

Message

**From:** FARMER, DONNA R [AG/1000] [/O=MONSANTO/OU=NA-1000-01/CN=RECIPIENTS/CN=180070]  
**Sent:** 12/1/2010 6:53:51 PM  
**To:** Amy Williams [REDACTED]@exponent.com]  
**Subject:** second half and additional articles  
**Attachments:** Glyphosate Dev Repro Review rest.docx; mladinic\_2009.pdf; prasad\_2009.pdf; cavalcante\_2008.pdf; conners\_2004.pdf; holeckova\_2006.pdf; piesova\_2005.pdf; bolognesi\_2009.pdf; pazymino\_2007.pdf

Amy,

See attached.

Donna

**From:** Amy Williams [REDACTED]@exponent.com]  
**Sent:** Wednesday, December 01, 2010 10:38 AM  
**To:** FARMER, DONNA R [AG/1000]  
**Cc:** John DeSesso; SALTMIRAS, DAVID A [AG/1000]  
**Subject:** RE: First half - second reply

Donna and David,

We've received the disk with the Knapp studies and will work on verifying/revising the related text in the manuscript. Can you let us know when to expect comments back on the second half of the paper? Thanks so much.

Best regards,

Amy Lavin Williams, PhD, DABT

Exponent

1800 Diagonal Road, Suite 500

UNITED STATES DISTRICT COURT  
NORTHERN DISTRICT OF CALIFORNIA

**TRIAL EXHIBIT** 467

Case No. 3:16-cv-0525-VC

Date Entered \_\_\_\_\_

By \_\_\_\_\_  
Deputy Clerk

MONGLY02406325

Alexandria, VA 22314

[REDACTED]

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**From:** FARMER, DONNA R [AG/1000] [REDACTED]@monsanto.com]  
**Sent:** Tuesday, November 23, 2010 4:35 PM  
**To:** Amy Williams  
**Cc:** John DeSesso; SALTMIRAS, DAVID A [AG/1000]  
**Subject:** RE: First half - second reply

Amy,

See responses below.

Have a few other things to send will do tomorrow.

Need to go get my daughter from a retreat.

Donna

**From:** Amy Williams [REDACTED]@exponent.com]  
**Sent:** Tuesday, November 23, 2010 9:51 AM  
**To:** FARMER, DONNA R [AG/1000]  
**Cc:** John DeSesso; SALTMIRAS, DAVID A [AG/1000]  
**Subject:** RE: First half - second reply

Donna,

MONGLY02406326

When can we expect comments on the rest of the document? I'm trying to get the document finalized and out the door as quickly as possible. Along those lines, I have a few more questions to ask:

- In the abstract, we listed 2 mg/kg/d as the RfD based on what was proposed in the glyphosate RED. In the revisions, someone changed that to 1.75 mg/kg/d. I realize this is based on a dose of 175 mg/kg/d in a developmental study with an uncertainty of 100, but the RED did not propose an RfD of 1.75 mg/kg/d; it listed the RfD as 2 mg/kg/d. In the IRIS database, the RfD is listed as 0.1 mg/kg/d. What value do you believe we should use? I need to be able to back the number up with a citation as well.

See attachment

For many years the only dataset that was available for glyphosate was Monsanto's ....today there are a number of other full data sets.

The original Monsanto set of studies that supported glyphosate used high doses of ~ 30 mg/kg/day. These studies were reviewed by the WHO in 1986  
<http://www.inchem.org/documents/jmpr/jmpmono/v86pr08.htm>

They concluded that there were no effects related to treatment and set the ADI at 0.3 mg/kg/day. In the US, IRIS reviewed the same studies and came to a different conclusion on the 3 gen rat repro study and concluded the effects at the high-dose were related to treatment and in 1990 they set the ADI at 0.10 mg/kg/day based on that study. <http://www.epa.gov/iris/subst/0057.htm> The effects observed in that first study were not reproduced in the second repro study by Monsanto nor in any other repro study by any other manufacturing at much higher doses.

Glyphosate was re-registered in 1993. Monsanto conducted several new studies for that re-registration to meet the guidelines in place at that time and the two key studies were the 2-gen rat repro and the rat chronic study with high doses of 20,000 ppm.

The EPA selected the **lowest NOEL** in the glyphosate toxicology data base to set the ADI which was based on the maternal effects observed in the rabbit teratology study. In the 1993 RED the RfD was 2 mg/kg/day ...this was "rounded up" from 1.75. Lately we have been seeing them use the non-rounded post- FQPA cPAD of "1.75 mg/kg/day" value - <http://www.federalregister.gov/articles/2004/11/10/04-25098/glyphosate-pesticide-tolerance>

**The IRIS database and website for glyphosate is completely out of date**, in the Revision History section the last entry for tox information was 1990 – they did not revise after the RED!. Note that the cancer classification is a “D”...in 1991 it was concluded to be an “E” – Evidence of non-carcinogenicity in humans. We have repeatedly asked them to update and revise because the discrepancies have been problematic for us – note that the last entry in the revision history it is no longer being assessed by IRIS. The MCL for glyphosate of 700 ppm is based on the IRIS database <http://www.epa.gov/safewater/pdfs/factsheets/soc/glyphosa.pdf> From their “What are the health effects?” they state: “Short-term: EPA has found glyphosate to potentially cause the following health effects when people are exposed to it at levels above the MCL for relatively short periods of time: congestion of the lungs; increased breathing rate.” [We have no idea how they came to this conclusion since the inhalation study for glyphosate was waived.]

Long-term: Glyphosate has the potential to cause the following effects from a lifetime exposure at levels above the MCL: kidney damage, reproductive effects.” The subsequent studies do no support this conclusion at all. We also asked that the MCL be revised based on the new data and they also said no...why because they saw no need as glyphosate is never found in finish drinking water therefore they saw no concern to put resources to the review.

In Canada and Japan...which is based on Monsanto data only the RfD is 0.75 mg/kg/day...same rabbit teratology study different conclusion on high-dose effects.

EU - 0.3 mg/kg/day – based on our first chronic study even though there were multiple submitters

WHO 2004 - 1.0 mg/kg/day multiple submitters based on another companies chronic rat study.

If you use the RED as your reference – the correct value for the RfD is 2 mg/kg/day (as edited is not correct because the RED does not use 1.75 mg/kg/day....understand your confusion)

If you use the post-FQPA cPAD then I would use 1.75 mg/kg/day.

Do not use the IRIS value it is of no value today.

- More discussion was added to the introduction on dermal absorption, including data from Nielsen, 2009. The paper you sent to me earlier this week was Nielson, 2010. I cannot find the data that was added to the introduction in the 2010 paper and I cannot find a Nielsen, 2009 paper. Are the numbers wrong or is there another reference? If the numbers are right and based on the 2010 paper, can someone lead me through the calculations?

David...you added this section , please respond.

- For the study of Reyna, 1990, some doses in mg/kg/d were added to the document corresponding with the ppm amounts administered in the diet. I am not clear on from where those doses are derived. Can someone show me where these values came from?

They were what was published in the William's et al. paper page 127. Below is a table lifted from a Monsanto Summary of that study. I am okay with going with approximate mg/kg/day.

| Generation/Sex    | Dietary concentration of glyphosate (ppm) |        |        |
|-------------------|---|--------|--------|
|                   | 2,000                                     | 10,000 | 30,000 |
| F0 Parents: Males | 132                                       | 666    | 1,983  |
| Females           | 160                                       | 777    | 2,322  |
| F1 Parents: Males | 140                                       | 711    | 2,230  |
| Females           | 163                                       | 804    | 2,536  |

- Can you forward the studies from Knapp (2007, 2008)? We do not have those in-house to verify the data. Plus, we will need to make some revisions since, as noted in the comments, at least some of the text was lifted directly from the report summaries.

David ...where are you on getting these studies to them?

- In the section on genotoxicity, one of the reviewers mentioned a study by Kier and Stegman (1993) in which AMPA was tested. I do not have this study and cannot find it in Pubmed. Can you forward it so it can be added to the paper?

David ..can you send them a copy of this report as well with the others?. The WHO 1997 review of AMPA <http://www.inchem.org/documents/jmpr/jmpmono/v097pr04.htm> and in 2004 ...the the WHO in the glyphosate review...reviewed a few new studies and came to the same conclusion on AMPA

- An additional 7 papers on genotoxicity were recommended to be added to the report. These include Mladinic et al (2009), Prasad et al (2009), Cavalcante et al (2008), Conner and Black (2004), Holeckova (2006), Piesova et al (2005), and Bolognesi et al (2009). Would you like these added, and if so, can you forward these papers for review? Alternatively, we can order them in-house. Let me know.

Will forward in another email.

- A bit of text was added to the report for a study by Benachour et al (2007). After some review, I realized that the year given is incorrect. It should be Benachour et al (2009). Earlier this week, you forwarded the 2007 paper. Can you forward the 2009 paper for our review? The text that was added is very limited and needs to be revised. I will also be moving discussion of this study to a more appropriate section of the document.

See attached – the text was only a place holder please revise upon your review.

- The single name 'Pagenelli' was added to the report. I did a search to find that this is reference to a study, Paganelli et al 2010. I assume you would like discussion of this study added to the paper. Can you provide the paper for review?

See attached.

Thanks for your assistance.

Best regards,

Amy Lavin Williams, PhD, DABT

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**From:** FARMER, DONNA R [AG/1000] [REDACTED]@monsanto.com]

**Sent:** Friday, November 19, 2010 10:41 AM

**To:** Amy Williams

**Subject:** RE: First half - second reply

Amy,

Attached are the external publications listed below – the Romano paper was one that was reviewed by Bill Kelce...so you should already have a copy and an extensive review of that publication.

