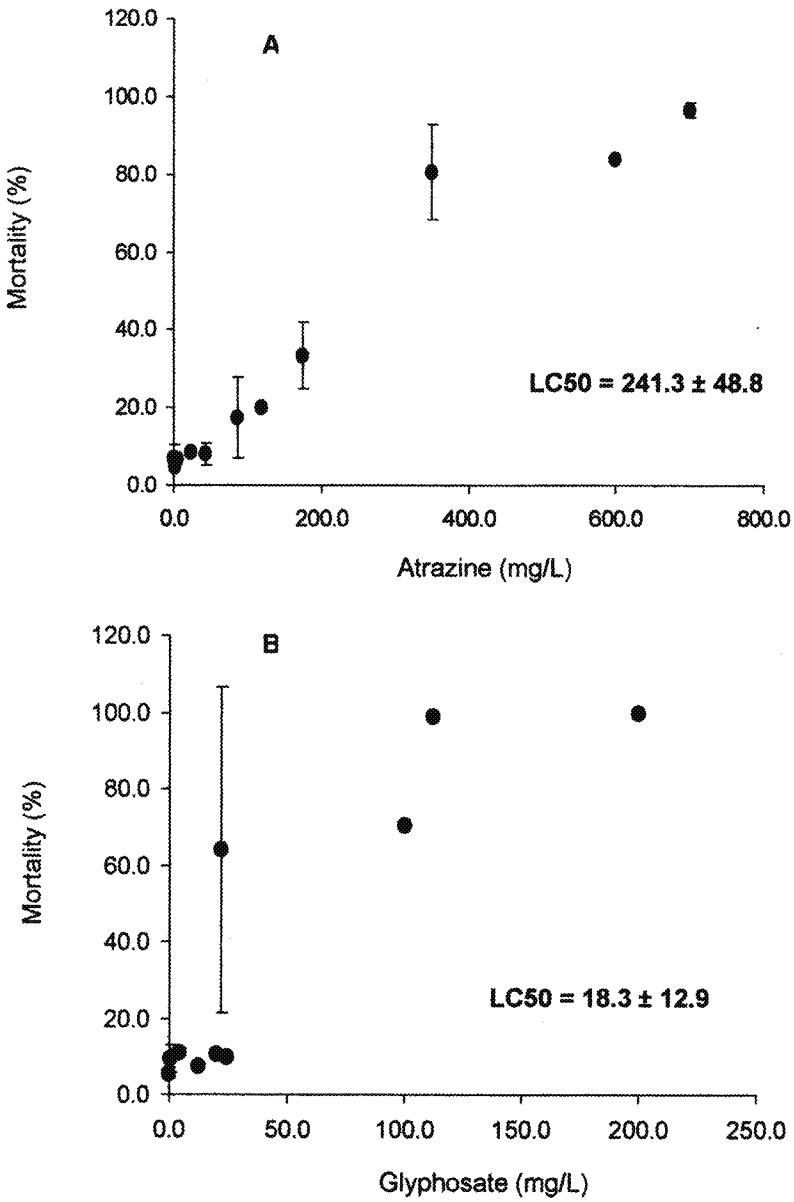


**Fig. 2.** Mortality of *U. imbecillis* glochidia exposed to the herbicides atrazine (**A**) and glyphosate (**B**). Data are means (SD) and LC50s are means ± 95% confidence intervals from three and four replicate tests, respectively

used in toxicity tests (*e.g.*, amphipods, chironomids, and cladocerans), *U. imbecillis* glochidia appear to be as or more sensitive to copper, glyphosate formulated as Roundup, and carbaryl (Table 1). In contrast, glochidia appear to be less sensitive to commercial formulations of diazinon than amphipods, chironomids, and cladocerans. No acute toxicity data were found in the literature for aquatic invertebrates exposed to commercial formulations of atrazine. Mixtures of lawn care chemicals in environmentally realistic proportions (LC50 = 0.59 toxic units) were slightly more toxic to glochidia than chemicals in equitoxic proportions (LC50 = 0.68 toxic units) (Fig. 4). Additive indices (95% confidence intervals) were 0.47 (−0.60, 1.88) for the equitoxic mixture and 0.41 (−0.10, 0.48) for the environmental mixture. Toxic interactions among chemicals in both equitoxic and environmentally realistic mixtures to mussel glochidia were likely additive, as 95% confidence intervals overlapped zero (Marking 1977).

*Genotoxicity*

To assess genotoxicity of lawn care chemicals to freshwater mussels, glochidia were exposed to chemicals at ¼ and ½ of their respective NOECs (Table 2). DNA damage in glochidia exposed to the positive control, 4-nitroquinoline, was significantly greater than controls, and DMSO, the vehicle used only for 4-nitroquinoline, did not induce significant DNA damage (average tail moment for control = 0.44 ± 0.13, DMSO = 1.36 ± 1.15, and 4-nitroquinoline = 11.38 ± 3.5) ($p < 0.05$). Significant increases in DNA damage were observed in glochidia exposed to copper, atrazine and diazinon however, these levels of damage were much lower than the damage observed in 4-nitroquinoline exposed glochidia (Fig. 5). Concentration-dependent trends in DNA damage were variable in that increasing copper concentrations did not cause a corresponding increase in DNA damage, and lower levels of DNA damage were

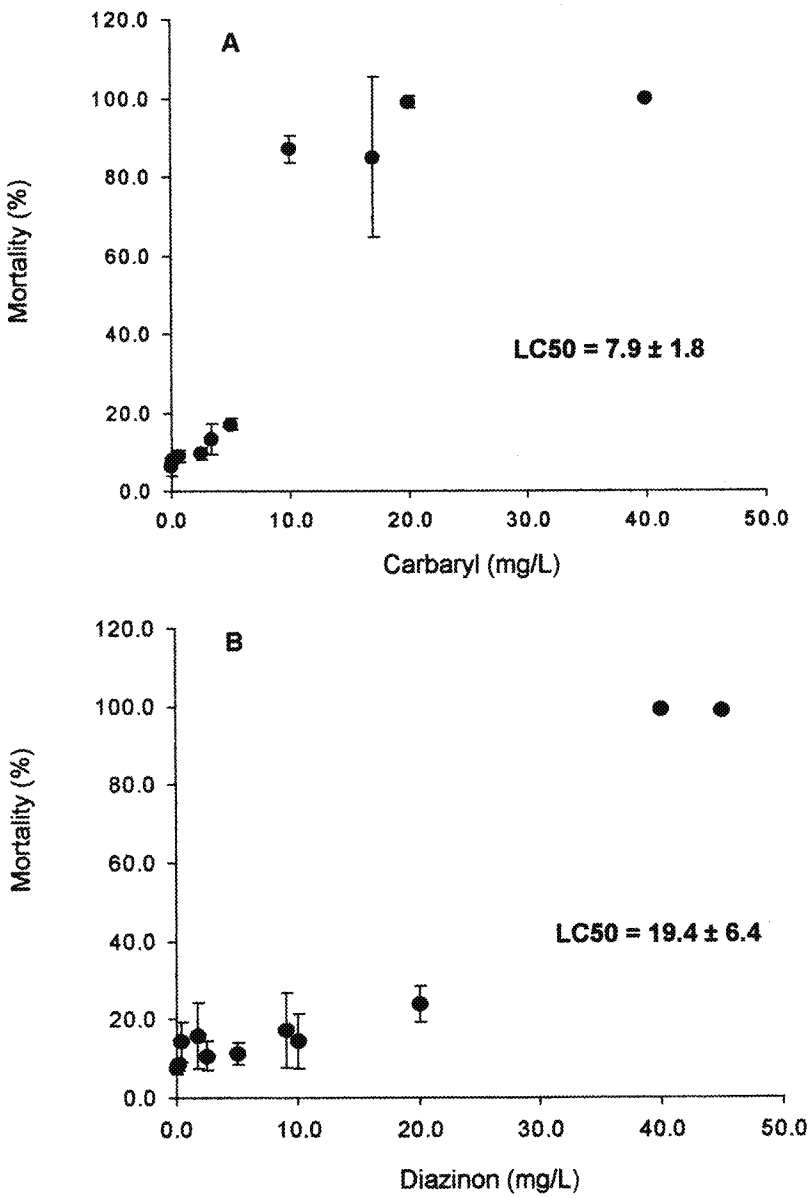


**Fig. 3.** Mortality of *U. imbecillis* glochidia exposed to the insecticides carbaryl (**A**) and diazinon (**B**). Data are means (SD) and LC50s are means ± 95% confidence intervals from four replicate tests

observed at the highest concentration of diazinon tested. The genotoxicity of atrazine at levels equal to ¼ NOEC could not be assessed with accuracy because three of the four Comet assay slides were ruined when the gels ripped during the cell lysis procedure. However, DNA damage of the one remaining slide was high (tail moment = 6.65), possibly indicating that increasing concentrations of atrazine also produced lower levels of DNA damage. Apoptosis, which can be distinguished in the Comet assay by distinct micronuclei, was encountered infrequently (approximately <1% of all cells evaluated), and was not quantified in this study.

## Discussion

Few pesticides have been tested on freshwater bivalves to evaluate their sensitivity relative to other aquatic invertebrates routinely used in toxicity tests for developing water quality criteria. Studies that have been conducted suggest that early life stages of mussels are less sensitive to many pesticides than cladocerans (Johnson *et al.* 1993; Keller 1993; Keller and Ruessler 1997). This contrasts with other studies that demonstrate that freshwater bivalves are highly sensitive to toxic insult by metals (Keller and Zam 1991; Jacobson *et al.* 1997), other inorganic contaminants (*e.g.,* chlorine and ammonia) (Goudreau *et al.* 1993; Augspurger *et al.* 2003), and some organics (*e.g.,* pulp and paper mill effluents and fluoranthene) (McKinney and Wade 1996; Weinstein 2001). In our research, we have observed that *U. imbecillis* glochidia appear to be less sensitive to toxicity caused by the organophosphate insecticide, diazinon, compared with amphipods, chironomids, and cladocerans, and this is congruent with past research on pesticide toxicity in freshwater bivalves. However, our research also demonstrates that *U. imbecillis* glochidia are equally or more

**Table 1.** Acute toxicity of lawn care chemicals to freshwater aquatic invertebrates commonly used in toxicity tests

| Chemical | Taxon | Species | 24-h LC50 (mg/L) | Formulation | Study |
|---|---|---|---|---|---|
| Copper[a] | Amphipod | *Gammarus* sp. | 1.2 | Technical | Rehwoldt *et al.* (1973) |
| | Chironomid | *Chironomus* sp. | 0.65 | Technical | Rehwoldt *et al.* (1973) |
| | | *Polypedium nubifer* | 2.05 | Technical | Hatakeyama (1988) |
| | Cladoceran | *Ceriodaphnia dubia* | 0.0201 | Technical | Kim *et al.* (1999) |
| | | *Ceriodaphnia dubia* | 0.0196 | Technical | Nelson and Roline (1998) |
| | Bivalve | *Pyganodon grandis*[b] | 0.046 | Technical | Jacobson *et al.* (1997) |
| | | *Lampsilis fasciola*[b] | 0.046 | Technical | Jacobson *et al.* (1997) |
| | | *Villosa iris*[b] | 0.0546 | Technical | Jacobson *et al.* (1997) |
| | | *Anodonta anatina*[b] | 0.0418 | Technical | Hanstén *et al.* (1996) |
| | | *Utterbackia imbecillis*[b] | 0.0374 | Technical | This study |
| Atrazine[c] | Bivalve | *Utterbackia imbecillis*[b] | 241.3 | Atrazine 4L | This study |
| Glyphosate[d] | Amphipod | *Gammarus pseudolimnaeus* | 100.0 | Roundup | Folmar *et al.* (1979) |
| | Cladoceran | *Daphnia magna* | 24.0 to 37.0 | Roundup | Material Data Safety Sheet |
| | Bivalve | *Utterbackia imbecillis*[b] | 18.3 | Roundup | This study |
| Carbaryl | Amphipod | *Gammarus lacustris* | 0.04 | Technical | Sanders (1969) |
| | Chironomid | *Chironomus thummi* | 0.127 | Technical | Fisher and Lohner (1986) |
| | Cladoceran | *Daphnia magna* | 22.9 | Technical | Lejczak (1977) |
| | Bivalve | *Utterbackia imbecillis*[b] | 30.1 | Technical | Johnson *et al.* (1993) |
| | | *Utterbackia imbecillis*[b] | 7.9 | Sevin | This study |
| Diazinon | Amphipod | *Hyalella azteca* | 0.03 | Technical | Werner and Nagel (1997) |
| | | *Gammarus lacustris* | 0.8 | Technical | Sanders (1969) |
| | Chironomid | *Chironomus tepperi* | 0.036 | Gesapon | Stevens (1992) |
| | Cladoceran | *Moina marcopa* | ~0.010 | NS[e] | Wong (1997) |
| | | *Ceriodaphnia dubia* | 0.0006 | Technical | Bailey *et al.* (1997) |
| | | *Daphnia magna* | 0.0009 | Technical | Fernández-Casalderrey *et al.* (1994) |
| | Bivalve | *Utterbackia imbecillis*[b] | 19.4 | Diazinon | This study |

[a] Hardness ranged from 30 to 85 mg/L in all studies evaluated.
[b] Mussels used in toxicity tests were glochidia.
[c] No comparative toxicity data were found for commercial formulations of atrazine, which substantially alter the solubility of the active ingredient.
[d] Review data for glyphosate were restricted to Roundup because different formulations are known to vary in toxicity (Solomons and Thompson 2003).
[e] Not specified: the specific formulation was not specified, however, a commercial formulation was used in the study.

sensitive to some commonly used pesticide formulations (carbaryl and glyphosate) and other components of lawn care products (copper) than other aquatic invertebrates.

