EXHIBIT 120
Comparison of the in vivo and in vitro genotoxicity of glyphosate isopropylamine salt in three different organisms

Carlos Alvarez-Moya1, Mónica Reynoso Silva1, Carlos Valdez Ramírez1, David Gómez Gallardo1, Rafael León Sánchez2, Alejandro Canales Aguirre3 and Alfredo Feria Velasco4

1Environmental Mutagenesis Laboratory, Departamento de Biología Celular y Molecular, University of Guadalajara, Zapopan, Mexico.
2Banco Nacional Genómico de Tilapia, Departamento de Ingeniería de Proyectos, University of Guadalajara, Guadalajara, Mexico.
3Unit of Medical and Pharmaceutical Biotechnology, Jalisco Center for Research and Assistance in Technology and Design, Guadalajara, Mexico.
4División de Ciencias Biológicas, University of Guadalajara, Zapopan, Mexico.

Abstract

There is considerable controversy with regard to the genotoxicity of glyphosate, with some reports stating that this compound is non-toxic for fish, birds and mammals. In this work, we used the comet assay to examine the genotoxicity of glyphosate isopropylamine (0.7, 7, 70 and 700 µM) in human lymphocytes, erythrocytes of Oreochromis niloticus and staminal nuclei of Tradescantia in vitro and in vivo. Cells, nuclei and fish that had and had not been exposed to 5 mM N-nitrosodiethylamine (NDEA) were used as positive and negative controls, respectively. Significant (p < 0.01) genetic damage was observed in vivo and in vitro in all cell types and organisms tested. Human lymphocytes and Tradescantia hairs showed lower genetic damage in vivo compared to in vitro, possibly because of efficient metabolization of the herbicide. In O. niloticus erythrocytes, significant (p < 0.001) genotoxicity was observed at :: 7 µM, whereas in vitro, glyphosphate was genotoxic in human lymphocytes and Tradescantia hairs at :: 0.7 µM. These results indicate that glyphosate is genotoxic in the cells and organisms studied at concentrations of 0.7-7 µM.

Key words: comet assay, DNA damage, genotoxicity, glyphosate.

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Introduction

Glyphosate (N-(phosphonomethyl) glycine) is usually produced as glyphosate isopropylamine salt, one of the most widely used herbicides (Cox, 1998). The US Environmental Protection Agency (EPA) classified glyphosate as category E, indicating “evidence of no carcinogenicity for humans” (EPA, 1993) and, according to the US Forest Service (1997), glyphosate has no adverse effects in humans. Glyphosate is also reportedly not genotoxic in soil microorganisms (Owczarek et al., 1999; Busse et al., 2001; Conner and Black, 2004; De Roos et al., 2005; Dimitrov et al., 2006). Chronic feeding studies of glyphosate have provided no evidence of a carcinogenic effect in mice or rats (Williams et al., 2000). The risk of genotoxicity in humans is low in areas where glyphosate is applied for cocoa and poppy eradication (Bolognesi et al., 2009), but an association with multiple myeloma has been suggested (De Roos et al., 2005). In contrast, several studies using different tests have reported high genotoxicity for glyphosate (Sivikova and Dianovsky, 2006; Sparling et al., 2006; Cavas and Könen, 2007; Alvarez et al., 2011; Guilherme et al., 2012), with the genotoxicity observed being related to the test system used (Zúñiga, 2001), e.g., plants (Dimitrov et al., 2006; Alvarez et al., 2011; Truta et al., 2011), fish (Cavas and Könen, 2007) and human cells (De Roos et al., 2005; Bolognesi et al., 2009) are reportedly very sensitive to glyphosate.

