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# Genotoxicity of glyphosate assessed by the comet assay and cytogenetic tests

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ABSTRACT

It was evaluated the genotoxicity of glyphosate which up to now has heterogeneous results. The comet assay was performed in Hep-2 cells. The level of DNA damage in the control group (5.42 ± 1.83 arbitrary units) for tail moment (TM) measurements has shown a significant increase ($p < 0.01$) with glyphosate at a range concentration from 3.00 to 7.50 mM. In the chromosome aberrations (CA) test in human lymphocytes the herbicide (0.20–6.00 mM) showed no significant effects in comparison with the control group. In vivo, the micronucleus test (MNT) was evaluated in mice at three doses rendering statistical significant increases at 400 mg/kg (13.0 ± 3.08 micronucleated erythrocytes/1000 cells, $p < 0.01$). In the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400 mg/kg in mice. Thiobarbituric acid reactive substances (TBARs) levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in their organs. The results showed an increase in these enzyme activities.
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## 1. Introduction

Glyphosate is a widely used, broad-spectrum, foliar-applied herbicide for vegetation control, introduced in the early 1970s. Today, glyphosate and several of its commercial presentations are registered in more than 100 countries (Williams et al., 2000). Glyphosate is more mobile and persistent in aquatic environments than earlier researches have indicated (Kolpin et al., 2006). Reviews on the safety of glyphosate, and Roundup herbicide have been conducted by several regulatory agencies and have concluded that there is no indication of any human health concern (EPA, 1993; WHO, 1994; Williams et al., 2000). However, recent researches indicate that this herbicide may not be as safe as previously thought (Isenring, 2004; Çağlar and Kolankaya, 2008). Other authors suggested a connection between aerial spraying with glyphosate added to a surfactant solution and genetic alterations in a population from the Ecuadorian Colombian frontier (Paz-y-Miño et al., 2007), and cancer incidence among glyphosate-exposed pesticide users (De Roos et al., 2005). In spite of the extensive use of this herbicide there is no clear definition regarding the subject of its genotoxic capacity; the literature shows contradictory results.

The single cell gel electrophoresis (comet) assay and the chromosome aberrations (CA) assay were used to evaluate primary DNA damage and chromosome breakage, respectively. The comet assay is a technically simple and fast method which detects in vitro and in vivo genotoxicity in any cell type (Moller, 2006). Studies analyzing CA have shown great accuracy in predicting cancer risk, since there is a connection between CA level and some types of cancer (Boffetta et al., 2006). On the other hand, the micronucleus test (MNT) is required by regulatory agencies (OECD, 1997b) and has emerged as one of the preferred in vivo methods for the assessment of chromosome damage.

The purpose of this study was to provide knowledge in order to clarify the contradictory data about in vitro and in vivo genotoxicity information of glyphosate. The comet assay in Hep-2 cells and the CA test in human peripheral lymphocytes were performed in vitro. The micronuclei formation after intraperitoneal injection was evaluated in mice. An increase in micronuclei level was obtained with the highest dose of glyphosate used, so oxidant markers were evaluated in different organs in terms of thiobarbituric acid reactive substances (TBARs) and by enzymatic activities of superoxide dismutase (SOD) and catalase (CAT) as possible genotoxicity mechanisms.

## 2. Materials and methods

### 2.1. Chemicals

Analytical grade glyphosate [*N*-(phosphonomethyl) glycine], CAS 1071-83-6 (96%), and Mitomycin C (MMC), CAS 50-07-7, were purchased from Sigma–Aldrich, Argentina. Cyclophosphamide was purchased from Laboratorio Filaxis, Argentina.