Selective toxicity of pesticides among different species is primarily mediated by a species' ability to metabolize the parent compound to less or more toxic forms, and by the susceptibility of the target site to the actions of the chemical (Feyereisen 1995; Narahashi 1996). This has been eloquently demonstrated in fish, where Keizer *et al.* (1995) observed that the guppy (*Poecilia reticulata*) was highly sensitive to diazinon toxicity in comparison to three other fish species because it can actively convert diazinon to more toxic metabolites via Phase I oxidative biotransformation reactions and possesses a relatively sensitive cholinesterase, the target site of organophosphate and carbamate insecticides. Adult mollusks are known to have reduced oxidative biotransformation capabilities compared to crustaceans and insects (Baturo and Lagadic 1996; Livingstone 1998), and detoxification is often reduced or absent in juvenile aquatic organisms (Andersson and Förlin 1992), this may partially explain the observed insensitivity of freshwater mussels to organophosphates requiring bioactivation in this study and in studies by Keller and Ruessler (1997) and Doran *et al.* (2001). Likewise, target site insensitivity may explain why Keller (1993) observed that *U. imbecillis* juveniles were tolerant of organochlorine cyclodiene insecticides (chlordane and toxaphene), as these chemicals do not require bioactivation and target GABA receptors, which are highly reduced in bivalves (Karhunen *et al.* 1993) and dissimilar in structure to arthropod receptors (Xue 1998). Importantly, we observed that *U. imbecillis* glochidia were sensitive to the commonly used pesticides, carbaryl and glyphosate. Carbaryl, a carbamate insecticide, targets cholinesterases as do organophosphates but does not require bioactivation to do so. Hence, the sensitivity of *U. imbecillis* to carbaryl suggests that glochidia of this species possess a sensitive cholinesterase. A freshwater mussel die-off attributed to anticholinesterase poisoning by insecticides has been documented in the field (Fleming *et al.* 1995). While Johnson *et al.* (1993) directly compared the acute toxicity of carbaryl between freshwater mussels and cladocerans, and found the mussels to be less sensitive, juveniles were used in the tests. Juveniles, unlike the glochidia lifestage used in this study, are able to close their shells in response to toxic insult, and this may temporarily protect them during short-term exposures (Jacobson *et al.* 1993; Keller and Ruessler 1997; Black *et al.*, unpublished data). Glyphosate inhibits the enzyme 5-enolpyruvyl shikimate-3-P synthetase that controls aromatic amino acid synthesis in plants and is relatively nontoxic to animals (reviewed by Solomon and Thompson 2003). The selective toxicity of glyphosate that we observed in freshwater mussels is likely due to surfactants, as commercial formulations of glyphosate are known to be more toxic to aquatic organisms than the active ingredient (Folmar *et al.* 1979;

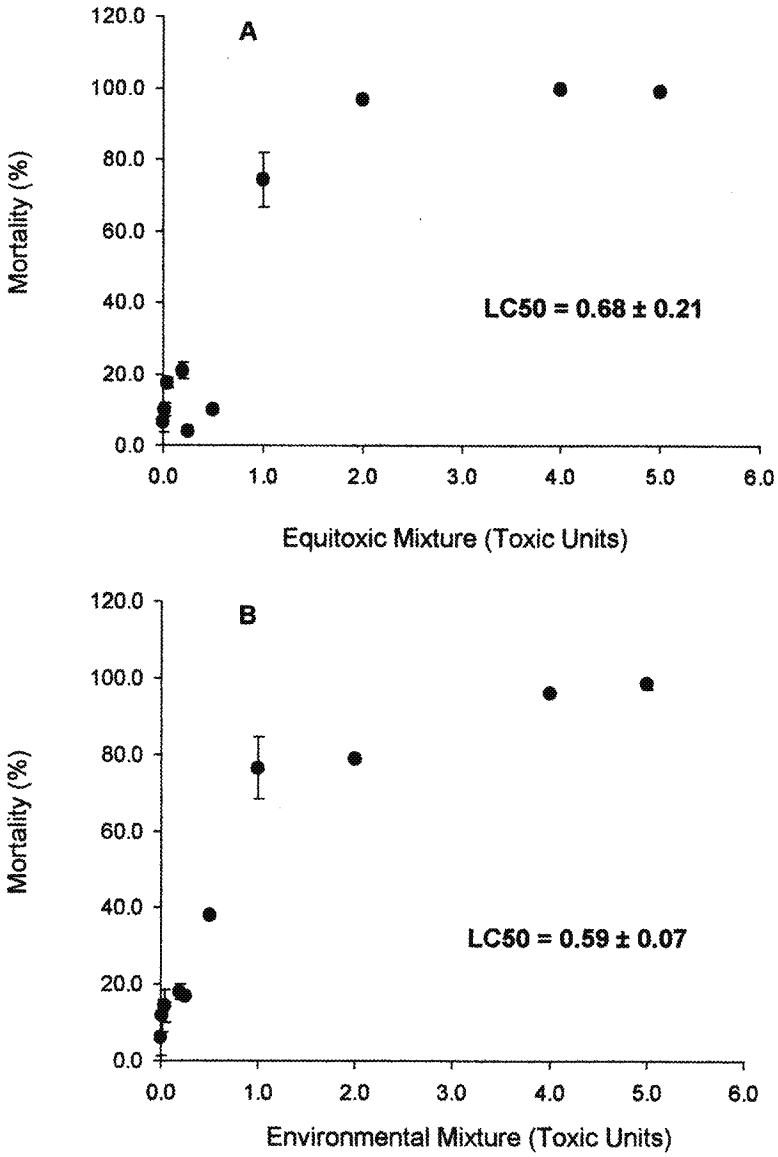


**Fig. 4.** Mortality of *U. imbecillis* glochidia exposed to an equitoxic (**A**) and an environmentally realistic (**B**) mixture of lawn care chemicals. Data are means (SD) and LC50s are means ± 95% confidence intervals from three replicate tests

Perkins *et al.* 2000; Solomon and Thompson 2003). Clearly, more research on pesticide toxicity in freshwater bivalves is needed to adequately protect this imperiled fauna from chemicals currently used in lawn care.

In the environment, non-target organisms are exposed to mixtures of chemicals, and we have observed that equitoxic and environmentally realistic mixtures of copper, atrazine, glyphosate, carbaryl, and diazinon were adequately described by an additive model in producing lethal effects in *U. imbecillis* glochidia. In the literature, few studies exist that explore the combined toxicity of chemicals similar to those used in this study on aquatic invertebrates. Of these, Belden and Lydy (2000) observed that atrazine may synergistically affect diazinon toxicity in chironomids by increasing its bioactivation, and Kaushik and Kumar (1993) observed antagonism between carbaryl and an organophosphate (monocrotophos) in freshwater crabs, whereas carbaryl toxicity was not affected by copper in the Microtox test and in protozoa (Vasseur *et al.* 1988). Furthermore, copper can complex with glyphosate and reduce its toxicity to plants (Sundaram and Sundaram 1997). Clearly, the type of toxic interaction observed in mixture studies will vary depending on the number, type, and concentration of chemicals

**Table 2.** No-observed effect concentrations (NOECs) of lawn care chemicals to *U. imbecillis* glochidia and concentrations used for genotoxicity screening

| | | Genotoxicity test concentration | |
|---|---|---|---|
| Chemical[a] | NOEC | ¼ NOEC | ½ NOEC |
| Copper (μg/L) | 12.62 | 3.12 | 6.30 |
| Atrazine (mg/L) | 45.12 | 11.28 | 22.55 |
| Glyphosate (mg/L) | 10.04 | 2.50 | 5.00 |
| Carbaryl (mg/L) | 3.49 | 0.88 | 1.75 |
| Diazinon (mg/L) | 1.07 | 0.28 | 0.55 |

[a]Pesticides used were commercial formulations, and NOECs represent amount of active ingredient.

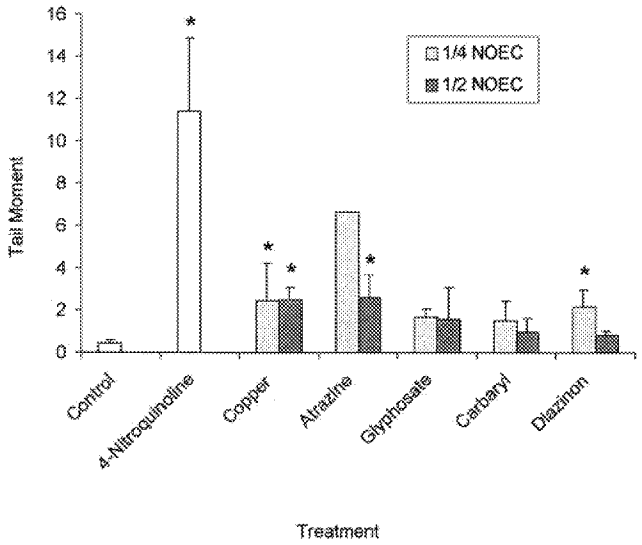


**Fig. 5.** DNA damage (tail moment) in *U. imbecillis* glochidia exposed to sublethal concentrations of lawn care chemicals. Data are means (SD). $n = 4$ for all treatments except atrazine at ¼ NOEC, where $n = 1$. Asterisks indicate significant differences from controls ($p < 0.05$)

used, the test organisms evaluated, and the endpoint of toxicity. Present research suggests that mixture studies on many pesticides with different modes of action have a tendency to give additive results (Broderius and Kahl 1985; Deneer 2000), and this would likely be the case for our observations of additive toxicity with the equitoxic mixture. In contrast, the observed additive toxicity in the environmental mixture may have been the result of copper driving toxicity, as confidence intervals for copper concentrations in mixture levels producing 50% mortality (18 to 22 μg/L) were not substantially different from copper concentrations producing 50% mortality singly (19.7 to 48.3 μg/L). While these observations suggest that diverse lawn care chemicals may act independently to produce additive-like effects in mussels, the difficulty in extrapolating from laboratory studies to field exposures warrants the exploration of other methods to assess combined toxicities such as effluent testing and sediment bioassays.