I thought David had sent you copies of the Knapp and Moxon study reports. I will check with him on those and the Ward studies.

Donna

**From:** Amy Williams [REDACTED]@exponent.com]  
**Sent:** Friday, November 19, 2010 8:53 AM  
**To:** FARMER, DONNA R [AG/1000]; John DeSesso  
**Subject:** RE: First half - second reply

Donna,

You have added significant text to the document with regard to the following references:

- Bo Nielsen et al., 2009
- Ward, 2010
- Moxon, 2000
- Knapp, 2007
- Knapp, 2008
- Benachour et al., 2007
- Romano, 2010

- Gasiner et al., 2009

Unless someone from Monsanto plans to be listed as an author, we need to see these references in order to verify that we are in agreement with the newly added text. As such, could you forward these papers to us? Thanks so much.

Best regards,

Amy Lavin Williams, PhD, DABT

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**From:** FARMER, DONNA R [REDACTED]@monsanto.com]  
**Sent:** Friday, November 19, 2010 8:20 AM  
**To:** John DeSesso  
**Cc:** Amy Williams  
**Subject:** RE: First half - second reply

John,

Can you provide me the specific references? Is it the surfactant studies or the additional references for the gasiner paper.



Thanks,

Donna

**From:** John DeSesso [REDACTED]@exponent.com]  
**Sent:** Thursday, November 18, 2010 3:01 PM  
**To:** FARMER, DONNA R [AG/1000]  
**Cc:** Amy Williams  
**Subject:** RE: First half - second reply

Hi Donna,

Since there will not e authors from Monsanto on the manuscript, can we get copies of the papers that are summarized in the new text? We need to independently verify that our conclusions coincide.

Thanks,

John

**From:** FARMER, DONNA R [AG/1000] [REDACTED]@monsanto.com]  
**Sent:** Thursday, November 18, 2010 2:50 PM  
**To:** John DeSesso  
**Subject:** First half

John,

Attached is the first 46 pages.

I added a section in genotox from the Gasnier study...see a attached a critique we did that I took that from. Am working on a section for gasiner in the

mechanistic section. Also we cut and pasted in summaries of the POEA surfactant studies. Attached are more detailed summaries – see Knapp. For right now I think we should go with POEA surfactants. I am checking to find out if there are any concerns with using MON 0818 and MON 8109 as well as indicating they are tallow and coco-derived – will get back to you on that as well as sending the remaining pages. Hope to have them done this afternoon if not will send tomorrow.

<<Glyphosate Dev Repro Review Part I.docx>> <<Publication 4 Gasnier 2009.docx>>  
<<Knapp studies.docx>>

Regards,

Donna

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### Endocrine Disruption

In recent years, many environmental pollutants have been suspected to contribute to endocrine disruption; however, only a few have been scientifically proven to disrupt the endocrine system at environmentally relevant concentrations (WHO, 2002). Mechanistic studies to ascertain whether glyphosate can cause adverse developmental or reproductive effects by interfering with the functioning of the endocrine system have been conducted (Table 11). These studies are varied in their approach and examine potential effects on steroid hormone production and placental enzyme activity. In a number of cases, glyphosate-based formulations containing surfactant systems were evaluated for aromatase activity using microsomes. These studies are flawed from the outset because microsomes are denatured by very low concentrations of surfactants and detergents. This is noted in the US EPA Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1200: Aromatase (Human Recombinant), which clearly warns that all glassware and apparatus used in the microsome preparations should be free of detergent residue. Furthermore, if detergent residues compromise study viability, testing measurable concentrations of detergent like substances would overload such *in vitro* systems and is certainly not a viable approach to investigating endocrine disruption. Levine et al (2007) evaluated a variety of surfactants using an *in vitro* systems and determined results were due to the a non-endocrine mechanism of compromised mitochondrial membrane potential and altered permeability of cell membranes.

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Petit et al. (1997) screened various herbicides, fungicides, insecticides, xenobiotics, and phytoestrogens for estrogenic potency using two *in vitro* systems: a recombinant yeast system expressing the rainbow trout estrogen receptor and rainbow trout hepatocyte cultures. Yeast cells containing a *lacZ* reporter gene linked to two estrogen-responsive elements were treated in culture at  $10^{-5}$  to  $10^{-4}$  M of each test agent for four hours. 17  $\beta$ -Estradiol was used as the positive control.  $\beta$ -Galactosidase activity, dependent on expression of the *lacZ* gene, was measured in Miller units using a colorimetric substrate. To ensure that the absence of a response was not due to toxicity, cell density measurements were made before and after treatment, although the data for agents that were not estrogenic were not shown. Glyphosate treatment had no effect on the basal level of  $\beta$ -galactosidase activity. Only those test agents shown to be positive for estrogenicity in the yeast system, plus eleven other randomly selected test compounds, were evaluated in the trout hepatocyte cultures for expression of the vitellogenin gene, as determined by slot blot analysis; glyphosate was not among those tested. One

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weakness of this study is that the description of methods is not clear as to whether pure glyphosate or a glyphosate-based herbicide was tested. Nevertheless, these data provide no evidence of estrogenic activity.

Lin and Garry (2000) investigated whether certain herbicides and fungicides commonly used in the Red River Valley of Minnesota could induce proliferation of the estrogen-responsive MCF-7 cell line. MCF-7 cells were seeded in media containing either regular fetal bovine serum (FBS) or steroid growth-factor-deficient FBS (produced through prior treatment with 10% charcoal dextran). Following a 48-hour incubation, the cells were then treated with different dilutions of test chemicals,  $10^{-9}$  M estradiol (positive control), or solvent vehicle (negative control). After seven days in culture, cell numbers and viability of harvested cells were assessed using a fluorescence-activated cell sorter. In separate experiments, cytotoxicity (following 72-hour incubation of MCF-7 cells in various concentrations of test agents) and apoptosis (using propidium iodide staining) were evaluated by flow cytometry. Both the "Roundup" branded formulation (identified as containing 0.99% glyphosate) and its active ingredient, glyphosate, were shown to induce proliferation of MCF-7 cells. This occurred in media containing either regular or steroid growth-factor-deficient FBS, suggesting that the proliferative effect was mediated through a nonestrogenic pathway. Maximum induction levels ranged from  $121 \pm 10.3\%$  for  $10 \mu\text{g/ml}$  "Roundup" in regular FBS and  $135 \pm 3.5\%$  for  $2.28 \times 10^{-4}$  M glyphosate in steroid growth-factor-deficient FBS. None of the test agents used in these experiments was shown to be cytotoxic at the concentrations used in the seven-day proliferation studies. Also, neither glyphosate nor "Roundup" was shown to induce apoptosis. While these results suggest that glyphosate may be able to induce cell proliferation, this response is not mediated through an estrogenic pathway.

**Comment [drf1]:** This suggest this was a L&G product... maybe a good idea to go back and check all ref. for how they report the test materials and use the terminology used by the authors.

Using an *in vitro* system, Meulenberg (2002) tested the ability of various endogenous steroids, pharmaceutical agents, pesticides, and pollutants to displace estradiol ( $E_2$ ) from human sex hormone-binding globulin (SHBG), a high affinity, but low capacity, hormone-binding protein found in the blood that functions in the transport of sex hormones and protects against their degradation. Changes in the binding capacity of SHBG will affect the free concentrations of various sex hormones. Because it is assumed that only the free fraction of such hormones can exert biological activity, such changes will likely result in hormonally-mediated changes in the organism. Microtiter plates were coated with rabbit anti-SHBG antibody, and using these plates, SHBG was isolated overnight from the serum of pregnant women. Following several washes, tritiated  $E_2$ , along with the test compound, was added to the microtiter plates. Following 48 hours incubation, supernatant was removed from the plates and the amount of radioactivity in the media was measured using a scintillation counter. Because testosterone is known to have a three times greater affinity for SHBG than  $E_2$ , it was used as a positive control. The binding of varying concentrations of test agents was referenced to the standard curve for testosterone. Affinity of these compounds for SHBG was defined as an ability to displace tritiated  $E_2$  to an extent comparable to that of testosterone. The study authors indicate that glyphosate showed ambiguous

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results for displacement of E<sub>2</sub> from SHBG, although actual experimental data were not shown. These results indicate that glyphosate should not affect the ability of SHBG to bind sex hormones in the blood.

In Xie et al. (2005), the estrogenic potency of glyphosate, three non-glyphosate-based herbicides, and two types of ethoxylate-containing surfactants (R-11 and Target Prospreader Activator [TPA]) was determined using the *in vivo* rainbow trout vitellogenin assay. In fish, adult female production of vitellogenin is mediated by estrogenic activity; thus, vitellogenin expression is thought to serve as a biomarker for chemicals likely to alter estrogenic activity in fish and other animals. In this study, exposure of the fish for seven days to 0.11 mg/L glyphosate had no effect on vitellogenin levels, suggesting that glyphosate is unlikely to alter estrogenic activity. Recommended concentrations of both 2,4 dichlorophenoxyacetic acid and trichlopyr increased vitellogenin production, suggesting that these chemicals can exert estrogenic activity. Mixtures of both these pesticides with the surfactants led to a significant increase in vitellogenin levels.