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**Table 1**
DNA damage level detected in Hep-2 cells exposed to different concentrations of glyphosate using the comet assay.

| Treatment | Tail mean intensity, mean ± SEM (arbitrary units) | Tail length, mean ± SEM (arbitrary units) | Tail moment, mean ± SEM (arbitrary units) |
|---|---|---|---|
| Control | 3.95 ± 0.53 | 6.07 ± 1.05 | 5.42 ± 1.83 |
| Mitomycin C 0.01 mM | 51.80 ± 5.48** | 117.10 ± 7.47** | 292.30 ± 35.10** |
| Glyphosate | | | |
| 3.00 mM | 68.73 ± 6.70** | 183.60 ± 12.26** | 624.90 ± 70.22** |
| 4.50 mM | 109.60 ± 11.09** | 217.50 ± 13.24** | 803.60 ± 75.02** |
| 6.00 mM | 127.90 ± 13.54** | 220.30 ± 21.16** | 740.70 ± 95.44** |
| 7.50 mM | 109.30 ± 12.71** | 223.40 ± 12.80** | 963.20 ± 88.01** |

SEM: standard error mean.
** $p < 0.01$ Dunnett's test.

Eagle's minimum essential medium with Earle's balanced salt solutions (EMEM), L-glutamine, fetal bovine serum, antibiotics, phytohaemagglutinin (Gibco), RPMI with hepes (Gibco) and Colcemid (Gibco) were purchased from Invitrogen Argentina, Argentina. Normal melting point agarose (NMP) and low melting point agarose (LMP) were purchased from Promega, Argentina. All other chemicals and solvents were of analytical grade.

### 2.2. *Chromosome aberrations test*

Heparinized human blood samples were obtained from six healthy donors, three females and three males, from 18 to 33 years old. The donors had no history of pesticide exposure and were non-smokers. Lymphocytes were cultured for 72 h at 37 °C according to conventional methods (Moorhead et al., 1960). Glyphosate 0.20, 1.20 or 6.00 mM concentrations were added during the last 48 h of culture. The herbicide was used previously dissolved in 1 ml of culture medium and adjusted to pH 7.2–7.4. Mitomycin C (0.89 μM) was used as a known CA inductor (OECD, 1997a). Control untreated cultures were established as well. Two thousand cells/culture were examined for mitotic index (MI), calculated as the number of dividing cells/2000 cells. One hundred metaphases were analyzed for number and type of CA and classified according to the International System of Cytogenetic Nomenclature (ISCN, 1985). Chromatid breaks (ctb) and gaps (ctg), chromosome breaks (csb) and gaps (csg), dicentric chromosomes (dic), acentric fragments (ace) and endoreduplicated (end) cells were considered. The slides were scored blind by two observers.

### 2.3. *Single cell gel electrophoresis assay (comet assay)*

Cell line Hep-2 maintained in EMEM, supplemented with 200 mM L-glutamine, 2% (v/v) fetal calf serum, and antibiotics were used in this study. Cells were settled in a 96-well tissue culture plate at $8.25 \times 10^5$ cells/ml with glyphosate at concentrations of 3.00, 4.50, 6.00, 7.50, 9.00, 12.0 and 15.0 mM. In all cases the cultures were diluted to 200 μl final volume/well and carried out in duplicate. Positive (Mitomycin C 0.01 mM) and negative (only MEM medium) controls were included. In preliminary experiments, a 4 h exposure to glyphosate was chosen for this assay, because in this condition in 70% of the concentrations tested the viability of Hep-2 cells never decreases below 80%, evaluated through trypan blue exclusion technique. The protocol followed the general guidelines proposed by Singh et al. (1988), with slight modifications. Volumes of 50 μl of Hep-2 cells from each culture well were added to 100 μl of 0.75% LMP agarose at 37 °C. The mixtures were layered onto slides precoated with 0.75% NMP agarose, covered with a coverslip. The slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), supplemented with 1% Triton X-100 and 10% DMSO just before use) for at least 1 h, right after the slides immersed in a freshly prepared alkaline buffer pH > 13 and electrophoresis was conducted for 30 min. The slides were fixed in absolute ethanol, stained with ethidium bromide, and scored using a fluorescent microscope. From each treatment, images from 100 "nucleoids" were captured with a camera attached to the fluorescent microscope and linked to the Comet Score 1.5 software. Highly damaged cells were not included in the scoring (clouds were not analyzed). Tail moment (TM), Tail mean intensity (TI) and tail length (TL), were used to estimate DNA damage (arbitrary units).