Comparisons between the cells of different organisms in vivo and in vitro using the comet assay system can be important in assessing the genotoxicity of glyphosate. The comet assay system, which was first used in human lymphocytes, is very efficient in detecting genotoxicity (Singh et al., 1988) because it allows the visualization of damage directly in the genetic material of individual cells. This test has also been used in plants and fish (Koppen and Verschaeve, 1996; Alvarez et al., 2001; Guilherme et al., 2012).
In this study, the genotoxicity of glyphosate was assessed by applying the comet assay to *Tradescantia* (clone 4430) staminal nuclei, tilapia (*Oreochromis niloticus*) erythrocytes and human lymphocytes. The findings in the latter cells were compared with the genetic damage in lymphocytes from workers occupationally exposed to glyphosate based on data from a previous report (Paz-y-Miño et al., 2007).

**Materials and Methods**

**Chemicals**

Glyphosate (N-(phosphonomethyl)-glycine), 96% (CAS No. 1071-83-6, lot 09816 PE) was obtained from Aldrich Chemical Co. (St. Louis, MO, USA) (Cox, 1995). The concentrations tested were 0.7, 7, 70 and 700 µM, with 5 mM N-nitrosodiethylamine (NDEA, CAS No. 55-18-5) being used as a positive control. These concentrations were used in a previous investigation (Alvarez et al., 2011).

**Organisms studied**

For each concentration, eight tilapia (*O. niloticus*) and 30 *Tradescantia* plants were studied, in addition to the corresponding negative and positive controls, *in vivo* and *in vitro*. In the case of human lymphocytes *in vitro*, cells from eight individuals were also used. Paz-y-Miño et al. (2007) studied the direct effect of glyphosate in occupationally exposed humans. Their study was done in similar conditions to ours with respect to the control groups. Blood samples were tested using the alkaline comet assay as described (Singh et al., 1988; Paz-y-Miño et al., 2007). Comets were analyzed on a Zeiss fluorescence microscope fitted with a 50 W mercury lamp and an excitation filter of 515-560 nm. While the above cited authors used a calibrated ocular micrometer, we used a comet assay program. Nonetheless, the use of either method did not affect the final tail length measurements because the comparison was in microns.

**Preparation of cells and nuclei**

For the preparation of human lymphocytes, peripheral blood samples were obtained from a finger puncture in young students who initially responded to a questionnaire to rule out exposure to genotoxic agents. Individuals on medical treatment, smokers, drug users and inhabitants of the contaminated area of Guadalajara, Jalisco, Mexico were excluded from the study. Each blood sample was placed in a test tube containing 3 mL of phosphate-buffered saline (PBS; 160 mM NaCl, 8 mM Na₂HPO₄, 4 mM NaH₂PO₄ and 50 mM EDTA; pH 7) and immediately centrifuged at 3000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in phosphate buffer and immediately stored at 4 °C until used.

Tilapia erythrocytes were from blood collected from specimens 10 ± 3 cm in length obtained from Banco Nacional Genómico de Tilapia Oreochromis niloticus. The fish were acclimatized in 5000 L aquaria under a natural photoperiod in aerated, recirculating tap water, with the following physicochemical conditions: salinity 0, temperature 20 ± 1 °C, pH 7.3 ± 0.2 and dissolved oxygen 8.1 ± 0.5 mg/L. During this period, the fish were fed with fish food every other day. The experiment was done in 20 L aquaria, in static mode. The fish were deprived of food for one day before the experiment and during the experimental period. Thirty-two fish were divided into four aquaria (groups of eight fish per treatment) and exposed to different concentrations of Roundup® (Monsanto; Roundup is the trade-mark name for a glyphosphate product) (*in vivo* treatment). Another two aquaria with clean water served as a negative control and positive control (5 mM NDEA). Each glyphosate concentration was tested during a 20 h exposure and there was no mortality at any of the concentrations. After each exposure, 0.5 mL of erythrocytes was obtained by branchial puncture and the cells then washed and centrifuged in ice-cold PBS, as mentioned for human lymphocytes.