Kojima et al. (2004) tested over 200 pesticides for their ability to act as agonists and antagonists to two human estrogen receptor (hER) subtypes, hER $\alpha$  and hER $\beta$ , and a human androgen receptor (hAR). For each hormone receptor of interest, Chinese hamster ovary cells were transfected with the appropriate cDNA expression vector, along with a reporter plasmid containing either an estrogen-responsive element or an androgen-responsive element, and a *Renilla* luciferase expression vector (used as an internal control for determining transfection efficiency). After three hours transfection, cells were dosed for 24 hours with varying concentrations of test agent. To assess antagonistic activity to hER $\alpha$ , hER $\beta$ , and hAR, test agents were co-administered to the appropriate transfected cells with either 10<sup>-11</sup> M E<sub>2</sub>, 10<sup>-10</sup> M E<sub>2</sub>, or 10<sup>-10</sup> M 5 $\alpha$ -dihydroxytestosterone (DHT), respectively. Following incubation, expression of the response element-linked luciferase reporter was measured and normalized against that of the *Renilla* transfection control vector. Agonist activity was measured as the concentration showing 20% relative effective activity (REC<sub>20</sub>) as 10<sup>-10</sup> M E<sub>2</sub>, 10<sup>-9</sup> M E<sub>2</sub>, and 10<sup>-9</sup> M DHT at the hER $\alpha$ , hER $\beta$ , and hAR, respectively. Antagonist activity was expressed as the 20% relative inhibitory concentration (RIC<sub>20</sub>); that is, the concentration of test agent causing 20% inhibition of activity of 10<sup>-11</sup> M E<sub>2</sub>, 10<sup>-10</sup> M E<sub>2</sub>, or 10<sup>-10</sup> M at the hER $\alpha$ , hER $\beta$ , and hAR, respectively. Although not completely clear from the methods section of the paper, it appears that the authors deemed a test agent positive for agonist or antagonist activity when, at the range of concentrations tested (10<sup>-5</sup> to 10<sup>-6</sup> M), the test agent showed greater activity than the REC<sub>20</sub> or RIC<sub>20</sub>, respectively. The values presented in the paper are the mean and standard deviations derived from at least three independent experiments. Although glyphosate was tested, it was not identified as a chemical having agonist or antagonist activity at any of the three receptor sites evaluated. It must be noted that specific tests for cell toxicity were not conducted, although assays were conducted at concentrations  $\leq$  10<sup>-5</sup> M to minimize cytotoxicity. Based on these results, glyphosate does not appear to affect hormone binding at the hER $\alpha$ , hER $\beta$ , or hAR.

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In Walsh et al. (2000), researchers assessed whether glyphosate or Roundup could affect the synthesis of the steroidogenic acute regulatory (StAR) protein. The StAR protein, located on the outer mitochondrial membrane, transports cholesterol to the inner mitochondrial membranes (Granot et al., 2002). The study authors hypothesized that this protein might be particularly sensitive to environmental toxicants in general because its active precursor form is both highly labile and critically dependent on trophic hormone stimulation. Translocation of cholesterol across the mitochondrial membranes is a rate-limiting step in steroidogenesis, so slight disruptions of StAR function and/or synthesis can potentially cause adverse effects. In this study, the authors showed that Roundup (180 g/L glyphosate) significantly inhibited steroidogenesis (as seen by decreased progesterone production in MA-10 cells) by inhibiting StAR protein expression. The authors note that glyphosate alone, however, did not have any effect on steroidogenesis or protein production at any concentration tested (0-100 µg/mL), indicating that the effect on StAR was dependent on other components of the herbicide formulation.

Levine et al. (2007) investigated the potential role of the surfactant in a Roundup-branded formulation in the inhibition of progesterone production upon treatment of MA-10 mouse Leydig cells. In this study, MA-10 cells were exposed for two hours to various surfactants (LAS D-40 [a linear alkylbenzene sulfonate], alcohol ethoxylate, lauryl sulfate [SDS], and benzalkonium chloride), as well as a concentrated Roundup-branded Lawn and Garden herbicide (with 180 g/L glyphosate isopropylamine, and 6.53 g/L surfactant [primarily POEA]), and Roundup blank (formulation without glyphosate). Both the Roundup-branded formulation and Roundup blank decreased the hCG-stimulated increase in progesterone production. In both cases, the median inhibition concentration (IC<sub>50</sub>) was approximately 5 mg/mL. IC<sub>50</sub> values for the four other surfactants were similar to that of the Roundup branded formulation and Roundup blank, indicating that: 1) the effect on progesterone is largely attributable to the surfactant, and not glyphosate; and 2) surfactants, in general, decrease hCG-stimulated progesterone production. The impact of the various surfactants on StAR protein levels was also assessed by Western Blot analysis on hCG-stimulated and non-stimulated MA-10 cells. Exposure to the surfactants, Roundup-branded formulation, and Roundup blank resulted in decreased levels of the 30 kDa form of StAR protein, but not the 37 kDa precursor form. Because formation of the 30 kDa form requires mitochondrial import and processing of the 37 kDa precursor, the effect of treatment on mitochondrial potential, an indicator of proper mitochondrial membrane function, was measured using the JC-1 cationic dye. Treated MA-10 cells demonstrated a loss of normal mitochondrial membrane potential, meaning that proper import and processing of the 37 kDa form of the StAR protein was disrupted upon treatment. This finding explains the previously observed decrease in the 30 kDa form of the StAR protein. Additionally, this effect on mitochondrial membrane potential was made for benzalkonium chloride and the alcohol ethoxylate surfactants, the Roundup branded formulation, and Roundup blank at concentrations below those that affect steroidogenesis. Overall, these results strongly support the concept that the adverse effects of Roundup



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branded herbicidal formulations on steroidogenesis are not mediated by glyphosate exposure, but rather, by the effect of surfactants on unprotected cells in culture.

Richard et al. (2005) examined aromatase activity and mRNA levels in JEG3 cells (derived from a human placental choriocarcinoma cell line) exposed to pure glyphosate or unspecified Roundup. Because glyphosate affects the cytochrome P450 activity of plants (Lamb et al., 1998), the study authors hypothesized that mammalian aromatase (also a cytochrome P450 enzyme) could be adversely affected. Additionally, the authors wished to further investigate claims made in other studies that glyphosate and/or an unspecified Roundup branded formulation cause reproductive/ developmental problems. The "Roundup" formulation was diluted in water to concentrations of  $\leq 2\%$  based on the recommended concentration for agricultural use of 1-2% in water. Concentrations of pure glyphosate equivalent to those present in the range of Roundup dilutions tested were also used. Aromatase activity was measured at one and 18 hours post treatment by determining the amount of tritiated water released from the radiolabeled aromatase substrate,  $[1\beta\text{-}^3\text{H}]$ -androstendione. RT-PCR to amplify aromatase and GAPDH (as an endogenous control) mRNA was performed. General cell viability was also measured. Roundup had a more pronounced effect on cell viability than equivalent concentrations of pure glyphosate, indicating that the formulation ingredients played an important role in cytotoxicity, as discussed previously for in vitro systems where surfactants are added. Pure glyphosate did not affect aromatase activity at one or 18 hours at any concentration tested ( $\leq 0.8\%$ , or the highest dose at which marked cytotoxicity was not observed). Likewise, aromatase mRNA levels were unaffected by 18 hour treatment with  $\leq 0.1\%$  glyphosate. Incubation of the cells in Roundup for one hour, however, increased aromatase activity at all concentrations examined (0.02-0.2%). In contrast, incubation in Roundup for 18 hours caused a dose-dependent decrease in aromatase activity at all doses tested ( $\leq 0.8\%$ ). Levels of aromatase mRNA were also significantly decreased upon 18 hour incubation with 0.02 and 0.06% concentrations of Roundup. It was noted that, if glyphosate was combined with 0.02% Roundup, a greater decrease in aromatase activity after 18 hour incubation was seen than with 0.02% Roundup alone; however, the concentration of pure glyphosate used in this experiment was not indicated. The authors also measured aromatase activity in microsomes prepared from human full-term placental tissues incubated for 15 minutes with higher concentrations of Roundup and glyphosate ( $\leq 10$  and  $1.1\%$ , respectively). In this case, Roundup and glyphosate significantly decreased aromatase activity at concentrations of  $>0.05\%$  and  $\geq 0.5\%$ , respectively. Because significant cytotoxicity would not be expected at 15 minutes post treatment, the decrease in aromatase activity likely is not due to cell death. Based on additional experiments using microsomes derived from equine testis, the study authors conclude that the rapid decrease in microsomal aromatase activity is due to competitive inhibition; however, only data using Roundup are presented in the paper. Based on these results, the authors conclude that the additives in Roundup play a key role in its effect on aromatase, but that glyphosate itself can elicit toxic effects as well. Although it was shown that pure glyphosate added to Roundup further decreased aromatase activity, the concentration of glyphosate required

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to elicit this effect was not indicated. Finally, in interpreting such findings for human health risk assessment, one must consider that the internal glyphosate concentration anticipated to reach sensitive tissues is several orders of magnitude lower than those used in this study. Because these experiments were all conducted in an unvalidated *in vitro* system using physiologically irrelevant concentrations and the authors were thought to have greatly over-interpreted the results of their studies, the French Ministry of Agriculture and Fish concluded that the study of Richard et al. (2005) provided no information that was of use for human health risk assessment (Committee for Study of Toxicity, 2005). As discussed previously, it is now recognized that testing surfactant like substances in such a test system is not valid.