### 2.4. *Micronucleus test*

Male and female Balb C mice of 8–12 weeks old were maintained on rodent diet and water *ad libitum*, kept under controlled conditions and according to the criteria established by the Institutional Ethics Committee. Groups of five animals each were employed for the micronucleus test (OECD, 1997b). The groups received ip injections, of 50, 100 and 200 mg/kg of glyphosate. All doses were repeated after 24 h. Cyclophosphamide positive control group received one injection of 20 mg/kg and negative control group equivalent volume of saline solution. All the animals were sacrificed 24 h after the second administration and bone marrow smears were prepared from the femoral bones. Smears were stained with May Grunwald–Giemsa as originally described by Schmid (1975). About 1000 erythrocytes were scored for the presence of micronuclei for each animal. To evaluate bone marrow toxicity, the ratio polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) was calculated by counting 500 erythrocytes.

### 2.5. *TBARs, SOD and CAT determinations*

Three groups of five mice each one were employed for the TBARs, SOD and CAT determinations. Glyphosate 400 mg/kg single dose was intraperitoneally injected and mice sacrificed after 1 and 2 h. Control group was injected with equivalent volume of saline solution during 1 h. Animals were sacrificed by cervical dislocation. Livers, kidneys, hearts and lungs were removed and were stored at −20 °C until the analysis. Tissue homogenates (10%) were prepared in chilled 0.05 M potassium phosphate buffer, pH 7.4. TBARs concentrations, expressed as nmol of malondialdehyde (MDA)/g of tissue were measured spectrophotometrically at 532 nm in liver and kidney homogenates using the method of Marcincak et al. (2003). The concentrations were determined using standard curves of MDA. Superoxide dismutase activity was assayed spectrophotometrically in the supernatants of liver homogenates by the method of Misra and Fridovich (1972). One unit of enzymatic activity has been defined as the amount of enzyme which causes 50% inhibition of auto oxidation of epinephrine. Catalase activity was measured at 240 nm by the decomposition of the $H_2O_2$ by the method of Glick (1954).

### 2.6. *Statistical analysis*

Statistical analysis was performed using Prism software (PRISM, 1997). One way ANOVA and Dunnett as "a posteriori" test were used in all the experiments. The Pearson statistical test was used to examine possible dose-response effects. In all cases, the level of significance was set at $\alpha = 0.05$.

## 3. Results

Table 1 presents the level of DNA damage in Hep-2 cells (5.42 ± 1.83 arbitrary units) for TM measurements in the comet assay. For glyphosate TM DNA damage scores increased from 624.90 ± 70.22 to 963.20 ± 88.01 at a range of concentrations from 3.00 to 7.50 mM respectively ($p < 0.01$). Mitomycin C as the positive control induced a significant increase in DNA migration when compared to negative controls ($p < 0.01$). Glyphosate exhibited dose-dependant genotoxic effect in the three parameters analyzed in Hep-2 cells exposed to 0.00–7.50 mM of glyphosate ($r \geq 0.90$, $p < 0.05$). Viability was lower than 80% with concentrations above 7.50 mM for glyphosate, as determined by the trypan blue dye exclusion method and these were not assayed for genotoxicity.