Many pesticides are not classified as genotoxins because they do not interact directly with DNA and cause carcinogenesis; rather they often promote cancers indirectly via DNA damage by mechanisms such as peroxisomal proliferation, endocrine disruption, and oxidative stress, which are threshold-based (Rakitsky *et al.* 2000). This presents a challenge for genotoxicity testing in aquatic invertebrates where neoplasias rarely develop and there is a need for correlations between DNA damage and other endpoints (*e.g.,* impaired metabolism, tissue atrophy, reduced growth) (Kurelec 1993). In this study, weak genotoxic responses to chemicals were observed in mussel glochidia exposed singly to copper, atrazine, and diazinon. Clements *et al.* (1997) also observed genotoxicity of commercial formulations of atrazine with the comet assay in tadpoles. Other studies indicate that atrazine is not mutagenic in bacteria (Kappas 1988) but may be genotoxic in mammalian systems, especially when S9 detoxification systems are absent (Ribas *et al.* 1995; Lioi *et al.* 1998). As mussel glochidia likely have reduced or absent phase I detoxification capabilities, this could possibly explain species and life-stage sensitivity to atrazine. In contrast to studies indicating that glyphosate and carbaryl formulations may be weakly genotoxic in vertebrates (Rank *et al.* 1993; Amer *et al.* 1996), we did not find any evidence of genotoxicity in glochidia. We did, however, observe that low concentrations of diazinon were genotoxic to glochidia, and similar results have been observed in mudminnows (Vigfusson *et al.* 1983) and *in vitro* mammalian assays (Matsuoka *et al.* 1979; Sobti *et al.* 1982). Additionally, we observed that DNA damage in glochidia exposed to ¼ NOECs was comparable to or higher than that in glochidia exposed to ½ NOECs, and such a decrease in DNA damage at higher concentrations is suggestive of cytotoxicity, which may inhibit metabolism and further expression of DNA damage as has been observed in mammalian test systems (Lodovici *et al.* 1994; Lioi *et al.* 1998). Henderson *et al.* (1998) studied the ability of the Comet assay to discriminate between genotoxins and cytotoxins and recommended that measurements of cellular viability >75% should be used concomitantly with demonstration of DNA damage in the Comet assay to avoid false positives. While we did not obtain cytotoxicity data in our study, such information would be useful for evaluating the genotoxic potential of xenobiotics with the Comet assay in aquatic invertebrates. Likewise, future research on freshwater mussels should explore the consequences of DNA damage observed in glochidia to ecologically relevant endpoints such as transformation success into fully formed juveniles.

In conclusion, these data indicate that glochidia of freshwater mussels are among the most sensitive aquatic organisms tested for some chemicals commonly used in lawn care and that measurements of DNA damage may be useful as screening tools to evaluate potential sublethal effects of chemicals on non-target aquatic organisms.

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# EVALUATION OF THE *IN VITRO* EFFECT OF GLYPHOSATE-BASED HERBICIDE ON BOVINE LYMPHOCYTES USING CHROMOSOME PAINTING

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## Abstract

The induction of bovine chromosome 1 aberrations was investigated in cultivated peripheral lymphocytes of cattle after an application of a glyphosate-based herbicide formulation. Fluorescence *in situ* hybridization with bovine chromosome 1-specific painting probe was used for metaphase cell examination. The cultures were exposed to herbicide at concentration levels, ranging from 28 to 1 120 µmol/L for the last 24 h. Structural chromosome aberrations of chromosome 1 (acentric fragments) were detected, but without a statistical significance. Slight bovine chromosome 1 aneuploidy increase (monosomy, trisomy) was found in cultures after 24 h treatment with a dose of 56 µmol/L. Statistically significant elevation in polyploidy induction was shown at the same concentration ($P<0.05$).

**Key words:** cattle, glyphosate, herbicide, chromosomal aberrations.

Glyphosate (N-phosphonomethyl glycine) is a broad-spectrum, non-selective herbicide; widely applied in agriculture in the production of soybeans, corn, hay, and pasture, as well as on fallow land. It has been documented that some of the used patterns of glyphosate can lead to detectable residues (1, 5) that may consequently enter the food chain (26).

Glyphosate-based formulations have become the most frequently applied pesticides in the world, because of the introduction of crops, which are genetically engineered to be tolerant of the herbicide. They are approved throughout Europe for a range of agricultural uses (9).

The results from genotoxicity studies of glyphosate have been conflicting. A week increase of both *in vivo* (mice bone marrow cells) and *in vitro* (human lymphocytes) genotoxic activity was evident, using the technical glyphosate formulation in contrast to glyphosate itself (3). In the study of Lioi *et al.* (13), positive clastogenic and genotoxic effects of glyphosate (98% purity) were documented in cultivated bovine peripheral lymphocytes. Garry *et al.* (7) reported that unlike commercial herbicide products, all surfactant mixtures tested showed positive dosage response effects after *in vitro* genotoxicity examination. According to the findings of Grisolia (8), Roundup® induced statistically significant erythrocyte MN frequency in *T. rendalli*, at three dose levels while in mice it presented negative results.

Since it has been shown that surfactant mixtures, which help glyphosate to penetrate plant cells, and which are often the part of inert ingredients in glyphosate-containing products (such as commercial herbicide Roundup®), are more toxic than glyphosate itself, the reason for testing entire glyphosate-based formulation instead of pure active ingredient has emerged. In addition, when the pesticides are applied, the environment as well as farm animals are exposed to formulations and not pure active ingredients. The presence of herbicides in animal diets could affect not only the health of livestock but also the quality of animal products (17).

Cattle are known as a very sensitive animal species to the exposure to various environmental pollutants. As demonstrated in several studies, cytogenetic analysis of bovine peripheral lymphocytes is a useful tool for the estimation of the exposure of cattle to environmental pollution (6, 21, 24). Fluorescence *in situ* hybridization (FISH) using chromosome painting probes is a sensitive method, particularly for detecting stable chromosomal aberrations, which should be heritable.

At present, there is a scarcity of data about the glyphosate-based herbicides effect on the chromosome aberration induction in farm animals detected by specific chromosome painting. Therefore, the aim of the study was to evaluate the *in vitro* effect of technical herbicide, containing isopropyl amine salt of glyphosate on

cultivated peripheral lymphocytes of cattle by means of bovine chromosome 1 whole chromosome painting probe.

## Material and Methods

The peripheral blood from two clinically healthy bull calves (Slovak spotted cattle, 6-8 month old) were used in the experiment. All the blood specimens were cultivated for 72 h at 38°C in 5 ml of RPMI 1 640 medium, supplemented with L-glutamine and 15 mmol/L HEPES (Sigma, USA), 15% foetal calf serum (Sigma, USA), antibiotics (penicillin 250 U/mL and streptomycin 250 µg/mL), and phytohaemagglutinin (PHA, 180 µg/mL Welcome, England).

Isopropylamine salt of N-phosphonomethylglycine (glyphosate, approximate 62% by weight) with 38% inert ingredients of a not specified composition (Monsanto Europe S. A., Belgium), was dissolved in sterile water and added to the lymphocyte cultures for the last 24 h, at concentrations of 28, 56, 140, 280, 560 and 1 120 µmol/L. The doses were chosen, referring to the highest dose causing a reduction in the mitotic index (MI) of more than 50%.

Fluorescent *in situ* hybridization technique (FISH) was performed, for the chromosome aberrations detection. Orange-red labelled whole chromosome painting probe, specific for the bovine chromosome 1 as the largest physical unit of cattle genome (prepared in Veterinary Research Institute, Brno, Czech Republic) was used for hybridization. The painting probe in hybridization mixture (50% formamide, 2xSSC, 10% dextran sulphate, salmon sperm DNA, competitor DNA) was denatured at 72°C for 10 min and reannealed at 37°C for 80 min. The denaturation of slides was performed in 70% formamide, 2xSSC (pH 7.0) at 72°C for 2 min and followed by a dehydration procedure (70%, 90%, and 96% ethanol, -20°C). After overnight hybridization at 37°C, the slides were washed in 50% formamide, 2xSSC (pH 7.0) at 42°C, in 0.1xSSC (pH 7.0) at 42°C, and in TNT (Tris-NaCl-Tween 20 buffer, pH 7.0) at 42°C. The slides were counterstained in DAPI/Antifade (4′, 6′-diamino-2-fenolindol, Q-BIOgene, UK).

A fluorescent microscope Nikon Labophot 2A/2, equipped with dual band pass filter FITC/TRITC, was used for probe visualization. Chromosome aberrations were scored according to PAINT nomenclature (27). The statistical analysis of the results was performed using a chi-square test.

## Results

The frequencies of bovine chromosome 1 aberrations in cultivated lymphocytes after 24 h exposure to glyphosate-based herbicide, evaluated by means of bovine chromosome 1 specific painting probe (Fig. 1) are shown in Table 1.

**Fig. 1.** Metaphase plate of cattle (60; XY) after FISH with bovine chromosome 1 painting probe.

Acentric fragments of chromosome 1 were the most common type of structural chromosome-type of aberrations at each concentration tested, but the level of aberrations was not statistically significantly increased, as compared with untreated cultures. Stable chromosomal aberrations, such as translocations, were not observed under conditions of our experiment.

**Table 1**

The frequencies of chromosome aberrations in bovine peripheral lymphocytes after 24 h glyphosate-based herbicide treatment evaluated *in vitro* by means of bovine chromosome 1 painting (% mean ± SD)

| Dose (µmol/L) 24h | Number of cells analysed | Chromosome 1 aneuploidy (2n±1) | Polyploidy (4n) | Acentric fragments of chromosome 1 |
|---|---|---|---|---|
| Control | 1000 | 0.3 ± 0.055 | 0.4 ± 0.063 | 0.4 ± 0.063 |
| 28 | 900 | 0.6 ± 0.074 | 0.9 ± 0.094[a] | 0.8 ± 0.088 |
| 56 | 900 | 1.0 ± 0.090 | 1.2± 0,110[*] | 0.6 ± 0.074 |
| 140 | 650 | 0.9 ± 0.095 | 1.1 ± 0.103[a,b] | 0.5 ± 0.074 |
| 280 | 610 | 0.8 ± 0.090 | 0.8 ± 0.090[a,b] | 0.3 ± 0.057 |
| 560 | 400 | 1.0 ± 0.099 | 1.0 ± 0.099[a,b] | 1.2 ± 0.111 |
| 1120 | 350 | 1.1 ± 0.106 | 0.3 ± 0.053[a,b] | 0.8 ± 0.092 |

[*] $P<0.05$ according to chi-square test; a – without statistical significance; b – insufficient number of cells

Aneuploidy of bovine chromosome 1, particularly monosomy (2n-1) and trisomy (2n+1) were shown in bovine cell cultures. The slightly increased frequencies of bovine chromosome 1 monosomies and trisomies were obtained after treatment with a dose of 56 μmol/L, but without a statistical significance (Table 1).

Polyploidy (4n) was the statistically significant increased type of numerical aberration ($P<0.05$), induced after exposure to glyphosate product at the concentration of 56 μmol/L.

## Discussion

In our study, all of the chromosome-painting probe for bovine chromosome 1 (BTA 1) was applied to evaluate the involvement of this chromosome in the formation of chromosomal aberrations, after *in vitro* exposure to glyphosate-based herbicide. Taking into account the fact that chromosome 1 is the largest chromosome in bovine genome, as well as the assumption of Russell *et al.* (22) that if chromosome specific sensitivities exist, the recognition of their order is important, we decided to start the analysis just with this chromosome.

For this chromosome, the loci are responsible for some serious hereditary diseases (11, 16) as well as genes included in *de novo* purine biosynthesis (29) have recently been mapped. Moreover, it has been shown in humans that the distribution of chemically induced chromosomal aberrations does not appear to be random (23), and that different clastogens may induce a different pattern of chromosomal aberrations (2).

Although the FISH technique using all the chromosome painting probes is a sensitive method for detecting chromosomal rearrangements, Kubickova *et al.* (12) reported that the use of chromosome painting in farm animals is mainly limited by the fact that chromosome specific probes are not commercially available for individual animal species.

The stable chromosomal aberrations, which are well known as good indicators of chronic exposure to ionic radiation or clastogen, were not detected under conditions of our experiment. This might be probably explained by relatively low proportion of the painted genome in cattle examined by means of bovine probe available. It has been well documented in humans that analysis of chemically as well as radiation-induced chromosome aberrations by FISH-painting is restricted to parts of human genome (2, 4). We assumed that glyphosate product tested did not induce enough double-strand breaks in one cell to form chromosome exchanges. As reported by Marshall and Obe (15), the stable aberrations are seen at only relatively low frequencies even after treatment with very potent clastogens. Moreover, it has been recently indicated that cattle have a reduced sensitivity to the chromosomal mechanisms, which can cause structural chromosomal aberrations (20).

Our results showed that induction of chromosome aberrations (CAs) by glyphosate herbicide observed by using FISH is similar as induction of CAs, observed by using conventional chromosomal analysis (25). The authors did not find significantly increased frequency of CA in bovine peripheral lymphocytes after technical glyphosate-treatment for 24 h *in vitro*. Based on a small increase in the frequencies of CA after treatment with a threshold dose (1120 μmol/L) in comparison to the control; they assumed that an indirect mechanism in chromosome damage could be considered. This is in agreement with Rakitski *et al.* (19), who declared that, with very rare exceptions, pesticides do not react with DNA directly.