Cell viability was assessed with the Trypan Blue test using 20 mL of peripheral blood lymphocytes and 20 mL of circulating erythrocyte suspension. The mean percentage viability for each group was > 89%.

*Tradescantia* (clone 4430, a *T. subacaulis* X *T. hirsutiflora* hybrid that is highly sensitive to environmental mutagens) was used to prepare stamen nuclei. The plants were grown under controlled conditions, at a daytime temperature of 22 °C and night-time temperature of 16-18 °C. For each concentration, 30 inflorescences (~10 flowers containing 1500-3000 stamen hairs) were immersed for 3 h in 250 mL of the desired concentration of glyphosate isopropylamine salt (*in vivo* study). The same number of inflorescences and time interval were used for the negative and positive controls (Hoaglands solution and 5 mM NDEA, respectively). The experiment was done in duplicate. After treatment, the inflorescences were washed with distilled water and placed in Hoagland’s solution. On day 6 after treatment (the point at which the mutation became evident (Underbrink et al., 1973), indicating that it had not been repaired), the stamen nuclei from treated plants were used for the comet assay and were separated according to Alvarez et al. (2001).

For the *in vitro* study, stamen nuclei from ten flowers of untreated plants for each experimental condition were placed in a cold mortar with 500 µL of Honda buffer (0.44 M sucrose, 2.5% Ficoll (type 400), 5% Dextran T-40, 25 mM Tris-HCl (pH 8.5), 10 mM MgCl₂, 10 mM 1-mercaptoethanol and 2.5% Triton X-100) and homogenized for 2 min, after which the mixture was filtered through a nylon mesh (80 µm). The nuclei were separated by centrifugation (3000 rpm, 4 °C, 5 min) and washed three times in 5 mL of wash solution (0.4 M sucrose, 50 mM Tris base and 5 mM MgCl₂, pH 8.5), re-suspended in 200 µL of the same solu-
tion and stored at -20 °C until electrophoresis (Alvarez et al., 2001).

Slides of *Tradescantia* nuclei, fish erythrocytes and human lymphocytes were prepared according to Singh et al. (1988). Slides with nuclei from untreated plants were exposed to each concentration of glyphosate isopropylamine salt for 20 h at 25 °C, washed and then stored at 4 °C to prevent repair of the induced damage. Other slides with nuclei from cells that had or had not been exposed to 5 mM NDEA were used as positive and negative controls, respectively. Slides with stamen nuclei from treated plants (*in vivo* experiment) were also prepared.

Slides of human lymphocytes and fish erythrocytes from the *in vitro* experiments were exposed to each concentration of glyphosate isopropylamine salt for 20 h at 25 °C, washed and then stored at 4 °C, as described above. Slides of cells that had or had not been exposed to NDEA were used as positive and negative controls, respectively. Cells from fish that had or had not been exposed to 5 mM NDEA were used as positive and negative controls, respectively, in the *in vivo* experiments. The entire experiment was repeated twice.

Comet assay

The nuclei from *Tradescantia* stamens and human lymphocytes were used in the comet assay, as described by Singh et al. (1988) and Alvarez et al. (2001). The slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% sodium lauryl sarcosine, 1% Triton X-100 and 10% DMSO, pH 10) for 20 h at 4 °C to ensure nuclear lysis and then placed in a horizontal electrophoresis system with a high pH buffer (30 mM NaOH, 1 mM Na₂EDTA, pH 13) for 45 min to allow DNA unfolding before electrophoresis for 20 min (*Tradescantia* nuclei and human lymphocytes) or 10 min (fish erythrocytes) at 1.0 V/cm with an amperage of ~300 mA. The same electrophoretic unit and power supply were used throughout the study (Hartmann et al., 2003). All of the steps described above were done under yellow light to prevent additional changes to the DNA.