The herbicide glyphosate was also tested for CA on six different human blood samples after a 48 h treatment. The MI ± SEM has not shown any statistical differences between the various treatments and control groups (Table 2). The results of the CA analysis in blood exposed in vitro to glyphosate are shown in Table 2. The total aberrant cells in control group was 4.4 ± 1.4 and 2.2 ± 0.7 CA/100 metaphases including and excluding gaps respectively. No statistically significant clastogenic effect was quantitatively detected in any glyphosate treatments. Total 50.3 ± 25.2 and 48.5 ± 38.3 aberrant cells, including and excluding gaps respectively were analyzed in Mitomycin C treatment. As it is shown in Table 2 most of the analyzed aberrations after different treatments were of chromatid type, considering gaps and breaks. Dic and ace were only detected in glyphosate cultures and multiple breaks in Mitomycin C cultures.

**Table 2**
Chromosome aberrations in human lymphocytes cultivated with glyphosate. *n*: 6 adult healthy donors of both sexes for each treatment. ctb: chromatid break; csb: chromosome break; ctg: chromatid gap; csg: chromosome gap; dic: dicentric; ace: acentric fragment; end: endoreduplication. MI: mitotic index. Data are expressed as media ± standard error mean (SEM).

| Treatment | ctb | csb | ctg | csg | dic | ace | end | Aberrant cells (%) with gaps | Aberrant cells (%) without gaps | Mitotic index |
|---|---|---|---|---|---|---|---|---|---|---|
| Without | 1.6 ± 1.5 | 0.0 ± 0.0 | 2.0 ± 2.3 | 0.6 ± 0.5 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.2 ± 0.4 | 4.4 ± 1.4 | 2.2 ± 0.7 | 7.0 ± 3.2 |
| Mitomycin C 0.89 μM | 23.5 ± 9.2 | 18.0 ± 15.6 | 3.0 ± 4.2 | 4.0 ± 2.8 | 0.0 ± 0.0 | 27.0 ± 26.0 | 0.0 ± 0.0 | 50.3 ± 25.2* | 48.3 ± 38.3* | 3.5 ± 2.1 |
| Glyphosate 0.20 mM | 1.3 ± 1.1 | 0.3 ± 0.6 | 2.7 ± 3.0 | 0.3 ± 0.6 | 0.0 ± 0.0 | 0.3 ± 02 | 0.0 ± 0.0 | 5.0 ± 2.6 | 2.3 ± 1.2 | 13.0 ± 2.8 |
| Glyphosate 1.20 mM | 2.0 ± 1.3 | 0.5 ± 0.8 | 2.2 ± 1.9 | 0.7 ± 0.6 | 0.2 ± 0.1 | 0.3 ± 0.2 | 1.0 ± 0.9 | 6.8 ± 1.6 | 4.0 ± 0.7 | 10.3 ± 4.5 |
| Glyphosate 6.00 mM | 1.3 ± 0.8 | 0.1 ± 0.4 | 1.8. ± 1.5 | 0.7 ± 1.2 | 1.7 ± 0.4 | 1.7 ± 0.4 | 0.5 ± 0.4 | 5.4 ± 1.0 | 2.6 ± 0.4 | 6.0 ± 1.9 |

* $p < 0.01$, Dunnett's test.

**Table 3**
Number of micronucleated erythrocytes (MNE)/1000 analyzed erythrocytes (MNE) and the ratio polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) in bone marrow of mice after glyphosate ip treatment.

| | MNE/1000 erythrocytes | PCE/NCE |
|---|---|---|
| Saline solution | 3.8 ± 0.8 | 0.85 ± 0.17 |
| Cyclophosphamide 20 mg/kg | 19.2 ± 3.9* | 0.80 ± 0.20 |
| Glyphosate 100 mg/kg (2 × 50 mg/kg) | 3.7 ± 0.5 | 0.77 ± 0.09 |
| Glyphosate 200 mg/kg (2 × 100 mg/kg) | 4.2 ± 0.5 | 0.89 ± 0.17 |
| Glyphosate 400 mg/kg (2 × 200 mg/kg) | 13.0 ± 3.5* | 0.84 ± 0.15 |

The results are expressed as mean ± SD. *n*: 5 animals/treatment.
* $p < 0.01$ Dunnett's test.