We observed slight bovine chromosome aneuploidy increase (monosomy as well as trisomy) after treatment with a dose of 56 μmol/L. At the same, but not at higher concentrations, statistically significant elevation in polyploidy induction was shown ($P<0.05$), as compared with the control. One of the possible explanations of this fact could be the decreased mitotic index in exposed cultures with the subsequent impossibility to examine the sufficient number of metaphases. It is known that also in healthy cattle lymphocytes cultivation the insufficient lymphocyte proliferation stimulation by mitogen is often the case. As reported by Řezáčová *et al.* (20), it was sometimes problematic to obtain more than 800 analysable cells per cow in the course of FISH experiments. Furthermore, in some pesticides, the significant immunotoxic effect (a decrease in lymphocyte activation with phytohaemagglutinin) was observed (18).

The results of Piesova (17) indicated the possible aneugenic and/or clastogenic effect of glyphosate-based herbicide tested by using cytokinesis block micronucleus assay. The author observed weak induction of micronuclei in bovine lymphocytes after prolonged 48 h exposure to herbicide *in vitro*.

It is generally accepted that aneuploidy in somatic cells is associated with the development of several cancers. One of the targets of aneugens, are molecules involved in cell cycle control (10), which was reported in case of various commercial glyphosate products by Marc *et al.* (14). Glyphosate formulations affected the cell division at the level of CDK1/cyclin B activation that is a universal regulator of the G2/M transition of the cell cycle.

In conclusion, glyphosate based herbicide did not induce statistically significant level of bovine chromosome 1 structural aberrations in cultivated bovine lymphocytes. The presence of chromosome 1 aneuploidies as well as polyploidies could indicate rather aneugenic than clastogenic effect of herbicide tested under conditions of our experiment. However, to confirm this assumption the micronucleus assay (*e.g.* with fluorescent labelled probes) should also be performed. Nevertheless, a direct DNA effect of this herbicide cannot be definitely excluded without performing experiments with longer incubation periods.

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# THE EFFECT OF GLYPHOSATE ON THE FREQUENCY OF MICRONUCLEI IN BOVINE LYMPHOCYTES *IN VITRO*

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(Received 12. January 2005)

*Glyphosate is a widely used broad-spectrum herbicide that has expanded its applications on plant varieties that are genetically modified to tolerate glyphosate treatment.*

*The aim of this study was to determine the frequency of micronuclei (MNi) in bovine peripheral lymphocytes after exposure to glyphosate in vitro. The cytokinesis block micronucleus assay (CBMN) for estimation of genotoxic activity was used. The obtained results indicate that glyphosate weakly induced micronuclei in bovine peripheral lymphocytes. Significant elevations of MNi ($p < 0.05$) were observed at concentrations of glyphosate of 280 μM and 560 μM, respectively. Treatment of bovine lymphocytes did not result in the induction of micronuclei in a dose-dependent manner. From cytotoxicity data it is evident that CBPI does not reflect the reduction of cell proliferation.*

*The influence of metabolic activation on the genotoxic activity of glyphosate was investigated, too. When lymphocyte cultures were treated with glyphosate together with a liver membrane fraction (S9) from Aroclor 1245-induced rat liver, the number of micronuclei in binucleated cells did not increase significantly.*

*Key words: bovine peripheral lymphocytes, glyphosate, micronucleus, S9*

## INTRODUCTION

Glyphosate is a broad-spectrum, non-selective systemic herbicide. Its herbicidal activity is expressed through direct contact with the leaves with subsequent translocation throughout the plant. Today, a variety of glyphosate-based formulations are registered under different trade names such as: Roundup, Rodeo, Accord, Sting, Spasor, Muster, Tumbleweed and other. In pure chemical terms glyphosate is an organophosphate, however it does not affect the nervous system in the same way as organophosphate insecticide, and is exploited for its anticholinesterase effects (Marrs, 1993).

Glyphosate inhibits plant growth through interference with the production of essential aromatic amino acids by inhibition of the enzyme enolpyruvylshikimate phosphate synthase, which is responsible for the biosynthesis of choristmate,

which is an intermediate in phenylalanine, tyrosine, and tryptophan biosynthesis (Williams *et al*. 2000). The plant varieties have been inserted with a gene from a bacterium that makes them resistant to the herbicide glyphosate and then the weeds are killed, leaving the crop unaffected.

While glyphosate itself may be relatively harmless (Haughton *et al*. 2001; Smith and Oehme, 1992) some of the products with which contain it have a less benign reputation. Marketed formulations of glyphosate generally contain a surfactant. The purpose of this is to prevent the chemical from forming into droplets thus rolling off leaves that are sprayed. The most widely used type of surfactants in glyphosate formulations are known as ethylated amines. Members of this group of surfactants are significantly more toxic than glyphosate. In a recent study Adam *et al.* (1997) compared the toxicities of Roundup and its component chemicals following administration to rats. They found that POEA (polyoxy-ethyleneamine) and preparations that contained POEA were more toxic than glyphosate alone.

The acute toxicity of glyphosate itself is very low. According to the World Health Organisation, for pure glyphosate the oral $LD_{50}$ in the rat is 4.320 mg/kg. In spite of low toxicity, some laboratory studies have reported adverse effects in each standard category of testing (subchronic, chronic, carcinogenicity, mutagenicity, and reproduction). These signs included eye and skin irritation (Temple and Smith, 1992), cardiac depression (Tai, 1990; Lin *et al.* 1999) vomiting (Lee *et al.* 2000; Burgat *et al.* 1998), and pulmonary edema (Lee *et al.* 2000; Martinez *et al.* 1990). Hietanen *et al.* (1983) reported that glyphosate could disrupt functions of enzymes in animals. In rats it was found to decrease the activity of cytochrome P-450 and monoxygenase activities as well as the intestinal activity of aryl hydrocarbon hydrolase. Other studies have shown some reproductive problems after glyphosate exposure (Savitz *et al.*1997). A study was undertaken to investigate the effect of chronic treatment of glyphosate on body weight and semen characteristics in mature male New Zealand white rabbits. Yousef *et al.* (1995) reported that glyphosate effects included reduced ejaculate volume, and increased abnormal and nonviable sperm. The potential of glyphosate to cause non-Hodgkin's lymphoma has been analyzed by Hardell *et al.* (2002). A variety of organisms have shown that glyphosate-containing products cause genetic damage: in *Salmonella bacteria*, in onion root cells (Rank *et al.* 1993) and in human lymphocytes (Vigfusson and Vyse, 1980). In other studies glyphosate was not mutagenic in the mouse bone marrow, *Salmonella* and Allium anaphase-telophase tests (Rank *et al.* 1993).

A primary purpose of short-term tests for mutation is to provide information on the production of heritable changes (mutations) that could lead to further adverse biological consequences. In the present study, the ability of glyphosate to induce genetic damage was evaluated by the cytokinesis block micronucleus (CBMN) assay.

## MATERIALS AND METHODS

### *Chemicals*

Isopropylamine salt of glyphosate, Monsanto, Antwerp, Belgium

| Components | CAS No. | EINECS/ELINCS No. | % by weight (approximate) |
|---|---|---|---|
| Isopropylamine salt of glyphosate | 38641-94-0 | 254-056-8 | 62 |
| Inert ingredients | | | 38 |

Glyphosate was dissolved in sterile water and added to the lymphocyte cultures at concentrations of 28, 56, 140, 280, and 560 μM. The highest dose of glyphosate was chosen on the basis of the reduction in mitotic index by >50%. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 μM), cyclophosphamide (CP, Jenapharm, Ankerwerk, Rudolstadt, Germany, 0.1 mM) and ethylmethanesulphonate (EMS, Sigma, St. Louis, MO, USA, 250 μg/ml) were used as positive control agents in the assays in both the absence and presence of the metabolic activator (S9 mix).

### *Lymphocyte cultures*

Peripheral blood was drawn from the jugular vein of two clinically healthy donors, 5 months old. Lymphocyte cultures were set up by adding 0.5 ml of heparinized whole blood to 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 μM HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250 μg/ml) and phytohaemagglutinin (PHA, 180 μg/ml, Welcome, Dartford, UK).

For the CBMN test the cultures were incubated at 37° C for 72 h and 44 h from the start, cytochalasin B (Cyt. B) at a final concentration of 6 μg/ml was added to arrest cytokinesis. The test chemical was added 24 h after PHA stimulation.

The cultures treated for 2 h with S9 mix and those without S9 mix were set up without heat inactivated fetal calf serum. After the treatment, cultures were washed twice with PBS and reconstituted in the same way as cultures treated for 48 h.

For MN assay in the presence of S9 mix, a freshly prepared S9 mix (10% of the culture volume) from Aroclor 1254 (Supelco, Bellefonte, PA, USA) was prepared according to the method of Maron and Ames (1983).

Standard cytogenetic method was used for the obtained slides.

### *MN analysis*

A total 1000 binucleated cells (BN) with well-preserved cytoplasm were examined for each experimental concentration and donor. The cytokinesis block

proliferation index (CBPI) was evaluated by classifying 500 cells according to the number of nuclei (Surrallés *et al.* 1995).

The statistical evaluation of the results was carried out using Fisher's exact test for micronucleated cells and $\chi^2$ test for CBPI.

## RESULTS

Table 1 and 2 show frequencies of binucleated cells with micronuclei (BNMN) and cytotoxicity index (CBPI) obtained after treatment with glyphosate. In each table, the data obtained from the different experimental conditions is shown: treatment lasting forty-eight hours without microsomal fraction and treatment for two hours with and without S9 microsomal fraction, respectively.

Table 1. Induction of micronuclei in bovine lymphocyte cultures treated with glyphosate-donor A

| Treatment | Concentration μM | CBPI | Total BNMN |
|---|---|---|---|
| 48 h | Control | 1.63 | 20 |
| | 28 | 1.60 | 19 |
| | 56 | 1.69 | 24 |
| | 140 | 1.68 | 22 |
| | 280 | 1.51 | 39* |
| | 560 | 1.66 | 27 |
| MMC (0.4 μM) | | 1.52 | 55*** |
| 2h (-S9) | Control | 1.54 | 21 |
| | 28 | 1.48 | 17 |
| | 56 | 1.48 | 14 |
| | 140 | 1.53 | 19 |
| | 280 | 1.57 | 20 |
| | 560 | 1.55 | 20 |
| EMS (250 μg/ml) | | 1.48 | 48*** |
| 2 h (+S9) | Control | 1.52 | 20 |
| | 28 | 1.44 | 23 |
| | 56 | 1.65 | 20 |
| | 140 | 1.72 | 21 |
| | 280 | 1.71 | 19 |
| | 560 | 1.58 | 28 |
| CP (0.1 mM) | | 1.49 | 51*** |

1000 binucleated cells of each concentration were determined;
Statistical significance: *$p<0.05$, ***$p<0.001$

Table 2. Induction of micronuclei in bovine lymphocyte cultures treated with glyphosate-donor B

| Treatment | Concentration μM | CBPI | Total BNMN |
|---|---|---|---|
| 48 h | Control | 1.50 | 13 |
| | 28 | 1.40 | 16 |
| | 56 | 1.43 | 12 |
| | 140 | 1.41 | 19 |
| | 280 | 1.50 | 23 |
| | 560 | 1.47 | 26* |
| MMC (0.4 μM) | | 1.48 | 36*** |
| 2h (-S9) | Control | 1.54 | 12 |
| | 28 | 1.58 | 12 |
| | 56 | 1.62 | 14 |
| | 140 | 1.53 | 18 |
| | 280 | 1.57 | 11 |
| | 560 | 1.55 | 20 |
| EMS (25 μg/ml) | | 1.44 | 35*** |
| 2 h (+S9) | Control | 1.51 | 15 |
| | 28 | 1.58 | 14 |
| | 56 | 1.54 | 23 |
| | 140 | 1.45 | 20 |
| | 280 | 1.60 | 27 |
| | 560 | 1.62 | 22 |
| CP (0.1 mM) | | 1.48 | 40*** |

1000 binucleated cells of each concentration were determined;
Statistical significance: *$p<0.05$, ***$p<0.001$

The positive controls were MMC (0.4 μM) in the experiments without microsomal activation, CP (0.1 mM) in the experiments with S9 and EMS (250 μg/ml) in the experiments lasting 2 h without S9. Glyphosate treatments lasting for forty-eight hours appear to induce a very slight but statistically significant increase in BNMN frequency in cultures at higher tested concentration (280 μM and 560 μM, respectively). However, none of donors tested was able to induce a dose-dependent increase on micronuclei frequencies.

Treatments with glyphosate for 2 h did not show any positive response, probably due to the short time of exposure to the herbicide.