Despite the issues surrounding results reported by Richard et al. (2005), Benachour et al. (2007) conducted a similar study using both JEG3 cells and the human embryonic kidney 293 cell line. The effects of 1-2% concentrations of Roundup (~~formulation Bioforce<sup>®</sup> with~~ 360 g/l acid glyphosate) and equivalent glyphosate concentrations on cell viability and aromatase activity were assessed using cultures incubated in either serum-containing or serum-free media. The glyphosate solution used in many of these experiments was reported to have been pH adjusted to 5.8 (equivalent to the pH of 2% Roundup Bioforce solution). Following 1, 24 or 48 hours incubation, 293 cells were shown to be more sensitive to the cytotoxic effects of treatment than JEG3 cells; cells in serum-free media were more sensitive than those incubated in serum-containing media; and Roundup Bioforce was shown to be substantially more cytotoxic than glyphosate itself. In additional experiments, both Roundup Bioforce and glyphosate reduced aromatase activity in 293 cells cultured for 24 hours in serum-free medium and human placental microsomes treated for 15 minutes. Roundup Bioforce was also shown to affect aromatase activity in equine testicular microsomes and this effect appeared to be temperature responsive. The sensitivity of the cells incubated in serum-free media is not surprising. Serum supplementation of culture media provides cells with necessary nutrients and other protective elements. Along these lines, the authors report that cells grown in the absence of serum were not viable after 60 hrs, regardless of treatment. The authors interpret the results of their studies to suggest that glyphosate is cytotoxic and possesses endocrine-disrupting properties. Because many of these experiments were done using serum-free media and the pH of the glyphosate solution was only adjusted to be equivalent to that of Roundup and not to physiological pH, however, it is likely that many of the effects observed following treatment are due to changes in pH rather than a direct effect of glyphosate on the cells. Ideally, the pH of the glyphosate solution should have been adjusted to physiological pH for these experiments. Alternatively, a negative control treatment using media that was pH adjusted to 5.8 could have been included. Interestingly, in at least one of the experiments measuring the effects of Roundup treatment on aromatase activity in microsomal preparations, the pH of the Roundup was adjusted to physiological pH (7.4). Why the pH of the glyphosate solution was not similarly adjusted in these experiments is not clear. Given the confounding surfactant effects of damaging cell membranes, the value of these data is questionable.

Comment [drf2]: Roundup Bioforce is the name of the product

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Gasnier

A similar publications from Gilles Serailini's laboratory at the University of Caen, France by Gasnier et al. (2009), again focused exclusively on glyphosate and Roundup formulations in comparable test systems to their earlier publications. The same methodological flaws exist in this research, applying surfactants to cells in culture and treatment of cell lines in serum-free media. Their data interpretation not surprising, but incorrectly, suggests glyphosate based formulations exhibiting endocrine disrupting activity. To no surprise, glyphosate alone was innocuous. This is also reflected in the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) "Guidance on Identifying Endocrine Disrupting Effects" (2009), in which (i) glyphosate is exemplified as a non- endocrine disrupting substance and (ii) Richard et al. (2005) is discounted, citing methodological flaws.

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Hokanson et al. (2007) examined gene expression in MCF-7 cells in response to treatment with 0.0001 – 0.1% dilutions of a herbicidal formulation containing 15% glyphosate (exact formulation not specified). Following 18 hr exposure, the expression of 1,550 genes in treated and control cultures was evaluated using a DNA microarray platform. The authors report that 680 genes were either up-regulated or down-regulated in response to glyphosate treatment; however, it is not clear if the variability in gene expression of control cells was taken into account. The authors then examined expression of seven of the genes in more detail using quantitative PCR. In this analysis, only three of the seven genes evaluated continued to show up- or down-regulation; the other four failed to show dysregulation in response to treatment. The three genes that continued to demonstrate a treatment-related effect (hypoxia inducible factor 1 [HIF1], early growth response 1 [EGR1], and chemokine ligand 12 [CXCL12]) were said to also be affected by treatment with  $3 \times 10^{-10}$  M estrogen, which induced a response that was intermediate between that of control treatment and treatment with estrogen plus herbicide. The authors interpreted these results to mean that glyphosate treatment altered estrogen regulation of gene expression; however, it cannot be determined whether the gene response may be due to formulation ingredients besides glyphosate or an effect of treatment on pH of the cell culture media. Furthermore, no evidence exists in the study to suggest that the effect of herbicide treatment was mediated through an estrogen-related pathway.

**Summary - Endocrine Disruption**

Overall, these studies do not suggest that glyphosate is an endocrine disruptor. When tested alone, glyphosate was shown to be not estrogenic in a number of assay systems. Glyphosate did not activate the estrogen receptor or affect its ability to bind its normal endogenous ligand in either *in vitro* or *in vivo* test systems (Petit et al., 1997; Xie et al. 2005; Kojima et al., 2004); glyphosate also failed to displace estradiol from human sex hormone-binding globulin (Meulenberg, 2002). Although a Roundup-Roundup brand formulation was able to alter StAR protein function (Walsh et al., 2000), aromatase activity (Richard et al., 2005; Benachour et al., 2007), and inhibit progesterone production (Levine et al., 2007), these same effects generally were not observed when glyphosate was tested alone, suggesting that the responses were due to another component of

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the pesticide formulation – likely a surfactant as shown in the study by Levine et al. as a non-endocrine mechanism (2007). Finally, while both Round-up and its active ingredient, glyphosate, were able to induce the proliferation of estrogen-responsive MCF-7 cells in culture (Lin and Garry, 2000), the use of steroid growth factor-deficient serum suggested that this response was not mediated through an estrogenic pathway.

### **Reproductive Function**

Yousef et al. (1996) investigated the impact of glyphosate, as well as that of other pesticides, on the motility of human and rabbit sperm *in vitro*. This study was done, in part, to evaluate the utility of the motile rabbit spermatozoa assay as a test system for predicting human responses to male reproductive toxicants. The concentration of glyphosate used cannot be determined because the paper suggests that a glyphosate-based herbicide, and not pure glyphosate, was used in these experiments, and neither the commercial name nor the glyphosate concentration of this formulation is provided. Following incubation of sperm in varying concentrations of pesticides in either protein-free medium or medium containing bovine-serum albumin (BSA), a sperm motility index (SMI) was calculated. This index was based on the percentage of sperm that were motile and the motility grade of the sperm (with values ranging from zero in cases of no motility to four for cases of fast forward progressive movement). Fifteen minutes incubation in BSA medium containing what the authors report as 250, 500, or 1000  $\mu\text{M}$  of the glyphosate-based test solution resulted in rabbit SMI values of 2.4, 2.0, and 1.8, respectively, versus a control SMI of 3.5. In contrast, the glyphosate-based test solution administered in protein-free medium for 15 minutes resulted in a rabbit SMI value of 0, regardless of the concentration, versus a control SMI value of 2.7. Following 60-minute incubations with varying concentrations of the glyphosate-based test solution, the  $\text{IC}_{50}$  values for rabbit sperm were 23.3  $\mu\text{M}$  and 500  $\mu\text{M}$  in protein-free medium and BSA medium, respectively. Similarly, the  $\text{IC}_{50}$  values for human sperm motility were 48.2  $\mu\text{M}$  and 740  $\mu\text{M}$  in protein-free medium and protein-containing (BSA) medium, respectively. Although these results suggest that the protein present in BSA-containing medium partially protected the sperm from the harmful effects of treatment, little else can be concluded from this study. Because an herbicidal formulation was used rather than pure glyphosate, it is consistent with the aforementioned and reviewed studies that the observed results were due to the presence of surfactant rather than glyphosate. Furthermore, the study authors did not mention whether they corrected the pH of the media following the addition of the pesticides. Certainly, a pH outside the normal range would adversely impact sperm motility, regardless of treatment agent. Thus, the observed effects may have little to do with the actual agent administered in the study. Overall, this study provides no information regarding the potential adverse reproductive effects of glyphosate for men.

### **Conclusions**

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Overall, the aggregate of available mechanistic data does not provide a plausible mechanism of action by which glyphosate may cause adverse developmental or reproductive effects in humans. Many of these studies provide inadequate description of the test agent(s) – particularly, whether test systems were treated with pure glyphosate or a glyphosate-based commercial herbicide – and the final doses of glyphosate to which test models were exposed. These deficiencies make it impossible to determine whether the observed results can be attributed to glyphosate or another formulation ingredient, such as the surfactant. Furthermore, in the only study to test for this possibility (Levine et al., 2007), the results demonstrate that most of the observed effects are mediated through the surfactants present in the herbicidal formulations and consumer products. Finally, for the purposes of a human health risk assessment, these data provide very little relevant information. For one, the doses administered in these *in vitro* studies are substantially higher than those anticipated to be experienced as a result of dermal contact or oral ingestion of glyphosate. Additionally, these studies, by their very nature, do not take into account such factors as absorption, distribution, metabolism, and elimination, all of which play important roles in shaping human exposure responses. In conclusion, these data do not show a plausible and consistent mechanism by which glyphosate could cause developmental or reproductive problems in humans or animals.

**Comment [drf3]:** Most effects? What effects were observed that would not be considered related to the surfactants?

### EVALUATION OF BIOMONITORING DATA

Although the above hazard assessment for glyphosate fails to demonstrate any consistent evidence to indicate that glyphosate exposure may cause adverse developmental or reproductive health effects in humans, a review of the available biomonitoring data was considered pertinent to this evaluation in order to better understand the reasonably anticipated exposure levels for humans. To date, only a small body of biomonitoring data exists for assessing exposure levels associated with glyphosate field application (Table 12). These data are derived from studies looking at occupational pesticide levels in tree nursery workers (Lavy et al., 1992, 1993), those involved in the spray-clearing of brush (Cowell and Steinmetz, 1990a; Jauhainen et al. 1991), and members of farm and non-farm families (Baker et al., 2005; Acquavella et al., 2004, 2005; Mandel et al., 2005; Curwin et al., 2007a,b). Two other biomonitoring studies of glyphosate have been published (Abdelghani, 1995; Centre de Toxicology du Quebec, 1988), but neither study provides measures of individual systemic glyphosate concentrations, and thus, they are not discussed in this review. Studies that measured glyphosate exposures via passive dosimetry only (for example, on clothing, in air samples, or through hand-washes alone) were also excluded from analysis as these types of exposure measures do not provide a predictive indicator of internal dose.