In the MNT it was analyzed a basal level of 3.8 ± 0.8 micronucleated erythrocytes (MNE)/1000 analyzed cells in the animals of the negative control group without statistical differences with the treated animals at 100 mg/kg (3.7 ± 0.5 MNE/1000 analyzed cells) and 200 mg/kg (4.2 ± 0.5 MNE/1000 analyzed cells) (Table 3). The group exposed to the highest dose of glyphosate employed, 400 mg/kg, (13.0 ± 3.5 MNE/analyzed cells) and the positive control group treated with Cyclophosphamide (19.2 ± 3.9 MNE/1000 analyzed cells) showed significant differences ($p < 0.01$, test de Dunnett) respect to the control group. There were not clinical signs to inform.

The results of the TBARs, SOD and CAT determinations in tissues of mice after 1–2 h of glyphosate 400 mg/kg are shown in Table 4. Concentrations of MDA/g of tissue were 7.1 ± 2.9 nmol/g liver, 6.6 ± 0.8 nmol/g kidney, 8.9 ± 1.4 nmol/g lung and 7.0 ± 3.5 nmol/g heart in the animals of the control group without significant differences with the control group. SOD activity, expressed as U SOD/g of tissue, were 130.6 ± 39.7 in liver of the control group and 207.6 ± 32.1 in the 400 mg/kg 2 h treatment. CAT activity, expressed as nmol/g of tissue, were 52.5 ± 5.1 in the same control tissue and 81.7 ± 1.4, 1 h after glyphosate treatment ($p < 0.05$).

## 4. Discussion

The comet assay using Hep-2 cells has been used for an initial screening of the potential genotoxicity of glyphosate. The high sensitivity of the comet assay compared to the CA and MNT, and the need for only very small amounts of test chemicals, makes this assay a good alternative to screen the genotoxic potential of chemicals. This cell line was previously used by other authors to evaluate genotoxicity through the comet assay (Miyaji et al., 2004; Andrighetti-Fröhner et al., 2006). The assay showed a clear increase of DNA damage measured by TL, TI and TM in cells exposed to glyphosate for 4 h (Table 1). The herbicide increased the extent of DNA migration in a concentration–response ratio in Hep-2 cells exposed to the tested concentrations. This increase was not connected to a possible cytotoxicity by the herbicide, since the trypan blue method showed that the tested concentrations were not toxic to these cells. MMC 0.01 mM induced a significant increase in DNA migration when compared to negative controls ($p < 0.01$) in Hep-2 cell line (Table 1). Different authors have shown that MMC increase DNA migration in the comet assay (Frenzilli et al., 2000; Arutyunyan et al., 2004). We may say that although MMC is not a classic positive control, in this study we have proved it can be used for this purpose. Therefore MMC 0.01 mM resulted in a correct positive control for comet assay in Hep-2 cell line (Table 1). Former studies using in vivo and in vitro comet assay have also shown that glyphosate affects DNA. This assay has revealed DNA damage with glyphosate 4.0–6.5 mM in GM38 and HT1080 cells (Monroy et al., 2005). DNA damage through Comet Assay has been previously performed in mice ip treated with glyphosate 300 mg/kg (Bolognesi et al., 1997) obtaining positive results.

The other in vitro testing for glyphosate potential genotoxicity was performed through the CA assay. In this study, glyphosate 0.20, 1.20 or 6.00 mM showed not clastogenic effects in peripheral human lymphocyte cultures during a 48 h exposure. We have found only two previous researches that have analyzed CA on human blood cells exposed to glyphosate in vitro. Van de Waart (1995), evaluated cultures exposed to glyphosate 0.33–0.56 mg/ml using metabolic activation and obtaining negative results (unpublished report cited by Williams et al., 2000) . On the other hand, Lioi et al. (1998a) after employing glyphosate 1.4 mg/l, without metabolic activation, obtained positive results. In bovine blood cells, the same authors (Lioi et al., 1998b) have obtained CA increase with glyphosate 2.9 mg/l. Meanwhile Šiviková and Dianovský (2006) have reported no CA effect of the herbicide 140 μmol/l in the same system. Overall, results found in CA evaluations regarding its genotoxic capacity do not show conclusive data and in vivo studies are quite scarce.