The results from the experiments for two hours in the presence of S9 mix showed no significant increase in the MN levels.

One of the well known cytotoxicity indexes CBPI was used. From our cytotoxicity data it is obvious that the herbicide did not induce the reduction of cell proliferation.

In conclusion, our results indicate that glyphosate is able to exert a very weak effect on frequency of micronuclei in bovine peripheral lymphocytes *in vitro*.

## DISCUSSION

Environmental risk assessments require multidisciplinary knowledge to study the mechanisms of action, metabolism, genetic damage and detoxification. Optimal integration of chemical measurements and biomarker responses could lead to an improved understanding of adverse effects in both human and ecological assessment (Eason and O'Halloran, 2002). The formation of micronuclei in peripheral blood lymphocytes is a valuable cytogenetic biomarker in human populations exposed to genotoxic compounds (Bolognesi *et al.* 2004).

To our knowledge, there is a small number of available reports describing the cytotoxicity or genotoxicity effects of glyphosate on domestic animal cells even though many of environmental mutagens are associated with reduced productive and reproductive efficiency of livestock. The purpose of this study is to provide evidence of the genotoxic potential of glyphosate on *in vitro* cultures of bovine lymphocytes using CBMN assay.

Lioi *et al.* (Lioi *et al.* 1998a; Lioi *et al.* 1998b) reported that glyphosate produced an increased frequency of chromosomal aberrations in both cultured human and bovine lymphocytes. In experiments with bovine lymphocytes they chose herbicide concentrations ranging from 17 to 170 μM and applied on lymphocytes separated by Ficoll-Hypaque gradient density that were cultured for 72 h. Purity of tested glyphosate was = 98%. Their results indicate a statistically significant increase of structural aberrations and sister chromatid exchanges. However, according to data by Li and Long (1988) administration of glyphosate to rats did not produce an increase in frequency of chromosomal aberrations. Similarly, from the results of the studies by De Marco *et al.* (1992) and Rank *et al.* (1993) it seems evident that glyphosate alone was not responsable for chromosomal damage.

Both the mouse and rat bone marrow micronucleus assays were used to study the effects of exposure to glyphosate on dividing red blood cells. The micronucleus assay appears to be sensitive enough to detect both clastogenicity and aneuploidy.

Bolognesi *et al.* (1997) obtained in Swiss/CD-1 mice a weak positive glyphosate-induced increase in the bone marrow micronucleus assay. Their results were in contrast with those of Kier *et al.* (1997) that reported no increased micronucleus formation.

The information about genotoxic effects of glyphosate is both large and heterogeneous. Their primary goal is to determine whether the chemical interacts directly or indirectly with DNA and thus could lead to adverse biological consequences, including cancer.

In conclusion, glyphosate only weakly increased the frequency of micronuclei in bovine lymphocyte cultures. Further studies are needed in this area, as genetically modified plant varieties will be likely used more extensively throughout the food chain. The presence of herbicides in animal diets could affect not only the health of livestock but also the quality of animal products. Development of glyphosate resistance in weeds species could be also a serious risk from now. It will make farmers more dependent on other pesticides and will probably lead to their increased use.

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## EFEKTI GLIFOZATA NA FREKVENCIJU POJAVLJIVANJA MIKRONUKLEUSA U LIMFOCITIMA GOVEDA *IN VITRO*

PIEŠOVÁ ELENA

SADRŽAJ

Glifozati su herbicidi širokog spektra, čija se primena proširuje i na razne vrste biljaka, koje su genetski modifikovane tako da mogu tolerisati tretman glifozatima.

Cilj ovog rada je bio utvrđivanje frekvence pojavljivanja mikronukleusa (MNi) u perifernim limfocitima goveda, nakon njihovog izlaganja glifozatima *in vitro*. Analizirana je blokada citokineze mikronukleusa (CBMN) u cilju procene genotiksične aktivnosti. Dobijeni rezultati ukazuju da glifozati vrlo slabo utiču na pojavljivanje mikronukleusa u perifernim limfocitima goveda. Značajno povećanje MNi ($p<0.05$) je utvrđeno pri koncentraciji glifozata od 289 μM i 560 μM. Pri ovom tretmanu govođih limfocita nije utvrđen dozno-zavisni efekat na indukciju pojave mikronukleusa. Podaci o citotoksičnosti govore da se CPBI ne odražava na redukciju stepena ćelijske proliferacije.

Takođe je ispitivan i uticaj metaboličke aktivacije na genotoksičnu aktivnost glifozata. Kada se kultura limfocita tretira istovremeno sa glifozatom i frakcijom membrane jetre (S9) iz jetre pacova, tretirane Aroklorom 1245, ne dolazi do statistički značajnog povećanja broja mikronukleusa u ćelijama sa dva jedra.

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# Biomonitoring of Genotoxic Risk in Agricultural Workers from Five Colombian Regions: Association to Occupational Exposure to Glyphosate

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**In order to assess possible human effects associated with glyphosate formulations used in the Colombian aerial spray program for control of illicit crops, a cytogenetic biomonitoring study was carried out in subjects from five Colombian regions, characterized by different exposure to glyphosate and other pesticides. Women of reproductive age (137 persons 15–49 yr old) and their spouses (137 persons) were interviewed to obtain data on current health status, history, lifestyle, including past and current occupational exposure to pesticides, and factors including those known to be associated with increased frequency of micronuclei (MN). In regions where glyphosate was being sprayed, blood samples were taken prior to spraying (indicative of baseline exposure), 5 d after spraying, and 4 mo after spraying. Lymphocytes were cultured and a cytokinesis-block micronucleus cytome assay was applied to evaluate chromosomal damage and cytotoxicity. Compared with Santa Marta, where organic coffee is grown without pesticides, the baseline frequency of binucleated cells with micronuclei (BNMN) was significantly greater in subjects from the other four regions. The highest frequency of BNMN was in Boyacá, where no aerial eradication spraying of glyphosate was conducted, and in Valle del Cauca, where glyphosate was used for maturation of sugar cane. Region, gender, and older age (≥35 yr) were the only variables associated with the frequency of BNMN measured before spraying. A significant increase in frequency of BNMN between first and second sampling was observed in Nariño, Putumayo, and Valle immediately (<5 d) after spraying. In the post-spray sample, those who reported direct contact with the eradication spray showed a higher quantitative frequency of BNMN compared to those without glyphosate exposure. The increase in frequency of BNMN observed immediately after the glyphosate spraying was not consistent with the rates of application used in the regions and there was no association between self-reported direct contact with eradication sprays and frequency of BNMN. Four months after spraying, a statistically significant decrease in the mean frequency of BNMN compared with the second sampling was observed in Nariño, but not in Putumayo and Valle del Cauca. Overall, data suggest that genotoxic damage associated with glyphosate spraying for control of illicit crops as evidenced by MN test is small and appears to be transient. Evidence indicates that the genotoxic risk potentially associated with exposure to glyphosate in the areas where the herbicide is applied for coca and poppy eradication is low.**

Glyphosate (*N*-phosphonomethyl glycine), a nonselective herbicide, is the active ingredient of a number of herbicide formulations and one of the most widely used pesticides on a global basis (Baylis, 2000; Woodburn, 2000; Duke & Powles, 2008). It is a postemergence herbicide, effective for the control of annual, biennial, and perennial species of grasses, sedges, and broadleaf weeds. The relatively high water solubility and the ionic nature of glyphosate retard penetration through plant hydrophobic cuticular waxes. For this reason, glyphosate is commonly formulated with surfactants that decrease the surface tension of the solution and increase penetration into the tissues of plants (World Health Organization International Program on Chemical Safety, 1994; Giesy et al., 2000).

A large number of glyphosate-based formulations are registered in more than 100 countries and are available under different brand names. One of the most commonly applied glyphosate-based products is Roundup, containing glyphosate as the active ingredient (AI) and polyethoxylated tallowamine

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(POEA) as a surfactant. Glyphosate and its formulations have been extensively investigated for potential adverse effects in humans (Williams et al., 2000). This pesticide was reported to exert a low acute toxicity to different animal species. Experimental evidence showed that glyphosate did not bioaccumulate in any animal tissues (Williams et al., 2000). Chronic feeding studies in rodents did not find evidence of carcinogenic activity or any other relevant chronic effects (U.S. EPA, 1993; World Health Organization International Program on Chemical Safety, 1994).

With in vitro studies with tissue cultures or aquatic organisms, several of the formulated products are more toxic than glyphosate AI (Giesy et al., 2000; Williams et al., 2000). Differences in the response of test organisms to the AI and the commercial formulation, e.g., Roundup, are likely due to the toxicity of different formulants and surfactants contained in commercial products. There is a general agreement that adjuvants may be more toxic for animals than glyphosate itself (Giesy et al., 2000; Williams et al., 2000; Richard et al., 2005). Cytotoxicity of the commercial formulation Roundup to human peripheral mononuclear cells was 30-fold higher ($LC_{50}$ = 56 mg/L) than for the AI ($LC_{50}$ = 1640 mg/L) (Martinez et al., 2007). Several in vitro and in vivo studies with parallel testing of glyphosate AI and Roundup showed that only the commercial formulation was genotoxic (Rank et al., 1993; Bolognesi et al., 1997b; Gebel et al., 1997; Grisolia 2002). Cytotoxic and genotoxic effects were observed with Roundup and other formulations of glyphosate, but not with glyphosate AI alone in comparative studies involving different experimental systems (Peluso et al., 1998; Richard et al., 2005; Dimitrov et al., 2006). The observed differences were attributed to some ingredients of Roundup, mainly surfactants, and/or to a synergic effect of glyphosate and components of the formulation (Sirisattha et al., 2004; Peixoto 2005).

Epidemiological studies generally showed no consistent or strong relationships between human exposure to glyphosate or glyphosate-containing products and health outcomes in human populations. No statistically significant association in humans was found with spontaneous abortion, fetal deaths, preterm birth, neural tube defects (Rull et al., 2006), and cancer incidence overall, although a suggested association between cumulative exposure to glyphosate and the risk of multiple myeloma was reported (De Roos et al., 2005).The epidemiologic evidence is insufficient to verify a cause–effect relationship for childhood cancer (Wigle et al., 2008). Four case-control studies suggested an association between reported glyphosate use and the risk of non-Hodgkin's lymphoma (NHL) in age groups from 20 to 70 yr (Hardell & Eriksson, 1999; McDuffie et al., 2001; Hardell et al., 2002; De Roos et al., 2003; Eriksson et al., 2008).

Glyphosate AI and Roundup were extensively tested for genotoxicity in a wide range of in vitro and in vivo systems evaluating different genetic endpoints (gene mutation, chromosome mutation, DNA damage and repair) using bacteria and mammalian somatic cells (Williams et al., 2000). The active ingredient did not induce any relevant genotoxic effects such as gene mutations in a variety of in vitro bacterial assays including the *Salmonella typhimurium* reversion assay, with and without metabolic activation (Wildeman & Nazar 1982; Moriya et al., 1983; Li & Long, 1988) and *Escherichia coli* WP-2 (Moriya et al., 1983; Li & Long, 1988). The active ingredient was also negative in the Chinese hamster ovary cell HGPRT gene mutation assay and in primary hepatocyte DNA repair assay (Li & Long, 1988). The genotoxic potential of the formulation Roundup was investigated in a number of studies evaluating various genetic endpoints in different biological systems and was (1) negative in the *S. typhimurium* reversion assay (Kier et al., 1997), (2) negative in the sex-linked recessive lethal assay with *Drosophila melanogaster* (Gopalan & Njagi, 1981), and (3) negative for in vivo micronucleus (MN) induction in mouse bone marrow (Rank et al., 1993; Kier et al., 1997; Dimitrov et al., 2006). The Roundup formulation was reported in a number of studies to exert weak genotoxic effects in short-term assays.

Differences in the response of test organisms to the active ingredient glyphosate and the commercial formulation Roundup might be due to the toxicity of different co-formulants and surfactants contained in commercial products. Several studies with parallel testing of glyphosate and Roundup showed that only the commercial formulation was genotoxic (Rank et al., 1993; Bolognesi et al., 1997b; Gebel et al., 1997; Grisolia 2002). A recent study on the genotoxic potential of glyphosate formulations found that in some cases the genotoxic effects were obtained under exposure conditions that are not relevant for humans (Heydens et al., 2008).