In a study sponsored by the US Department of Agriculture, Cowell and Steinmetz (1990a) measured glyphosate concentrations in the urine of forestry workers involved in the mixing and backpack spray application of a Roundup herbicide at three different locations. Although all 16 workers were involved in spray

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application of the herbicide, only one worker at each site prepared and mixed the Roundup herbicide prior to application. Air samples from the breathing zone of each worker were collected using an air filter and portable pump. Passive monitoring was conducted using hand washes and gauze patches placed at various pre-determined locations on the workers' clothing. To determine the percent of clothing penetration, patches were also worn underneath the clothes at sites adjacent to those where outside patches were attached. Urine samples were collected on the day before, the day of, and three days following herbicide application. Twelve hour composite samples from each worker were analyzed. Following sample processing, glyphosate was quantified using high pressure liquid chromatography and fluorescence detection. The lower limit of method validation (LLOMV) was reported to be 0.01 µg/mL for the urine samples, 0.5 µg for each air filter, and 0.1 µg per patch. For the purposes of exposure assessment, data less than the LLOMV were assumed to be equal to ½ the LLOMV. Applicator body doses were calculated based on the first 72 hours following application. Only five of the 16 workers had measurable glyphosate concentrations in their urine on the day of application; all other urine samples were below the limits of detection. Based on analysis of the collected urine samples, the estimated average total body dose following spray application was 18.8 µg. In comparison, the estimated average total body dose based on passive dosimetry measures was 274 µg. These data show that passive dosimetry estimates are approximately one order of magnitude higher than those based on biological measures.

Jauhainen et al. (1991) measured glyphosate concentrations in air and urine samples from five workers employed in the spray-clearing of forest brush. Workers were involved in the daily mixing of their own herbicide sprays, wore limited protective equipment (primarily helmets and gloves), and did not have access to wash facilities during their workday. A control group of five forest workers involved in the planting of trees was also evaluated. Air samples from the breathing zone of the workers were taken daily for one week using a portable pump. Sampling times varied from one to six hours. Urine samples were collected over the test week at the end of each workday, as well as after a three-week follow-up period. Following sample processing, glyphosate concentrations were measured by gas chromatography, with a detection limit of 0.1 ng/µl (0.3 µg/m<sup>3</sup>). Mid-week air samples contained less than 1.25 µg glyphosate/m<sup>3</sup> air. The highest recorded air sample readings were 2.8 and 15.7 µg/m<sup>3</sup>. All urine glyphosate concentrations were below the limits of detection.

Lavy et al. (1992, 1993) measured glyphosate exposure levels among conifer seedling nursery workers.

Fourteen workers, including applicators, weeders, and scouts, were employed at two tree nurseries that used a Roundup herbicide. In this study, three different types of measurements were taken to assess potential and real exposures: dislodgeable residues, passive monitoring, and biological monitoring. To assess the amount of residual glyphosate that could be dislodged from conifer seedlings during contact with the plants, 100 gram samples of fresh seedlings were shaken and rinsed under water for 45 seconds each. These measurements were made twice weekly over four Spring/Summer months. Passive monitoring of exposures was conducted

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using gauze patches attached to the clothing of workers at nine potential exposure points and via hand-rinses of the workers taken at the end of the same workday. These measurements were taken one day per week over the entire course of study and composited for each day of measurement to provide total passive exposures for each worker. Biological monitoring involved collection of total daily urine for each worker over 12 consecutive weeks. Twenty-four hour samples were also collected once weekly for five months following the study period for each worker. Glyphosate concentrations were determined using the analytical procedures of Cowell and Steinmetz (1990b). The limit of detection for urine samples was 0.002 ppm and the lower limit of method validation was defined as 0.01 ppm. Of the 78 dislodgeable residue samples taken at 21 different sampling times, only one sample was positive for glyphosate residue, measuring 138.5µg glyphosate. This finding indicates that dislodgeable residues are not a significant source of glyphosate exposure for nursery workers. Passive exposure measurements indicated that ankles and thighs received the greatest exposure, with 98% of exposures occurring at or below the thigh. Applicators received greater exposures than weeders. Scouts showed minimal exposure, with only one of 23 hand washes and one of 34 composited patch samples being positive for glyphosate. Normalizing the composite exposure values for body weight and exposure period resulted in average exposure levels of  $7.2 \times 10^{-4}$ ,  $2.0 \times 10^{-4}$ , and  $1.6 \times 10^{-6}$  mg/kg/hr for applicators, weeders, and scouts, respectively. A total of 355 urine samples were analyzed from the 14 workers over the course of study; however, all samples were below the limits of detection for glyphosate. These results suggest that, despite the level of passive exposures measured, actual internal doses of glyphosate received by the workers were minimal to non-existent.

The Farm Family Exposure Study was initiated in 1999 and ultimately involved the biomonitoring of 95 families for glyphosate, 2,4-D, and chlorpyrifos exposure during years 2000 and 2001 (Baker et al., 2005; Acquavella et al., 2004, 2005; Mandel et al., 2005). Only the results related to glyphosate application are discussed herein. Families were randomly selected from listings of licensed pesticide applicators in South Carolina and Minnesota. Eligibility requirements were as follows: family had to consist of the farmer, spouse, and at least one child between the ages of four and eighteen; the family had to live on the farm and to farm at least ten acres within one mile of the home, onto which they planned to apply one or more of the study pesticides within the study period as a part of normal operations; the family members had to be willing to collect 24-hr urine samples over five days, starting one day prior to the pesticide application through three days following application. Parents filled out pre- and post-application questionnaires detailing family activities and application practices. Also, trained field staff were on hand to observe the pesticide application. Forty-eight of the 95 families provided specimens related to glyphosate application; these included specimens for 79 children. Urine samples were analyzed for glyphosate using chelation ion exchange to concentrate and isolate the pesticide, followed by high pressure liquid chromatography and fluorescence detection. Glyphosate findings were adjusted for recovery of the analyte using values obtained from spiked field- and travel-samples. Recovery was

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69% for a 10ppb sample and 78% for 100ppb samples. The detection limit was 1µg/l for a 100 ml urine sample.

Twenty-nine percent of the farmers applied glyphosate within one week prior to their participation in the Farm Family Exposure Study. Glyphosate was applied using a tractor and boom sprayer in all cases. Twenty-nine percent of these farmers did not wear rubber gloves during the application process, 15% spilled pesticide during the mixing and/or loading stages of application, and 27% worked on their equipment during the application process. Only 60% of farmers had detectable glyphosate levels in their urine on application day, the day of highest glyphosate readings. By three days post-application, this number had declined to 27%. Urine concentrations of glyphosate ranged from below the limit of detection to 233ppb. The geometric mean value for farmers was 3.2ppb on application day, and declined to 1.0ppb by post-application day 3. Use of rubber gloves had the greatest influence on urinary concentrations. Other factors associated with urine concentrations of glyphosate in the farmers included the number of times the farmers mixed and loaded the glyphosate, use of an open cab tractor, observed skin contact with the pesticide, and repair of the application equipment. The number of acres treated had no influence on urinary glyphosate concentrations.

Only two of 48 spouses had detectable glyphosate concentrations in their urine on application day. The highest urine concentration of glyphosate in a spouse was 3ppb. No spouses participated in the pesticide application process. Nine of 78 children had detectable glyphosate concentrations in their urine on the day of application; all but one of these were reported to either be present during or to have helped with the pesticide application. The highest glyphosate urinary value in a child was 29ppb.

Systemic doses of glyphosate were calculated for all participants with detectable urine glyphosate concentrations. For each individual, the total amount of glyphosate excreted during the study period was determined, adjusting for incomplete excretion and pharmacokinetic recovery; this value was then divided by each individual's body weight for determination of an individual's systemic dose. Using these calculations, the maximum systemic dose for farmers was estimated to be 0.004mg/kg and the geometric mean value was estimated to be 0.0001mg/kg. Maximum systemic doses for spouses and children were estimated to be 0.00004mg/kg and 0.0008mg/kg, respectively. These values are all well below the oral reference dose for glyphosate of 2mg/kg/day set by the U.S. Environmental Protection Agency (US EPA, 1993).

Curwin et al. (2007a,b) conducted a similar study of both farm and non-farm families residing in Iowa during the spring and summer of 2001. Exposure to seven target pesticides (atrazine, acetochlor, metolachlor, alachlor, chlorpyrifos, glyphosate, and 2,4-D) was examined; however, only the results for glyphosate are discussed herein. Study recruitment was done by convenience sampling. Study eligibility requirements were as follows: households had to reside in one of ten counties in central or eastern Iowa and have at least one child



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under the age of 16 years; non-farm families had to reside on land that was not used for farming and no one in the household could be employed in agriculture or the commercial application of pesticides; farm families had to use at least one of the seven target pesticides. Twenty-five farm families (66 farm children) and 25 non-farm families (52 non-farm children) were enrolled in the study. Each household was visited twice during the study period and two urine samples collected from participants at each visit (one from the evening and one from the following morning). Dust samples were collected during each visit according to standard practices established by the American Society for Testing Material (ASTM). Urine samples were kept cool, then shipped frozen to the laboratory, where they were analyzed for parent pesticides and metabolites by immunoassay. The limit of detection (LOD) for glyphosate was 0.9 µg/L. Urinary concentrations data were recorded as positive values at or above the LOD, positive values below the LOD, or non-detects. These data were then analyzed using two different approaches. In the maximum likelihood estimation, urinary concentrations reported as either non-detects or at levels below the LOD were set at the LOD for the assay. In the mixed-effects modeling approach, positive urinary concentrations below the LOD were used as reported and non-detects were set at one-half the lowest positive concentration measured. Urinary creatinine levels were also measured and used to normalize for total daily urinary voids when estimating daily pesticide exposures. Only 30% of absorbed glyphosate was assumed to be excreted in the urine and this information was used to correct for total glyphosate exposure.