As it was previously mentioned, different authors have obtained positive genotoxic results for glyphosate through comet assay, using a variety of cells and organisms. The results are more homogeneous than the ones reported with CA test which sometimes were posi-

**Table 4**
MDA (equivalent to TBARs) levels, units of SOD and nmol CAT activity in tissues of mice after 1–2 h glyphosate 400 mg/kg ip single dose. The results are expressed as mean ± SEM. *n*: 5 animals/treatment.

| Treatment | Liver | | | Kidney | Lung | Heart |
|---|---|---|---|---|---|---|
| | MDA (nmol/g) | SOD (U/g) | CAT (nmol/g) | MDA (nmol/g) | MDA (nmol/g) | MDA (nmol/g) |
| Control | 7.1 ± 2.9 | 130.6 ± 39.7 | 52.5 ± 5.1 | 6.6 ± 0.8 | 8.9 ± 1.4 | 7.0 ± 3.5 |
| Glyphosate 400 mg/kg 1 h | 10.9 ± 9.0 | 195.4 ± 71.9 | 81.7 ± 1.4* | 6.8 ± 2.2 | 10.8 ± 4.3 | 6.3 ± 2.9 |
| Glyphosate 400 mg/kg 2 h | 6.2 ± 0.9 | 207.6 ± 32.1 | 64.2 ± 8.9 | 6.4 ± 0.8 | 9.2 ± 2.3 | 6.5 ± 1.5 |

* $p < 0.05$ Dunnett's test.

tive and others negative. The comet assay is generally considered a more sensitive method than the cytogenetic tests in the assessment of DNA damage induced by genotoxic agents (Hartmann et al., 1998; Maluf and Erdtmann, 2000), and has been widely used in the identification of environmental genotoxins as an exposure biomarker. Furthermore, the comet measurement is an indicative of initial genome damage if repair does not happen (Feng et al., 2004). In this study, it could be possible that the type of target cells and the lasting time between exposure and analysis may have contributed to the difference shown by both tests. It can be supposed that the early positive effects noticed in a cell line by the comet assay at a 4 h exposure, differs from the effects which persist after one cell division in the chromosome aberrations test at an exposure of 48 h.

The MNT allows the detection of small fragments of chromosomes in the cytoplasm of immature erythrocytes, and it is able of detecting clastogenic and aneugenic chemical effects (OECD, 1997b). The administration of 400 mg/kg analytical grade glyphosate has not been previously assayed in vivo in genotoxicity evaluations. The intermediate dose (200 mg/kg) caused a slight increment, showing an evident increase at the third dose (400 mg/kg). We believe that in order to observe a dose-response some other doses should be evaluated (250–300–350 mg/kg) due to the fact that other authors have observed genotoxicity at 300 mg/kg. Rank et al. (1993) have published negative results in the MNT in mice at 200 mg/kg, in accordance with the results obtained in this study at the same doses. Bolognesi et al. (1997) have reported genotoxicity in the same assay employing 300 mg/kg. Our results allow us to suppose that glyphosate can produce cytogenetic damage in vivo. To evaluate whether the possible mechanism of clastogenic damage in mice bone marrow cells was connected with changes in the systemic oxidative status, TBARs was analyzed as a lipid peroxidation marker, SOD and CAT as indicators of endogenous antioxidant status in their different organs.