An in vitro study described a concentration-dependent increase of DNA single-strand breaks (SSB), evaluated by comet assay, in two different human cell lines treated with glyphosate at sublethal concentrations (Monroy et al., 2005). Roundup formulations were shown to affect the cell cycle by inhibiting the G2/M transition and DNA synthesis leading to a genomic instability (Marc et al., 2004a, 2004b). Evidence of DNA damage in peripheral lymphocytes from a small group of subjects potentially exposed to glyphosate was reported in a recent paper (Paz-y-Miño et al., 2007). The number of subjects (21 control and 24 exposed) was small and there were 23 females and only 1 male in the exposed group, making interpretation of the results difficult.

Frequency of MN in human lymphocytes has been widely used for biomonitoring exposure to pesticides (Bolognesi, 2003; Costa et al., 2006; Montero et al., 2006). The MN test, an index of chromosomal damage, is one of the most appropriate biomarkers for monitoring a cumulative exposure to genotoxic agents. Chromosomal damage, as a result of inefficient or incorrect DNA repair, is expressed during the cell

division and represents an index of accumulated genotoxic effects. The cytokinesis-block micronucleus (CBMN) methodology (Fenech & Morley, 1985) allows a distinction to be made between a mononucleated cell that did not divide and a binucleated cell that has divided once, expressing any genomic damage associated to recent exposure. The test in its comprehensive application, as was proposed by Fenech (2007) including a set of markers of gene amplification, cellular necrosis, and apoptosis, allows evaluation of genotoxic and cytotoxic effects induced by exposure to a genotoxic agent.

Colombia's anti-drugs strategy includes a number of measures ranging from aerial spraying of a mixture of a commercial formulation of glyphosate (Glyphos) and an adjuvant, Cosmo-Flux (Solomon et al., 2007b), to manual eradication, including alternative development and crop substitution programs (UNODC, 2007). In order to assess the potential genotoxic risk associated with the aerial spraying program with the glyphosate mixture, a cytogenetic biomonitoring study was carried out in subjects from five Colombian regions, characterized by different exposure to glyphosate formulations and other pesticides.



## MATERIALS AND METHODS

The study was carried out in five regions of Colombia, with different potential exposure to glyphosate as reported by Sanin et al. (2009). Briefly, the characteristics of the study areas are described here:

Sierra Nevada de Santa Marta—where organic coffee is grown without use of pesticides.

Boyacá—an area of illicit crops, where manual eradication is performed and the use of pesticides and other chemical agents is common.

Putumayo and Nariño—where aerial spraying of glyphosate is performed for coca and poppy eradication. The aerial application rate for eradication of coca is 3.69 kg glyphosate a.e. (acid equivalents)/ha (Solomon et al., 2007b). In order to maximize penetration and effectiveness of the spray formulation, Glyphos is tank-mixed with an adjuvant (Cosmo-Flux® 411F; Cosmoagro, Bogotá).

Valle del Cauca—where glyphosate is applied through aerial spraying for sugar cane maturation. Roundup 747 is the most commonly used product and is applied at a rate of 1 kg a.e./ha, and has no additional adjuvant (personal communication, ASOCAÑA, the Colombian Association for Sugar Growers, December 2008).

### Study Population

Two hundred and seventy-four individuals were included in the study. The objective was to sample 30 couples of reproductive age in each area and, where possible, the same couples in the study conducted by Sanin et al. (2009) were sampled. In Putumayo, Nariño, and Valle del Cauca, the population was selected based on the scheduled aerial spraying of glyphosate. This schedule was confidential and provided exclusively for the purpose of the study by the Antinarcotics Police (Putumayo and Nariño) or ASOCAÑA (Valle del Cauca). In Valle del Cauca, a sample size of 30 couples could not be achieved because spraying was not carried out in populated areas of the study region. Most spraying during the study period was carried out on sugar cane crops where no inhabitants were found. All reported areas to be sprayed in Valle del Cauca were visited to search for couples; however, only 14 could be included.

In Sierra Nevada de Santa Marta and Boyacá, the same areas investigated in a previous study (Sanin et al., 2009) were identified, although, due to the instability of the population and high migration, most couples from the previous study were not located. In all regions, the same strategy as described before (Sanin et al., 2009) was followed, visiting household by household until completing 30 couples who fulfilled the inclusion criteria, women of reproductive age (15–49 yr of age) and their spouses, who voluntarily accepted to participate in the study.

### Field Data Collection

Field data collection was carried out between October 2006 and December 2007. Epidemiologists and interviewers in the five regions who participated in the Sanin et al. (2009) study were informed about the objectives of the study and trained for data collection. The Ethical Committee of Fundacion Santa Fe de Bogotá approved the study protocol and the informed consent forms used for the study. All the subjects were informed about the aims of the study. All of them gave their informed consent and volunteered to donate blood for sampling. They did not self-report illness at the time of blood sampling and interviews. Every volunteer was interviewed with a standardized questionnaire, designed to obtain relevant details about the current health status, history, and lifestyle. This included information about possible confounding factors for chromosomal damage: smoking, use of medicinal products, severe infections or viral diseases during the last 6 mo, recent vaccinations, presence of known indoor/outdoor pollutants, exposure to diagnostic x-rays, and previous radio- or chemotherapy. A simplified food frequency questionnaire that had already been used in other regions of Colombia was also applied, in order to evaluate dietary folic acid intake. Folic acid intake was characterized because of the role of folic acid deficiency in baseline genetic damage in human lymphocytes (Fenech & Rinaldi, 1994). Specific information about exposure at the time of aerial spraying in Putumayo, Nariño, and Valle del Cauca was addressed in the questionnaire.

### Blood Sampling and Cell Culture

Blood samples were collected twice in Boyacá, at the beginning of the study and 1 mo after the first survey, and at 3 different times in Nariño, Putumayo, and Valle del Cauca: immediately before spraying, within 5 d after spraying, and 4 mo later. A sample of 10 ml whole blood was collected from each subject, by venipuncture, using heparinized Vacutainer tubes kept at room temperature and sent within 24 h for the establishment of the lymphocyte cultures. The samples were coded before culturing. The modified cytokinesis-blocked method of Fenech and Morley (1985) was used to determine frequency of MN in lymphocytes. Whole blood cultures were set up for cytogenetic analysis in Bogotá (Colombia) by personnel specifically trained by cytogeneticists from Environmental Carcinogenesis Unit of the National Cancer Research Institute (Genoa, Italy).

Three sterile cultures of lymphocytes were prepared. A 0.4-ml aliquot of whole blood was incubated at 37°C in duplicate in 4.6 ml RPMI 1640 (Life Technologies, Milano, Italy) supplemented with 10% fetal bovine serum (Gibco BRL, Life Technologies SrL, Milano, Italy), 1.5% phytohemoagglutinin (Murex Biotech, Dartford, UK), 100 units/ml penicillin, and 100 µg/ml streptomycin. After 44 h, cytochalasin B (Sigma, Milano, Italy) was added at a concentration of 6 µg/ml. At the end of incubation at 37°C for 72 h, cells were centrifuged (800 × g, 10 min), then treated with 5 ml of 0.075 m*M* KCl for 3 min at room temperature to lyse erythrocytes. The samples were then treated with pre-fixative (methanol:acetic acid 3:1) and centrifuged . The cellular pellets were resuspended in 1 ml methanol. At this step the samples were sent to the Environmental Carcinogenesis Unit (National Cancer Research Institute, Genoa, Italy). All the samples were centrifuged in methanol. Treatment with fixative (methanol:acetic acid, 5:1) followed by centrifugation was repeated twice for 20 min. Lymphocytes in fresh fixative were dropped onto clean iced slides, air-dried, and stained in 2% Giemsa (Sigma, Milano, Italy). MN analysis was performed blind only on lymphocytes with preserved cytoplasm. On average, 2000 cells were analyzed for each subject. Cells were scored cytologically using the cytome approach to evaluate viability status (necrosis, apoptosis), mitotic status (mononucleated, binucleated, multinucleated) and chromosomal damage or instability status (presence of micronuclei, nucleoplasmic bridges, nucleoplasmic buds) (Fenech 2007). The proliferation index (PI) was calculated as follows:

$$\begin{aligned} PI = &(\text{number of mononucleated cells} + 2 \\ &\times \text{number of binucleated cells} + 3 \\ &\times \text{number of polynucleated cells})/ \text{total number of cells.} \end{aligned}$$

### Statistical Analysis

Continuous variables were characterized using mean and standard deviation, while categorical variables were expressed as proportions. Dependent variables, micronuclei per binucleated cell (BNMN), and differences in MN between sampling were square-root transformed where required to comply with the required assumptions of normal distribution and equal variances. Comparison of MN between areas was made by one-way analysis of variance (ANOVA). A significance level at 5% was used to assess differences among areas. For multiple comparisons, the Bonferroni test was applied ($\alpha = .05$). Significance of differences in frequency of BNMN between first and second, and second and third sampling were tested by the unpaired *t*-test with equal variances. Difference and 95% confidence interval were used to compare between samplings.

Bivariate analysis between dependent variables and putative risk factors was performed by one-way ANOVA, comparing exposed and nonexposed subjects. In cases where risk factor was continuous, such as age, folic acid intake, alcohol consumption, and coffee consumption, the correlation coefficient was used.

A multiple linear regression was conducted to assess association with BNMN at the first sampling with different variables: region, age (as continuous variable as well as categorical age), ethnicity as a dichotomous variable, exposure to genotoxic products as defined earlier, gender (female vs. male), and intake of folic acid (categorized in quartiles). Regression analysis was conducted with transformed variables, with square root transformation of BNMN and natural logarithm of age, to obtain a normal distribution.

## RESULTS

Demographic characteristics and habits of the study groups are described in Table 1. The study population comprised 274 subjects (137 female and 137 male; average age 30.4 ± 7.8 yr). The mean age of the subjects was similar in the different regions. A large part of the studied population was mestizo, with the exception of the Nariño area consisting of individuals of African origin. In the total population, 38% of interviewees had not completed primary education. Putumayo had the largest proportion with education and Valle del Cauca the lowest as shown in Table 1. Only 10% of all subjects were smokers, (20% in Putumayo); a large majority of subjects were drinkers of beer or liquor with a consistent consumption of guarapo (traditional alcoholic beverage prepared by fermentation of maize) in Santa Marta and Boyacá. No statistically significant differences of folic acid intake were observed between different regions (the mean values ranged from 750 and 1189 µg/wk).

One hundred and nine (39.8%) of 274 participants reported current use of pesticides in their occupation or other activities. Nariño (76.6%) and Putumayo (61.7%) were the two regions where prevalence of use of genotoxic pesticides was higher; Boyacá (24.2%) and Valle del Cauca (28.6%) reported lower use. None of the study subjects in Santa Marta reported use of pesticides. No data regarding quantity of pesticide used were available. Fifty (18.3%) out of 273 who gave information

**TABLE 1**
Demographic Characteristics and Possible Confounding Exposures in the Study Populations

| Area | Santa Marta | Boyacá | Putumayo | Nariño | Valle del Cauca |
|---|---|---|---|---|---|
| Number of subjects | 60 | 62 | 60 | 64 | 28 |
| Age (mean (SD)) | 27.0 (5.6) | 29.1 (8.8) | 31.4 (7.2) | 32.5 (7.4) | 33.4 (8.7) |
| Ethnicity (%) | | | | | |
| Mestizo | 100 | 100 | 88.3 | 3.1 | 60.7 |
| African | | | 6.7 | 96.9 | 39.3 |
| Indian | | | 5.0 | | |
| Education (%) | | | | | |
| None | | 4.8 | 1.7 | | |
| Primary incomplete | 26.7 | 38.7 | 53.3 | 42.2 | 21.4 |
| Primary complete | 21.7 | 29.0 | 20.0 | 23.4 | 32.1 |
| High school incomplete | 25.0 | 8.1 | 20.0 | 25.0 | 28.6 |
| High school complete | 26.7 | 19.4 | 3.3 | 9.4 | 17.9 |
| Technical | | | 1.7 | | |
| Occupation (%) | | | | | |
| Agriculture | 10.0 | 41.9 | 60.0 | 62.5 | 7.1 |
| Housewife | 40.0 | 50.0 | 38.3 | 34.4 | 50.0 |
| Other | 50.0 | 8.1 | 1.7 | 3.1 | 42.9 |
| Health insurance (%) | | | | | |
| Uninsured | 50.0 | 9.7 | 36.7 | 71.9 | 7.1 |
| Subsidized | 38.3 | 83.9 | 60.0 | 18.7 | 50.0 |
| Insured | 11.7 | 6.4 | 3.3 | 9.4 | 42.9 |
| Coffee consumption (cups/day) | | | | | |
| Mean (SD) | 1.8 (2.3) | 1.7 (0.8) | 2.3 (4.1) | 1.3 (0.4) | 1.7 (1.2) |
| Percent of population | 80.0 | 67.7 | 88.3 | 76.6 | 82.1 |
| Smoking (%) | | | | | |
| Nonsmokers | 91.7 | 95.2 | 80.0 | 87.5 | 92.9 |
| Alcohol (%) | | | | | |
| Liquor | 28.3 | 25.8 | 53.3 | 78.1 | 78.6 |
| Beer | 51.6 | 67.7 | 63.1 | 82.8 | 64.3 |
| Guarapo | 6.7 | 59.7 | 1.7 | 3.2 | 10.7 |
| Users of illicit drugs (%) | 6.7 | 0 | 5.0 | 7.8 | 0 |
| Diet | | | | | |
| Folic acid intake (μg/wk) | 1189 | 873 | 750 | 1160 | 812 |

about x-ray examination reported to having been exposed at some time; however, only 21 out of 46 who gave information on dates of x-ray reported exposure in the last 6 mo before the interview and first blood sample. Sixty-one percent of population reported viral infections, the highest prevalence in Nariño (89.5%) and the lowest in Putumayo (49.2%). However, 89.3% of viral infections were the common cold and 6.1% dengue fever. Hepatitis was reported by six interviewees without any specification of the type of the infection.