In the case of glyphosate, urinary concentrations were above the limits of detection for 65-75% of the parent samples and for 81-88% of children's samples. Furthermore, farm and non-farm families did not significantly differ in their mean urinary concentrations of glyphosate. The study authors surmised that this may be because glyphosate use is not restricted to agricultural practices, but rather, may be commonly seen in residential settings as well. Geometric mean urinary concentrations of glyphosate (using the maximum likelihood model) were 1.4 µg/L (range: 0.13-5.4 µg/L) and 1.9 µg/L (range: 0.020-18 µg/L) in non-farm and farm fathers, respectively; 1.2 µg/L (range: 0.062-5.0 µg/L) and 1.5 µg/L (range: 0.10-11 µg/L) in non-farm and farm mothers, respectively; and 2.7 µg/L (range: 0.10-9.4 µg/L) and 2 µg/L (range: 0.022-18 µg/L) in non-farm and farm children, respectively. Mean urinary concentrations calculated using the mixed-effect model were similar. These estimated urinary concentrations of glyphosate from this study are all within the same approximate order of magnitude as those found in the Farm Family Health Study, discussed above.

Based on these data, the authors estimated the geometric mean doses of glyphosate for both farm and non-farm children. Again using the maximum likelihood model, the daily absorbed dose of glyphosate for farm children was estimated to be 0.11 µg/kg/day (range: 0.013-0.34 µg/kg/day). This was similar to the dose estimated for non-farm children: 0.13 µg/kg/day (range: 0.037-0.33 µg/kg/day). However, these values are approximately 8-fold lower than the 0.8 µg/kg/day glyphosate exposure estimated for farm children in the Farm Family Exposure Study and certainly lower than the oral reference dose for glyphosate of 2 mg/kg/day set by the US EPA (US EPA, 1993). The reason for the discrepancy in values between the two studies is not clear,

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but likely relates to differences in adjustments made to account for total urinary void and incomplete excretion of glyphosate.

**Summary – Biomonitoring Data**

The body of biomonitoring data available for glyphosate is limited at this time. Nevertheless, the data reviewed herein clearly show that the degree of systemic glyphosate exposure that occurs as a result of normal application practices is exceedingly small, often below the limits of detection (especially for those not intimately involved in the application process). In fact, the highest systemic dose estimated from these studies was 0.004mg/kg (Acquavella et al., 2004), a value 500 times below the daily oral reference dose for glyphosate of 2mg/kg/day (US EPA, 1993). These findings indicate that the risk of substantial exposure as a result of glyphosate application practices is minimal at best.

**CONCLUSIONS**

An extensive, in-depth analysis of the available scientific literature provides no evidence to indicate that exposure to glyphosate is associated with the potential to cause adverse developmental and reproductive effects in humans. While the body of epidemiological data for glyphosate is fairly limited, and none of the available studies were designed specifically to assess the potential effects of glyphosate exposure, the data as a whole reveal no developmental or reproductive health problems associated with exposure. A lone exception may be the study by Garry et al. (2002), which suggested that ADD/ADHD in children may be associated with parents' use of phosphonamino herbicides, ia class of herbicides that includes glyphosate; however, this paper was not included in the final evaluation because 1) there is wide non-uniformity of criteria used to classify such neurobehavioral disorders, and 2) the cases discussed in the paper were not confirmed diagnoses. Until additional study is done to specifically assess whether a causal relationship exists between parental exposure to glyphosate and various neurobehavioral disorders in children, the finding by Garry et al. (2002) should be given little, if any, weight in a evaluation of potential developmental effects.

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In contrast to the epidemiological data, the database of animal studies for glyphosate is relatively robust, including studies of mice, rats, and rabbits exposed to glyphosate, various glyphosate-based herbicidal formulations, the major glyphosate environmental breakdown product AMPA, and POEA, a surfactants included in some Roundup branded herbicides. Only one guideline-compliant study found potential effects associated with prenatal exposure to glyphosate (IRDC, 1980a). In this study, an increase in resorptions, a decrease in the number of fetuses per dam, and reduced fetal weights were found after gavage of pregnant rats with 3,500mg/kg/day on GD6-19. This treatment, which exceed the current limit dose by 350%, caused substantial maternal toxicity, however, suggesting that the developmental findings may be secondary to toxicity

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in the mother rather than a direct effect of glyphosate on the developing fetus. All other guideline-compliant studies reviewed found no effects of glyphosate treatment on reproductive health or the developing offspring at non-maternally toxic doses. (IRDC, 1980a, b; Holson, 1990, 1991; Schroeder, 1981, Reyna, 1990, JMPR; FAO/WHO, 1997, Knapp 2007; K Knapp 2008). It should be noted that, while a number of non-guideline-compliant studies have claimed developmental effects associated with glyphosate exposure (Dallegrave et al., 2003; Yousef et al., 1995; Dariuch et al., 2001; Beuret et al., 2004), these suffer from numerous inadequacies in design, which makes substantiation of their conclusions problematical. Furthermore, these studies all used commercially formulated glyphosate-based herbicides rather than pure glyphosate. Thus, findings reported in these studies cannot be definitively assigned to glyphosate exposure.

Comment [drf6]: This is the AMPA review.

Similarly, review of the available mechanistic data related to glyphosate fails to find a plausible mechanism of action by which glyphosate may be able to induce adverse developmental or reproductive outcomes. It should be noted, however, that the body of available studies suffers from numerous design inadequacies, particularly with regard to the type of test agents used (commercially available glyphosate-based herbicides versus pure glyphosate). Furthermore, other than hypothesizing possible mechanisms of action, these data provide little relevant information that can be used in a human health risk assessment.

Finally, a review of the limited body of available biomonitoring studies shows that, via reasonably anticipated exposure routes, human exposure to glyphosate is likely to be well below the daily oral reference dose for glyphosate of 2mg/kg/day, as set by the US EPA (US EPA, 1993). These data show that, regardless of any potential developmental and reproductive hazards that may be alleged based on misinterpretation of results from animal and mechanistic studies, the levels of glyphosate to which humans are likely to be exposed are far below the range of doses considered to be safe by the US and other regulatory agencies worldwide.

In conclusion, a thorough evaluation of the available data demonstrates that exposure to environmentally relevant glyphosate concentrations is not anticipated to cause adverse developmental and reproductive effects in humans.

### ACKNOWLEDGEMENTS

The authors would like to acknowledge the Monsanto Company for funding and for providing their unpublished glyphosate and surfactant toxicity study reports.

Comment [drf7]: Do you need to see a copy of the AMPA dev tox study or can you use the WHO review?

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**Table 1. Epidemiological Studies Assessing Glyphosate Exposure and Potential Developmental Effects.**

| <b>Study</b>          | <b>Agent</b>                                    | <b>Exposure</b>  | <b>Study Popl'n.</b>  | <b>Endpoints</b>                           | <b>Outcome</b>                                   |
|-----------------------|---|--|---|--|--|
| Savitz et al., 1997   | Glyphosate                                      | Male, self-reported (0-3 mo prior to conception)                         | 3,984 pregnancies<br><br>Ontario Farm Family Health Study (OFFHS)                           | Miscarriage, pre-term delivery, SGA births | No effect  |
| Arbuckle et al., 2001 | Glyphosate                                      | Male or female, self-reported (0-3 mo prior – 1 <sup>st</sup> trimester) | 395 spontaneous abortions<br><br>OFFHS  | Spontaneous abortion                       | No effect  |
| Bell et al., 2001a    | Phosphate pesticides                            | Female, via maternal address (1-20 wk gestation)                         | 74 fetal deaths due to congenital anomalies<br><br>20 wks gestation – 24 hr after birth     | Fetal death                                | Increased odds                                   |
| Bell et al., 2001b    | Phosphate pesticides                            | Female, via maternal address (1-20 wk gestation)                         | 413 fetal deaths due to causes other than above<br><br>20 wks gestation – 24 hr after birth | Fetal death                                | No effect  |
| Rull et al., 2006     | Glyphosate                                      | Female, via maternal address (peri-conception)                           | 731 NTDs (anencephaly, spina bifida cystica, other sub-types)                               | Neural tube defects                        | No effect  |
| Garry et al., 2002    | Herbicides, insecticides, fungicides, fumigants | Male or female, self-reported  | 1,532 live births   | Birth defects                              | Increased risk with exposure to all four classes |

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**Table 2. Epidemiological Studies Assessing Glyphosate Exposure and Potential Effects of Reproductive Health**

| <b>Study</b>          | <b>Agent</b>               | <b>Exposure</b>   | <b>Study Popl'n.</b>                                       | <b>Endpoints</b>   | <b>Outcome</b>                                      |
|-----------------------|----------------------------|---|--|--|---|
| Curtis et al., 1999   | Glyphosate                 | Male or female, self reported (0-2 mo prior through pregnancy attempts) | 2,012 planned pregnancies<br><br>OFFHS                     | Fertility  | ♀: Decrease<br>♂: Increase<br>Use on farm: Increase |
| Larsen et al., 1998   | "Spermatotoxic" pesticides | Male, self-reported (1 yr prior to child's birth)                       | 904 pregnancies  | Time to pregnancy  | No effect   |
| Greenlee et al., 2003 | Herbicides                 | Female, self-reported (2 yr prior to pregnancy attempts)                | 322 cases of female infertility                            | Infertility  | Increased   |
| Farr et al., 2004     | Glyphosate                 | Female, self-reported (12 mo previous)                                  | 3,103 premenopausal women<br><br>Agricultural Health Study | Menstrual cycle characteristics (cycle length, inter menstrual bleeding, missed periods) | No effect   |