Glyphosate 400 mg/kg in mice did not induce cellular changes by lipid peroxidation (TBARs) in mice livers, lungs and kidneys. In liver, the main metabolizing organ, it seems to be a not statistically significant increase in SOD activity, and an increase ($p < 0.05$) in the CAT activity, after an hour of ip 400 mg/kg glyphosate administration in mice. According to the obtained results we cannot discard the oxidative stress as a potential genotoxicity mechanism. Due to the fact that the results were not conclusive we believe it is necessary to carry on researching the possible connection between oxidative stress an genetic damage. In normal conditions, the antioxidant enzymes provide an adequate protection against free radicals and reactive oxygen species (ROS), nevertheless the deregulation of only one of these enzymes could seriously affect the cellular defense mechanisms (Gehin et al., 2005). Therefore an increment in the SOD activity would lead to a bigger $H_2O_2$ production, which would also increase the CAT enzyme activity. This would explain the statistically significant increase in the CAT levels (from $52.5 \pm 5.1$ to $81.7 \pm 1.4$ nmol/g tissue), that we have found in the liver of balb C mice, 1 h after ip glyphosate treatment, which could coincide with the results reported by Pieniazek et al. (2004) who found an increment in CAT levels in human erythrocytes after 1 h exposure to glyphosate and Roundup.

In this research the results obtained in the quantification of TBARs in the same mice, did not show significant differences compared to the control group. In this case although they were not statistically significant, the more important lipid peroxidation values, were found after 1 h exposure in the liver of mice with a mean of $10.9 \pm 9.2$ nmol MDA/g tissue, similar values were found in lungs ($10.8 \pm 4.3$). The higher levels of MDA in lung compared to those found in other control tissues could be related to the organ higher exposure to ROS. Nevertheless, the hepatocytes more than any other cell are dependant on the antioxidant enzymes. The increase in MDA levels which was registered in hepatic tissues 1 h after the treatment, although it was not statistically significant, could suggest that the liver would be an important target for ROS attack after glyphosate administration. This would coincide with the results obtained by Beuret et al. (2005), who detected a significant increase in the TBARs levels in pregnant rats livers and in at term fetus livers, 21 days after oral administration. Increases in the TBARs values in in vitro cells exposed either to glyphosate or Roundup, have been also reported by Gehin et al. (2005). Pieniazek et al. (2004) have also reported an increase in dose-response effect in the TBARs after an hour exposure to glyphosate and Round up. More researches in the oxidative stress as a mechanism of genotoxicity are needed. It would be also interesting to investigate in vitro oxidative stress in the hOGG1 modified comet assay (Smith et al., 2006).

The cytogenetic tests performed in this study seem not to be enough to assure the glyphosate genotoxicity. The highest tested dose of glyphosate in the MNT was able to increase the spontaneous micronuclei level in mice. This dose is equivalent to 1/4 of the glyphosate lethal dose for mice (WHO, 1994). The MNT has the advantage of considering the overall animal physiology, mainly absorption; tissue distribution, metabolism, excretion of xenobiotic and its metabolites and DNA repair processes. The positive and negative results must be analyzed through the nature of the assays performed in order to assure a correct interpretation. For instance, positive results obtained in the in vivo micronucleus test are of more importance than the positive results obtained in the in vitro comet assay. This MNT might be repeated with higher number of animals, different strains of mice and perhaps even by no conventional timing, like the MN application in sub chronic studies.

Glyphosate genotoxicity is our matter of interest, and its environmental detection (Kolpin et al., 2006) is also an important topic. At the beginning of its use, around 15 years ago (EPA, 1993; WHO, 1994), the genotoxicity effects of glyphosate were evaluated. The different ways in which this product is being applied are continuously increasing, as well as the resultant risks. Our biggest concern is the excessive amount of glyphosate in its many commercial presentations, especially those used for aerial spraying (Paz-y-Miño et al., 2007), which are incorporated to the soil and the ecosystems, not only in our country but also in the world. In the battery of tests employed in the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400 mg/kg in mice. The suitable, controlled, and regular use of herbicides is recommended, in order to obtain the beneficial effects of these resources without polluting the environment and without leaving their residues in food and water sources with potentially negative effects in human health.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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