The means and standard deviations of frequency of MN and related parameters according to regions are shown in Table 2 and presented graphically in Figure 1. Compared with Santa Marta, where people grow organic coffee without the use of pesticides and which is considered as a reference area, the baseline frequency of BNMN was significantly greater in subjects from the other four regions. The highest frequency of BNMN was in Boyacá, where no aerial eradication spraying of glyphosate was carried out, and Valle del Cauca, where aerial spraying was for maturation of sugar cane. There was no significant difference between mean frequency of BNMN in Boyacá and Valle del Cauca. There was no significant difference in frequency of BNMN between Putumayo and Nariño,

**TABLE 2**

Mean (SD) Frequency of Binucleated Cells with Micronuclei (BNMN), Total Micronuclei (MNL) per 1000 Binucleated Peripheral Lymphocytes, Frequency of Mononucleated Cells per 1000 Lymphocytes (MNMO), and Proliferation Index (PI) by Region before the Exposure (Phase 1), 5 d after Spraying (Phase 2) and 4 mo Later (Phase 3)

| Region | Santa Marta | Boyacá | Putumayo | Nariño | Valle del Cauca |
|---|---|---|---|---|---|
| Phase 1 | | | | | |
| Number of subjects | 60 | 62 | 58 | 63 | 28 |
| BNMN | 1.83 (0.97) | 5.64 (1.72) | 3.61 (1.51) | 4.12 (1.65) | 5.75 (2.48) |
| MNL | 1.97 (1.05) | 6.16 (1.91) | 3.90 (1.66) | 4.36 (1.85) | 6.02 (2.50) |
| MNMO | 0.41 (0.44) | 0.99 (0.64) | 0.47 (0.51) | 0.51 (0.39) | 1.12 (0.88) |
| PI | 1.54 (0.14) | 1.45 (0.14) | 1.68 (0.15) | 1.47 (0.12) | 1.51 (0.15) |
| Phase 2 | | | | | |
| Number of subjects | ND | 55 | 53 | 55 | 27 |
| BNMN | | 4.96 (2.00) | 4.64 (2.45) | 5.98 (2.03) | 8.64 (2.81) |
| MNL | | 5.41 (2.25) | 5.02 (2.95) | 6.35 (2.18) | 8.98 (2.93) |
| MNMO | | 0.87 (0.65) | 0.44 (0.46) | 0.70 (0.45) | 1.65 (0.62) |
| PI | | 1.72 (0.14) | 1.66 (0.20) | 1.40 (0.18) | 1.51 (0.14) |
| Phase 3 | | | | | |
| Number of subjects | ND | ND | 50 | 56 | 26 |
| BNMN | | | 5.61(3.08) | 3.91 (1.99) | 7.38 (2.41) |
| MNL | | | 5.96 (3.23) | 4.13 (2.20) | 8.17 (2.72) |
| MNMO | | | 0.82 (0.54) | 0.55 (0.42) | 0.98 (0.60) |
| PI | | | 1.43 (0.17) | 1.41 (0.14) | 1.45 (0.20) |



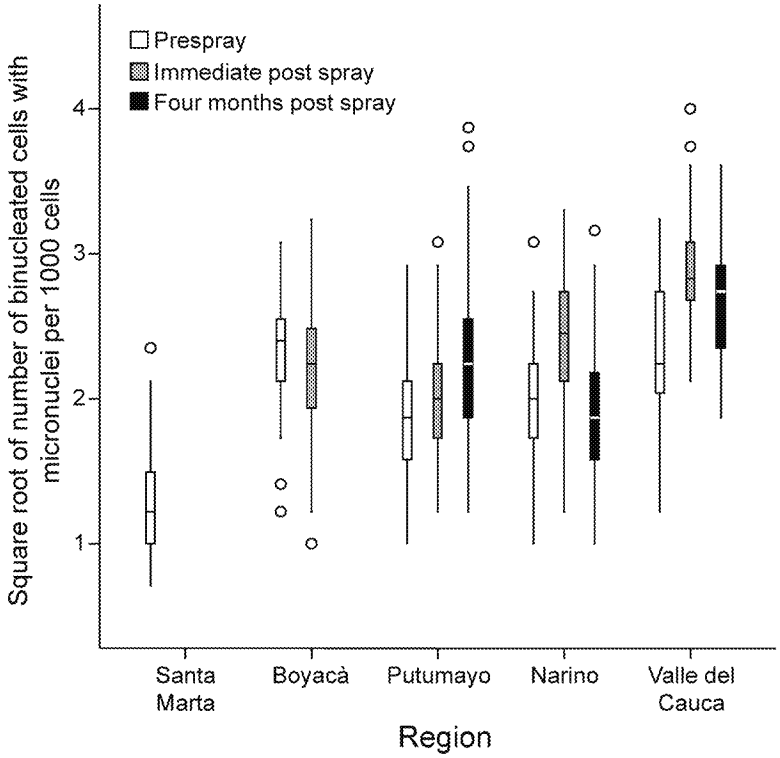


**FIG. 1.** Box plot of frequency of BNMN in the five study regions with samples taken prespray, 4–5 d post-spray, and 4 mo post-spray. Box plots: The center horizontal line marks the median of the sample. The length of each box shows the range within which the central 50% of the values fall, with the top and bottom of the box at the first and third quartiles. The vertical T-lines represent intervals in which 90% of the values fall. The O symbols show outliers. See text for description of statistically significant differences.

although Boyacá and Valle del Cauca showed a significantly higher frequency than Nariño and Putumayo. A higher frequency of BNMN in Boyacá was also observed in a second sampling 1 mo later.

There were differences in frequency of BNMN between sampling periods. A statistically significant difference in frequency of BNMN between first and second sampling was observed in Valle, Putumayo, and Nariño immediately (<5 d) after spraying. Four months after spraying in Nariño, there was a statistically significant decrease in the mean frequency of BNMN compared with the second sampling, but in Valle del Cauca the decrease was not significant nor was the increase observed in Putumayo significant (Figure 1 and Table 2).

The frequency of mononucleated cells with micronuclei (MOMN) was used as an index of background level of chromosomal damage accumulated in vivo (Table 2). The lowest frequency of MOMN for the first sampling was observed in Santa Marta; however, there was no marked difference in frequency of MOMN in Santa Marta, Putumayo, and Nariño and no statistically significant difference between Valle and Boyacá. However, Valle and Boyacá had a significantly higher frequency of MOMN than Putumayo, Nariño, and Santa Marta at first sampling. Immediately after spraying, Valle showed a significantly higher frequency of MOMN compared to Putumayo and Nariño, and Nariño was also higher than Putumayo. Between first and second sampling, the increase in frequency of MOMN in Nariño and Valle was statistically significant, but there was no difference in Putumayo nor in Boyacá 4 mo after the first sampling. Data suggest greater exposure to genotoxic agents in these populations is independent of the exposure to glyphosate products.

The proliferation index (PI) in all the studied groups was in the range of normal values described in the literature. No significant reduction of PI was observed in association with environmental exposures in groups of subjects from the different regions. A statistically significant correlation coefficient (0.288) between PI values from the first and the second samplings was observed, confirming the association with individual characteristics and not with any toxicity related to the exposure or to the culture techniques. Due to the low frequency observed, data with respect to other nuclear alterations, including in cytome analysis (Fenech, 2007), are not described in Table 2: the mean frequency of nucleoplasmic bridges (NPB) for all subjects was 0.010 per 1000 cells, that of nuclear buds was 0.022 per 1000 cells, and only rare necrotic and apoptotic cells were found in some samples.

Gender was the most important demographic variable affecting the BNMN index. Frequencies of BNMN in females were greater than those in males (mean 4.43 ± 2.36 vs. 3.61 ± 1.82, respectively, in total population) (Table 3). The groups of subjects were evenly matched for gender by including only couples in the study. No association was found between frequency of MN and age as a categorical variable, nor was there an association with smoking, but prevalence of smoking was low (~10% in the total population). A higher baseline frequency of MN was observed in subjects of African origin, suggesting greater susceptibility. Other lifestyle factors such as alcohol, coffee consumption, or illicit drug intake were not associated with initial measures of BNMN and MOMN.

One hundred and thirty-four of the 152 subjects in Nariño, Putumayo, and Valle reported information on contact with Glyphos and Cosmo-Flux after eradication spraying. The other 18 did not provide information in the second survey or blood samples were inadequate for testing micronuclei. Sixty-six (49.2.0%) reported no contact with the spray and 68 (50.8%) reported coming into contact with the spray because they entered sprayed fields or reported contact with the spray droplets. The mean BNMN in Nariño and Putumayo was greater in respondents who self-reported exposure, but differences were not statistically significant (Table 4). In Valle, only one respondent reported contact with glyphosate.

Region, gender, and older age (≥35 yr) were the only variables associated with the frequency of BNMN before spraying (Table 5). In fact, using Santa Martha, where no use of pesticides was reported, as reference, Boyacá, Valle del Cauca, Putumayo, and Nariño showed a statistically significant higher mean frequency of BNMN. There were also significant differences between Boyacá and Valle and Putumayo and Nariño. Females had a statistically higher mean frequency of BNMN than males after adjusting for all other variables. Greater age was also associated with greater frequency of BNMN. Neither exposure to genotoxic products, nor ethnicity, nor intake of folic acid was associated with frequency of BMMN at the first sampling. The multiple linear regression analysis of difference between second and first sampling only demonstrated statistically significant association with region after adjusting for all other variables, indicating that Putumayo, Nariño, and Valle had significantly greater differences between second and first sampling than Boyacá.