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Table 9. Genotoxicity/Clastogenicity Studies Assessing the Effects of Glyphosate Exposure.

| Study                     | Assays   | Basic Experimental Design   | Findings  |
|---------------------------|--|---|---|
| Chruscielska et al., 2000 | Ames test; <i>in vivo</i> micronucleus assay                           | Ames test: Perzocyd 10 SL (0.6-1.2 mg /plate; 3 plates/conc.) tested for induction of reverse mutations in various strains of <i>Salmonella</i> with & without S9 metabolic enzymes.<br><i>In vivo</i> micronucleus assay: Mice (6/group and time point) injected with 300 mg glyphosate /kg BW or 90 mg Perzocyd 10 SL /kg BW, and bone marrow samples taken 2, 24, & 48 hrs following exposure; 1,000 polychromatic cells /group scored for micronuclei.  | Ames test: Negative at all concentrations, both with & without S9 activation.<br>Micronucleus assay: Neither Perzocyd nor glyphosate induced an increase in micronuclei.  |
| Monroy et al., 2005       | Cytotoxicity tests (cresyl violet and trypan blue stains); Comet assay | Acute cytotoxicity: GM38 and HT1080 cells exposed to 4.0-6.5 mM glyphosate for 4 hr at 37 °C; viability measured with trypan blue stain.<br>Chronic cytotoxicity: GM38 cells exposed to 0.9-8.5 mM glyphosate, and HT1080 cells exposed to 0.6-3.3 mM for 72 hrs at 37°C; viability assessed with cresyl violet stain.<br>Comet assay: GM38 and HT1080 cells treated with 4.0-6.5 mM glyphosate for 4 hours; 25 cells per slide evaluated for tail length.  | Acute cytotoxicity: 80% viability observed at 5.5 and 6.5 mM glyphosate in the HT1080 and GM38 cells, respectively.<br>Chronic cytotoxicity: 80% cell viability reported at 0.6 mM and 5.5 mM glyphosate in the HT1080 and GM38 cells, respectively; 50% viability observed at 1.7 and 6.9 mM in the HT1080 and GM38 cells, respectively.<br>Comet assay: Average DNA migration distances significantly increased in cells treated with >4 mM and >4.75 mM glyphosate in GM38 and HT1080 cells, respectively. |
| Çavaş and Könen, 2007     | Micronucleus assay; Comet assay  | Goldfish treated to Roundup at concentrations equal to 0-15 ppm glyphosate for 2, 4, or 6 days (5 fish/concentration and duration).<br>Micronucleus assay: 1,500 peripheral erythrocytes/slide (5 slides/fish) scored manually.<br>Comet assay: 200 cells/slide (5 slides/fsh) scored manually under 400x magnification.  | Micronucleus assay: At 2 days, increased micronuclei at 15 ppm; at 4 and 6 days, dose-dependent increased micronuclei at all concentrations.<br>Comet assay: Dose-dependent increased DNA damage at all doses and durations of exposure.<br><i>Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate.</i>   |
| Poletta et al., 2009      | Micronucleus assay; Comet assay  | Caimans treated to 50-1,750 µg Roundup <i>in ovo</i> .<br>Micronucleus assay: 1,000 erythrocytes per slide (2 slides/animal) scored manually.<br>Comet assay: 50 cells per slide (2 slides/animal) scored manually for DNA damage on a 0-4 scale  | Micronucleus assay: Micronuclei increased at 500µg Roundup and above.<br>Comet assay: DNA damage increased at 500µg Roundup and above.<br><i>Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate.</i>   |
| Dimitrov et al., 2006     | Chromosomal aberration (CA) test; micronucleus assay                   | For plant assays, <i>Crepis capillaries L.</i> plants treated to 0.05-1% Roundup for 2 hrs, washed 1 hr, treated with colchicines, and fixed. For mammalian assays, 8 C57Bl mice/ group treated to 1/8, 1/4 or 1/2LD <sub>50</sub> of Roundup for 6-120 hrs.<br>CA test: For plants, 400 cells and 50 metaphases per slide per treatment evaluated. For mice, 50 metaphase polychromatic erythrocytes per animal evaluated.<br>Micronucleus assay: For plants, 4,000 cells and 1,000 interphases per treatment evaluated. For mice, 500 bone marrow cells per animal evaluated. | CA test: No increase in CAs with Roundup treatment.<br>Micronucleus assay: Slight, but non-statistically increased incidence of micronuclei in Roundup-treated plants; no increase in micronuclei in mice bone marrow cells.  |

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|------------------------------------|---|---|--|
| Lueken et al.,<br>2004             | Comet assay;<br>MTT<br>cytotoxicity<br>assay            | GM5757 cells treated to 40 and/or 50 µM H <sub>2</sub> O <sub>2</sub> plus various other chemicals for 1 hr in culture to assess any synergistic adverse impact on human fibroblast genotoxicity.<br>Comet assay: 25 cells per slide (2 slides per treatment) were evaluated.<br>MTT assay: Mitochondrial function measured by conversion of the yellow MTT tetrazolium salt to a blue, soluble formazan.                               | >75 mM glyphosate alone was noncytotoxic and nongenotoxic, but increased the genotoxicity of 40 and 50 µM H <sub>2</sub> O <sub>2</sub> .  |
| Piešová,<br>2004                   | Micronucleus<br>assay                                   | Bovine peripheral lymphocytes (from 2 donors) exposed to 28-1120 µM/l glyphosate-containing herbicide for 24 and 48 hrs; micronuclei formation assessed microscopically in at least 1000 binucleated lymphocytes.   | 24 hrs: No increase in micronuclei formation.<br>48 hrs: Micronuclei formation increased at 280 µmol/l (but not at 560 µmol/l) in one donor and only at 560 µmol/l in the other donor.<br><i>Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate.</i>  |
| Šiviková and<br>Dianovský,<br>2006 | CA test; sister<br>chromatid<br>exchange<br>(SCE) assay | Bovine peripheral lymphocytes (from 2 donors) exposed to 28-1120 µM/l glyphosate-containing herbicide for 24, 48, and 72 hrs at 36°C.<br>CA test: CAs measured in 100 metaphase cells after 24 hrs exposure.<br>SCE assay: SCEs examined microscopically in 50-100 metaphases after 24, 48, and 72 hrs exposure.  | CA test: No increase in treated cells.<br>SCE assay: Dose dependent increase in cells treated with 140 and 240 µmol/l herbicide at 2 hrs (higher concentrations not examined), 56-1120 µmol/l herbicide after 24 hrs exposure, and >280 and 560 µmol/l after 48 hrs of exposure.<br><i>Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate.</i>  |
| Kaya et al.,<br>2000               | <i>Drosophila</i><br>wing spot test                     | Glyphosate (0.1-10 mM) tested for ability to induce wing spots using standard and high bioactivation crosses of <i>Drosophila melanogaster</i> ; wings examined under 400X magnification.   | Standard cross: An increase in wing spots was observed at 2, 5, and 10 mM glyphosate.<br>High bioactivation cross: glyphosate had no impact on prevalence of wing spots.<br><i>Whether human metabolism is better represented by standard or high bioactivation cross is unknown, making extrapolation of results to humans difficult.</i>   |
| Mañas et al.,<br>2008              | Comet assay;<br>CA test;<br>micronucleus<br>test        | Comet assay: Hep-2 cells treated with 2.5-10 mM AMPA for 4 hrs; 100 nucleoids per treatment evaluated.<br>CA test: Human lymphocytes treated in vitro to 0.9 and 1.8 mM AMPA for 48 hrs; 2,000 cells per treatment assessed for mitotic index; 100 metaphases assessed for CAs.<br>Micronucleus test: Balb-c mice (5/group) treated with 2 x 100 or 200 mg/kg ip doses of AMPA; 24 hrs later, 1,000 bone marrow erythrocytes evaluated. | Comet assay: A dose-dependent increase in DNA damage observed with AMPA treatment.<br>CA test: 1.8 mM, but not 0.9 mM AMPA increased CAs.<br>Micronucleus test: Non-dose dependent increased incidence of micronuclei observed with AMPA treatment.<br><i>The AMPA concentrations and doses tested are 1,000-fold or more above those reasonably anticipated in the environment, making extrapolation of these results for human health risk assessment difficult.</i> |

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Table 10. Sea Urchin Embryo Assays Assessing the Ability of Glyphosate to Inhibit Cell Cycle Progression and Transcription.