After electrophoresis, the slides were gently washed to remove the alkaline solution and then immersed in neutralization buffer (0.4 M Tris base, pH 7.5) for 5 min. The gels were stained with ethidium bromide (100 µL at 20 µg/mL) for 3 min and then rinsed three times with distilled water. The preparation was subsequently covered with a coverslip and the slides were examined by fluorescence microscopy using a light microscope equipped with a 515-560 nm excitation filter. Nuclei and cells were observed at 10X magnification and tail length during migration was determined by using Comet assay software based on published protocols (Hartmann et al., 2003). Approximately 50 cells or nuclei per slide and two slides for each experimental point and controls were evaluated.

Data analysis

The results were expressed as the mean ± SD and were analyzed by one-way analysis of variance (ANOVA) using the CoStat program (Ma et al., 1994). All experimental groups were compared with the corresponding negative control using the Dunnett test. Fifty cells were used for all organisms. For *Tradescantia*, the number of hairs/nuclei tested in the treatments varied from 50 to 250, depending on flower availability. A value of p ≤ 0.05 indicated significance.

Results

Comet assay of human lymphocytes

Figure 1 shows that the migration (tail length) of human lymphocyte DNA in the comet assay increased with the concentration of glyphosate and was generally proportional to the latter. The responses to different concentrations of glyphosate differed significantly among themselves (p < 0.0001, ANOVA) and were significantly different from the negative and positive controls (p ≤ 0.01, Dunnett test).

Comet assay of *O. niloticus* erythrocytes

Figure 2 shows the comet assay results for tilapia erythrocytes exposed to different concentrations of glyphosate isopropylamine salt *in vitro* and *in vivo*. When tested *in vitro*, the increase in DNA migration was proportional to the glyphosate concentration (p ≤ 0.001), although no genotoxicity was observed at 0.0007 mM. The responses to different concentrations of glyphosate differed significantly among themselves (p < 0.0001, ANOVA) and were significantly different from the negative and positive controls (p ≤ 0.01, Dunnett test). Glyphosate was also genotoxic to fish erythrocytes *in vivo* (p ≤ 0.001), but the response was not concentration-dependent.

Comet assay of *Tradescantia* stamen nuclei

The comet assay results for *Tradescantia* stamen nuclei exposed to glyphosate *in vitro* and *in vivo* are shown in

![Figure 1](image-url) - Tail length in human lymphocytes exposed to different concentrations of isopropylamine glyphosate. The diagonal line indicates the relationship between glyphosate concentration and tail length in micrometers (µ). NDEA5 - 5 mM N-nitosodiethylamine. Negative control (C-). The values above the columns are the mean ± SD (n = 8). Tail length µc.
Human lymphocytes, cantia
Glyphosate was clearly genotoxic in all cases.

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Concentrations of isopropylamine glyphosate in vitro and
And was significantly lower than that observed
In vitro
And was not proportional to the concentration tested
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::
0.01)
Among the responses to these three concentrations of glyphosate, and between all glyphosate concentrations and the negative control (p : 0.01). Although glyphosate was also significantly (p : 0.05) genotoxic in vivo, this response was not proportional to the concentration tested and was significantly lower than that observed in vitro.

Table 1 compares the genotoxicity of glyphosate in human lymphocytes, O. niloticus erythrocytes and Tradescantia (clone 4430) stamen nuclei in vivo and in vitro. Glyphosate was clearly genotoxic in all cases.

Discussion
The comet assay is a valuable and sensitive tool for detecting genetic damage in individual cells (Singh et al., 1988). Alvarez et al. (2001) reported a protocol that simplifies this assay in Tradescantia stamen nuclei. Genetic damage induced by glyphosate has been reported (Mañas et al., 2009; Vera-Candioti et al., 2013) and the comet assay has been used to demonstrate genotoxicity in fish hepatic cells and Tradescantia nuclei (Alvarez et al., 2011; Guilherme et al., 2012). Although the evidence of glyphosate genotoxicity or carcinogenicity from in vitro and animal studies is poor (De Roos et al., 2005), a few epidemiological reports have indicated potential health effects (De Roos et al., 2003).