## DISCUSSION

The main objective of this study was to test whether there was an association between aerial spraying of glyphosate and cytogenetic alterations, evaluated as frequency of MN in peripheral leukocytes. Biomonitoring was carried out in three regions of Colombia in populations exposed to aerial spraying of glyphosate: Putumayo and Nariño, where the application was performed for eradication of coca and poppy, and Valle del Cauca where the herbicide was used for maturation of sugar cane. Two control populations not exposed to aerial spraying of glyphosate were also selected: the first one from Sierra Nevada de Santa Marta, where organic coffee is grown without the use of any pesticides, and the other from Boyacá, with a region of illicit crops, where manual eradication is performed and subjects were potentially exposed to several pesticides but not glyphosate for aerial eradication. The ex vivo analysis of leukocytes in the presence of cytochalasin B, added 44 h after the

**TABLE 3**
Association of Mean (SD) Frequency of Binucleated Cells (First Sampling) with Micronuclei (BNMN/1000 Binucleated Lymphocytes) and Demographic Variables

| Variable | Santa Marta | Boyacá | Putumayo | Nariño | Valle del Cauca | Total |
|---|---|---|---|---|---|---|
| Sex | | | | | | |
| Females | 1.98 (1.03) | 6.22 (1.79) | 3.91 (1.71) | 4.57(1.77) | 6.45 (2.82) | 4.43 (2.36) |
| Males | 1.68 (0.90) | 5.06 (1.46) | 3.31 (1.25) | 3.66 (1.39) | 5.05 (1.94) | 3.61 (1.82) |
| *p* | .236 | .007 | .131 | .028 | .138 | .002 |
| Age | | | | | | |
| 18–24 yr | 2.00 (1.14) | 5.50 (1.96) | 3.32 (1.25) | 3.64 (1.72) | 6.19 (2.15) | 3.67 (2.16) |
| 25–34 yr | 1.66 (0.87) | 5.70 (1.66) | 3.53 (1.17) | 4.20 (1.77) | 4.20 (0.76) | 3.97 (2.08) |
| 35 yr and older | 1.93 (0.67) | 5.62 (1.73) | 3.84 (1.86) | 4.25 (1.52) | 6.04 (2.84) | 4.41 (2.19) |
| *p* | .438 | .929 | .574 | .564 | .313 | .093 |
| Ethnicity | | | | | | |
| Mestizo | 1.83 (0.97) | 5.64 (1.72) | 3.72 (1.52) | 4.75 (1.06) | 5.82 (2.44) | 3.94(2.24) |
| Africa and Indian | 0 | 0 | 2.86 (1.31) | 4.10 (1.66) | 5.64 (2.65) | 4.20(1.90) |
| *p* | | | .162 | .588 | .850 | .368 |
| Smoking | | | | | | |
| Yes | 2.00 (1.06) | 5.33 (0.76) | 3.31 (1.00) | 4.77 (1.51) | 4.50 (1.41) | 3.83 (1.60) |
| No | 1.82 (0.97) | 5.65 (1.76) | 3.80 (1.56) | 4.03 (1.66) | 5.90 (2.57) | 4.07 (2.20) |
| p | .693 | .756 | .395 | .233 | .459 | .592 |
| Folic acid intake (quartiles) | | | | | | |
| 1 | 1.92 (0.99) | 6.11 (1.95) | 3.23 (1.12) | 4.50 (1.75) | 5.86 (2.34) | 3.89 (2.23) |
| 2 | 1.64 (0.66) | 5.70 (1.75) | 3.47 (1.49) | 3.80 (1.47) | 5.86 (2.74) | 3.97 (2.21) |
| 3 | 1.69 (0.92) | 5.69 (1.82) | 4.00 (1.37) | 3.85 (2.04) | 6.58 (2.84) | 4.47 (2.22) |
| 4 | 1.94 (1.20) | 4.94 (1.13) | 3.69 (2.429) | 4.28 (1.51) | 4.63 (2.05) | 3.75 (1.89) |
| *p* | .779 | .399 | .515 | .645 | .612 | .220 |



**TABLE 4**
Mean Frequency of Binucleated Cells with Micronuclei (BNMN) at the Second Sampling per 1000 Binucleated Lymphocytes and Self-Reported Exposures to the Glyphosate Spray in Three Areas Where Aerial Application Had Occurred

| | Nariño (*n* = 55) | | Putumayo (*n* = 53) | | Valle del Cauca (*n* = 26) | |
|---|---|---|---|---|---|---|
| Route of exposure | *n* | Mean BNMN (SD) | *n* | Mean BNMN (SD) | *n* | Mean BNMN (SD) |
| No exposure | 28 | 5.81 (1.85) | 13 | 3.84 (1.30) | 25 | 8.56 (2.90) |
| Spray in air | 5 | 7.30 (0.57) | 1 | 5.50 (0) | | |
| Spray on skin | 8 | 5.62 (1.60) | 15 | 4.90 (1.87) | 1 | 9.50 (0) |
| Entered sprayed field | 14 | 6.06 (2.77) | 24 | 4.87 (3.18) | | |
| *p* Value (ANOVA) | | 0.472 | | 0.612 | | 0.760 |
| Any exposure | 27 | 6.16 (2.22) | 40 | 4.90 (2.69) | 1 | 9.50 (0) |
| *p* Value (no exposure vs. any exposure) | | 0.525 | | 0.181 | | 0.760 |

*Note.* The data comprise respondents in the second survey from which blood samples were obtained.

**TABLE 5**
Multiple Linear Regression Analysis Adjusted for Region, Age, Gender, Ethnicity, and Folic Acid Intake

| Variable | Coefficient | *p* | 95% CI |
|---|---|---|---|
| Region | | | |
| Boyacá | 3.75 | ≤.0001 | 3.19, 4.31 |
| Putumayo | 1.58 | ≤.0001 | 1.00, 2.16 |
| Nariño | 2.06 | ≤.0001 | 1.49, 2.64 |
| Valle del Cauca | 3.65 | ≤.0001 | 2.92, 4.39 |
| Age (yr) | | | |
| 25–34 | 0.28 | .250 | –0.20, 0.76 |
| 35 and older | 0.75 | .008 | 0.20, 1.31 |
| Gender | | | |
| Females | 1.00 | ≤.0001 | 0.60, 1.40 |

start of cultivation, made it possible to distinguish between nondividing mononucleated cells—as an index of accumulated chromosomal damage—and binucleated cells, which had completed one nuclear division during in vitro culture and expressed MN associated with recent exposure to genotoxic agents.

The baseline level of chromosomal damage, evaluated as frequency of BNMN, was associated with the different regions considered in our study. The frequency of BNMN before spraying was also associated with region, gender, and age. Gender difference in the background incidence of MN in peripheral leukocytes, with the frequency being consistently higher in females, and a strong correlation between MN frequency and increasing age are well documented (Bonassi et al., 1995, 2001; Bolognesi et al., 1997a).

Data demonstrated no significant effect of smoking, confirming findings from the literature (Bonassi et al., 2003) although prevalence of smoking in our study population was small (7–20%, Table 1). No association with alcohol consumption was observed. A higher susceptibility of people of African origin compared to the mestizo group was suggested by a greater baseline frequency of BNMN and increased frequency at the second sampling period.

There was some indication of an association between BNMN and exposure to pesticides in general. The lowest frequency of BNMN was observed in Sierra Nevada de Santa Marta, where people self-reported that they did not use pesticides. The mean frequency of BNMN in this group of subjects ($1.83 \pm 0.97$) was similar to that observed in healthy unexposed subjects for the same range of age (Bolognesi et al., personal communication). The higher mean frequency of BNMN observed in Boyacá and Valle del Cauca ($5.64 \pm 1.72$ and $5.75 \pm 2.48$, respectively) and that in Nariño and Putumayo ($4.12 \pm 1.65$ and $3.65 \pm 1.51$, respectively), compared to Santa Marta, are in agreement with similar biomonitoring studies carried out in subjects exposed to pesticides using the MN test or other genetic endpoints (Bolognesi, 2003; Bull et al., 2006).

There was no clear relationship between BNMN and the reported use of pesticides classified as genotoxic. Participants in Boyacá and Valle del Cauca showed higher frequency of BNMN than those in Putumayo and Nariño. However, a greater proportion of participants in the latter regions self-reported the use genotoxic pesticides (76.6% in Nariño and 61.7% in Putumayo). There is no information available on other relevant factors such as frequency of use, rate applied, time of exposure, and protective measures used, and we could therefore not characterize exposures to explain the differences. There were further inconsistencies; for example, in Boyacá, where more frequent use of pesticides was expected, only 24.2% of participants self-reported use, compared with the greater values in Nariño and Putumayo. However, it is possible that in areas such as Boyacá, individuals might be potentially exposed to persistent pesticides applied in the past and still present in the environment.

There was no evidence of an association between BNMN and folic acid deficiency. An assessment of folic acid intake from the semiquantitative food frequency questionnaire showed that, according to accepted recommendations (Herbert, 1987), the diet of the study populations was not deficient in folic acid and there were only small differences between regions. Consistent with these data, no association was found between MN and folic acid intake, either as a continuous variable or by quartiles.

The frequency of BNMN increased after spraying with glyphosate but not consistently. The results obtained with a second sampling, carried out immediately after the glyphosate spraying, showed a statistically significant increase in frequency of BNMN in the three regions where glyphosate was sprayed. However, this was not consistent with the rates of application use in the regions. The increase in frequency of BNMN in Valle (application rate = 1 kg a.e. glyphosate/ha) was greater than that in Nariño and Putumayo (3.69 kg a.e. glyphosate/ha).

There was no significant association between self-reported direct contact with eradication sprays and frequency of BNMN. The frequency of BNMN in participants who self-reported that they were exposed to glyphosate because they entered the field immediately after spraying (to pick the coca leaves), felt spray drops in their skin, or they thought they were exposed because they had contact with the chemical in the air, was not significantly greater than in subjects living in the same areas but who were not present during spraying. Decreases in frequency of BNMN in the recovery period after glyphosate spraying were not consistent. The third sampling, 4 mo after spraying, demonstrated a statistically significant decrease in frequency of BNMN only in Nariño.

Overall, these results suggest that genotoxic damage associated with glyphosate spraying, as evidenced by the MN test, is small and appears to be transient. The frequencies of BNMN in Nariño and Putumayo during the second and the third sampling fell within the range of values observed in Boyacá, an area

where people were exposed to a complex mixture of different pesticides (including glyphosate). A greater increase in frequency of BNMN was observed in Valle del Cauca, but it cannot be attributed only to the glyphosate exposure, because the application rate of the herbicide in this area was one-third compared with that in Nariño and Putumayo. This conclusion is further supported by the frequency of MN in mononucleated cells (MOMN), which provides an indication of the background level of chromosome/genome mutations accumulated in vivo (Manteuca et al., 2006). A statistically significant increase of MOMN was observed in Boyacá and Valle del Cauca before and after the aerial spraying, suggesting exposure to other genotoxic compounds in these populations was independent of the exposure to glyphosate. Evidence indicates that the genotoxic risk potentially associated with exposure to glyphosate in the areas where the herbicide is applied for eradication of coca and poppy is of low biological relevance. One of the strengths of our study was the detection of a transient chromosomal damage, evaluated as MN frequency in peripheral blood of the exposed subjects, since it was possible to compare the baseline before spraying with the effects detected immediately after spraying. Glyphosate persists in the environment for only a short time (half-life for biological availability in soil and sediments is hours, and 1-3 d in water; Giesy et al., 2000), is rapidly excreted by mammals and other vertebrates (Williams et al., 2000; Acquavella et al., 2004) and chronic effects, if any, would not be expected.

One of the major drawbacks of environmental epidemiology studies is the characterization of exposures to the agents being investigated. In this study two approaches were used to characterize exposures to glyphosate: ecological and self-reported. In the ecological study design, frequency of BNMN in participants was compared from regions with different patterns of pesticide use. As previously discussed (Sanin et al., 2009), this ecological design may result in misclassification of exposures (Arbuckle et al., 2004), but as an exploratory assessment of exposure it is useful (Ritter et al., 2006).

Others have attempted to improve assessment of exposure to pesticides in epidemiological studies. One study used a self-administered questionnaire for the assessment of exposure to glyphosate, which was defined as (a) ever personally mixed or applied products containing glyphosate; (b) cumulative lifetime days of use, or "cumulative exposure days" (years of use times days/year); and (c) intensity-weighted cumulative exposure days (years of use times days/year times estimated intensity level) (De Roos et al., 2005). A pesticide exposure score based on self-reported work practices was recently developed to estimate annual exposure level (Firth et al., 2007). Based on an algorithm to estimate lifetime exposure to glyphosate from questionnaire information, a moderate correlation was found with concentrations of glyphosate in urine and no significant correlation with self-reported exposure (Acquavella et al., 2004).

In our study, questions related to whether there was direct contact with the spray were used but this did not consider area of skin exposed, region of skin exposed, differences in rates of penetration, or personal hygiene.

Given the situation, the best approach possible, a prospective cohort, was used but the need to use better procedures to estimate the exposure is acknowledged. Based on the applicable Bradford–Hill guidelines (Hill, 1965), it is not possible to assign causality to the increases in frequency of BNMN observed in our study. There was a smaller frequency of BNMN and MOMN in the region of no pesticide use compared with the regions where pesticides (including glyphosate) were used, which is consistent with other reports in the literature. Although temporality was satisfied in the increase in frequency of BNMN after spraying, this response did not show strength as it was not consistently correlated with the rate of application. Recovery was also inconsistent with decreases in frequency of BNMN in the areas of eradication spraying but not in the area where lower rates were applied on sugar cane.

Further studies are needed to better characterize the potential genotoxic risk associated with the application of glyphosate for sugar cane maturation. The smaller number of subjects recruited in this study and small amount of information about the exposure precluded any conclusions. Many pesticides are used in conventional agriculture in Colombia and many pesticides are used in the production of coca (Solomon et al., 2007a, 2007b); however, there is not sufficient information to correlate the frequency of MN to the pesticide exposure.

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