In this study, the comet assay clearly showed that glyphosate was genotoxic in the cells examined. Since the in vivo effect of glyphosate in humans was not examined in this work, we compared the data from a study of the direct effect of glyphosate in occupationally exposed humans (Paz-y-Miño et al., 2007) with the effect of direct exposure of human lymphocytes to glyphosate observed here. As shown in Table 2, glyphosate was genotoxic in both studies. The tail length was very similar in both cases and there was a positive relationship between genotoxicity and glyphosate concentration/dose.

The high genotoxicity of glyphosate in human lymphocytes, Tradescantia nuclei and fish erythrocytes in vitro may be indicative of direct DNA damage (Kirkland, 1998; Torstensson et al., 1989; Alvarez et al., 2011). The genotoxicity of glyphosate was lower in vivo than in vitro, perhaps because of efficient herbicide degradation in the former situation. This generalization did not apply to erythrocytes of O. niloticus since these fish were particularly sensitive to glyphosate, perhaps because of poor herbicide metabolism; this finding could also explain the lack of concentration-dependent genetic damage. Glyphosate has previously been reported to cause DNA damage in liver cells of Anguilla anguila (Guilherme et al., 2012) but differently, Oreochromis niloticus erythrocytes showed to be more susceptible to lower concentration.

Table 1 - Comparison of the genotoxicity of glyphosate in human lymphocytes in vitro and O. niloticus erythrocytes and Tradescantia stamen nuclei (clone 4430) in vivo and in vitro.

<table>
<thead>
<tr>
<th>Human cells</th>
<th>O. niloticus blood cells</th>
<th>Tradescantia (clone 4430) stamen nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro exposed cells</td>
<td>In vivo exposed fishes</td>
<td>In vitro exposed cells</td>
</tr>
<tr>
<td>PE</td>
<td>PE</td>
<td>PE</td>
</tr>
</tbody>
</table>

PE - positive effect.
Risk for a variety of organisms, including humans.

Of this herbicide, it is clear that glyphosate has a potential genotoxic, depending on the time and concentration used, to different organisms.

Glyphosate isopropylamine in cells and nuclei of different concentrations have been reported by Kültigin et al. (2009-500 mg/L) and Prasad et al. (2009) (25-50 mg/L). Our results suggest that the minimal concentration for glyphosate genotoxicity was close to 0.0007 mM (118 µg/L), although genotoxicity may start at even lower concentrations. This finding agrees with other studies that concentration for glyphosate genotoxicity was close to 0.0007 mM (118 µg/L), although genotoxicity may start at even lower concentrations. This finding agrees with other studies that


In conclusion, our results indicate that glyphosate is genotoxic, depending on the time and concentration used, as reported by Poletta et al. (2009). Given the extensive use of this herbicide, it is clear that glyphosate has a potential risk for a variety of organisms, including humans.

Acknowledgments

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References


Table 2 - Comparison between human lymphocytes from persons occupationally exposed to glyphosate (Paz-y-Miño et al., 2007) and human lymphocytes exposed directly to various concentrations of the compound.

<table>
<thead>
<tr>
<th>Study in vivo (Paz-y-Miño et al., 2007)</th>
<th>mM</th>
<th>Tail length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals exposed</td>
<td>35.5 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>Individuals not exposed</td>
<td>25.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Study in vitro (present study)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes exposed</td>
<td>0.7</td>
<td>51.0 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>48.9 ± 9.2</td>
</tr>
<tr>
<td></td>
<td>0.007</td>
<td>42.3 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>0.0007</td>
<td>42.9 ± 8.6</td>
</tr>
<tr>
<td>Lymphocytes not exposed</td>
<td>20.4 ± 4.1</td>
<td></td>
</tr>
</tbody>
</table>

The values are the mean ± SD (n = 8).


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