| Study               | Basic Experimental Design   | Findings  |
|---------------------|---|---|
| Medina et al., 1994 | 20 µl suspension of fertilized sea urchin eggs exposed to 480 g/L Roundup (containing $1.4 \times 10^{-4}$ M glyphosate) 3 minutes after appearance of the fertilization membrane; eggs observed for ~24 hrs until the pluteus (free swimming larvae) stage.  | Roundup-treated eggs exhibited deformed or destroyed nuclear elements, as well as a perforated nuclear membrane.<br><br><i>Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate. Also, concentrations of Roundup used in this study are not environmentally relevant.</i>  |
| Marc et al., 2002   | Effects of 0.8% Roundup (containing 8 M glyphosate), 8mM pure glyphosate, and 0.2% Roundup supplemented with concentrations of glyphosate up to 10 mM, on the 1 <sup>st</sup> cell division in sea urchin embryos were investigated; ~100 embryos were scored per treatment group. The kinetics of CDK/cyclin B activation were also measured using H1 protein as a substrate.  | Roundup exposure was associated with an increase in first cell division delay, and inhibits CDK/cyclin B activation; 8 mM glyphosate had no impact on urchin cell division; supplemental glyphosate added to 0.2% Roundup induced cell division delay, but no dose-response relationship was observed.<br><br><i>Concentrations of Roundup and glyphosate used in this study are not environmentally relevant.</i>  |
| Marc et al., 2003   | Impact of Roundup exposure on CDK/cyclin B activation at selected times following fertilization ( $\leq 120$ minutes) investigated using H1 histone as a phosphorylation substrate.   | Authors report that Roundup blocked CDK/cyclin B activation, but urchins underwent cell division, albeit delayed.<br><br><i>Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate. Also, concentrations of Roundup used in this study are not environmentally relevant.</i>   |
| Marc et al., 2004a  | Effect of various concentrations of glyphosate-containing herbicides (Roundup3Plus, Amega, Cargly, Cosmic, and Roundup Biovert) on time of 1 <sup>st</sup> cell division post-fertilization assessed.   | All herbicides tested delayed the 1 <sup>st</sup> cell division in a dose-dependent manner, but response across herbicides was independent of glyphosate concentration.<br><br><i>Because herbicidal formulations were tested, findings cannot be specifically attributed to glyphosate. Also, herbicide concentrations used in this study are not environmentally relevant.</i>  |
| Marc et al., 2004b  | Whether 10 mM Roundup inhibits CDK/cyclin B activation by preventing dephosphorylation of CDK1/cyclin B tyrosine 15 complex was examined using affinity purification and Western blot analysis; cells were examined at the time of 1st cell division post-fertilization. The effects of 10 mM pure glyphosate and Roundup 3 plus (at a conc. equiv. to 10 mM glyphosate) on phosphatase activity of the cdc25C recombinant protein and embryo extracts were also assessed.  | Roundup 3Plus exposure caused a 30 min. delay in CDK1 tyrosine phosphorylation and was associated with a 70% inhibition of DNA synthesis; neither 10 mM pure glyphosate nor the concentration of Roundup 3Plus (containing 10 mM glyphosate) was associated with a change in cdc25C protein.<br><br><i>Because only the herbicidal formulation was tested for effects on CDK1 phosphorylation, the findings cannot be specifically attributed to glyphosate. Also, concentrations of glyphosate and Roundup used in this study are not environmentally relevant.</i>  |
| Marc et al., 2005   | Impact of 0.2%, 0.4 %, and 0.6% Roundup (containing 2, 4, and 6 mM glyphosate), 30-900 mg/L POEA, and 0.2% Roundup with 8 mM supplemental glyphosate on the percentage of embryos hatching, and the delay in hatching time was observed with phase contrast microscopy. Transcriptional activity of embryo suspensions exposed to 0.2%, 0.4%, and 0.6% Roundup quantified by incorporation of 5-[3H] undine. Expression of sea urchin hatching enzyme mRNA (SgHE) in urchins exposed to 1% Roundup (10 mM glyphosate) measured by RT-PCR. | 0.2-0.6% Roundup was associated with a dose-dependent decrease in percentage of embryos hatching, and an increase in hatching delay; addition of 8 mM glyphosate to 0.2% Roundup increased the hatching delay (but no statistics are provided to show that this is significant) and 8 mM glyphosate alone had no effect; a dose-dependent decrease in urchin embryo transcription seen with 0.1-0.8% Roundup; 30-900 mg/L POEA led to irreversible embryonic damage or lethality.<br><br><i>Concentrations of Roundup and glyphosate used in this study are not environmentally relevant. Also, data suggest POEA, not glyphosate, is responsible for adverse effects of Roundup to sea urchin embryos.</i> |



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Table 11. Mechanistic Studies Assessing the Potential Endocrine-Disrupting Effects of Glyphosate Exposure.

| Study                  | Basic Experimental Design  | Findings  |
|------------------------|--|---|
| Petit et al., 1997     | Recombinant yeast system expressing the estrogen receptor (ER); Estrogenic potential of various chemicals, including $10^{-3}$ to $10^{-4}$ M glyphosate, tested in yeast cells expressing the rainbow trout ER linked to a <i>lacZ</i> reporter gene; cells treated to test agents for 4 hrs.   | Glyphosate did not demonstrate estrogenic activity.   |
| Lin and Garry, 2000    | Estrogen-responsive MCF-7 cells. Response of MCF-7 cells to Roundup or glyphosate exposure assessed; cell proliferation after a seven-day exposure period in presence and absence of steroid growth factor-deficient FBS examined by flow cytometry; cell viability and apoptosis examined after 72 hr incubation by flow cytometry and propidium iodide.  | Cell proliferation increased with exposure to both Roundup and glyphosate, but response was similar with and without FBS, suggesting it was mediated through a non-estrogenic pathway; no cytotoxicity or apoptosis observed due to glyphosate exposure.  |
| Meulenberg, 2002       | Displacement of estradiol ( $E_2$ ) from human sex hormone binding globulin (SHBG); Displacement of tritiated $E_2$ from SHBG by different concentrations of various test agents (including glyphosate) measured <i>in vitro</i> .   | Glyphosate reported to have shown ambiguous results for $E_2$ displacement from SHBG.   |
| Xie et al., 2005       | Rainbow trout vitellogenin assay: Ability of 0.11 mg/L glyphosate and other herbicides to induce vitellogenin expression in trout assessed.  | Glyphosate was not found to have estrogenic activity in this assay.   |
| Kojima et al., 2004    | Human ER $\alpha$ , ER $\beta$ , and androgen receptor (AR) binding: Over 200 pesticides were tested for agonist or antagonist activity at human ER $\alpha$ , ER $\beta$ , and AR transfected into Chinese hamster ovary cells; $< 10^{-5}$ M glyphosate tested.  | Glyphosate was not noted to affect hormone binding in any of the receptor subtypes tested.  |
| Walsh et al., 2000     | Steroidogenic acute regulatory (StAR) protein synthesis: Impact of Roundup (with 180g/L glyphosate) and other herbicides on steroidogenesis in MA-10 Leydig tumor cells was assessed by measuring progesterone production by radioimmunoassay; levels of StAR mRNA assessed using Northern blots.  | 20-100 $\mu$ g/ml Roundup, but not pure glyphosate, caused a significant dose-dependent decrease in progesterone production; 25 $\mu$ g/ml Roundup did not influence overall protein levels, but decreased levels of StAR mRNA.   |
| Levine et al., 2007    | Inhibition of progesterone production in MA-10 mouse Leydig cells: MA-10 cells were exposed for 2 hr. to Roundup with and without glyphosate, as well as to various surfactants; the hCG-stimulated increase in progesterone production was measured following incubation; impact of surfactants on StAR protein levels was assessed by Western Blot on hCG-stimulated and non-stimulated MA-10 cells; impact of treatment on mitochondrial membrane function was determined by JC-1 cationic dye. | Exposure to surfactants, as well as to Roundup with and without glyphosate, was associated with a decrease in hCG-progesterone production, decreased expression of the StAR protein, and a decrease in mitochondrial membrane function.   |
| Richard et al., 2005   | Aromatase activity and mRNA levels in JEG3 cells and placental and equine testicular microsomes: Aromatase activity in JEG3 cells treated 1 and 18 hrs with 0.2-2% Roundup (or corresponding concentrations of glyphosate) measured by radioimmunoassay; aromatase mRNA expression measured by RT-PCR. Aromatase activity in microsomes from full-term placentas and equine testes also assessed upon 15 min exposure to Roundup or glyphosate.  | JEG3 cells: 0.2-2% Roundup has significantly greater impact on cell viability than glyphosate of corresponding concentrations; aromatase activity significantly increased at 1 hr and significantly decreased at 18 hrs after exposure to 0.01% Roundup; aromatase mRNA also decreased at 18 hrs following Roundup exposure; $\leq 0.8\%$ glyphosate for 1 or 18 hrs had no effect on aromatase activity.<br>Microsomes: Aromatase activity decreased at $>0.05\%$ Roundup and $>0.5\%$ glyphosate.<br><br><i>Concentrations of Roundup and glyphosate used in this study are not environmentally relevant.</i> |
| Benachour et al., 2007 | Aromatase activity in JEG3 and human embryonic kidney 293 cells and placental and equine testicular microsomes: Cell viability and aromatase activity following 1, 24 or 48 hr treatment with 1-2% Roundup or equivalent concentrations of glyphosate assessed as above; cultures treated in either serum-containing or serum free media   | 293 cells were more sensitive than JEG3 cells; cells in serum-free media were more sensitive than those in serum-containing media; Roundup was substantially more cytotoxic than glyphosate; Roundup decreased aromatase activity in microsomes in temperature-responsive manner.<br><br><i>Concentrations of Roundup and glyphosate used in this study are not environmentally relevant. Also, the pH of the test agents were not adjusted appropriately.</i>  |
| Hokanson et al., 2007  | Gene expression in MCF-7 cells: Gene expression following 18 hr exposure to 0.001-0.1% of a glyphosate-containing herbicide was assessed by DNA microarray and RT-PCR.   | Treatment altered gene expression, but of seven genes selected for further study, dysregulation was confirmed by RT-PCR for only three.   |

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|  |  | <i>Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate. Also, no evidence indicates that these changes were mediated through endocrine-disruption.</i> |
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Table 12. Estimated Glyphosate Doses Associated with Herbicide Application.

| Study                      | Sample size          | Dosimetry method               | Estimated glyphosate dose | LLOMV <sup>a</sup>        |
|----------------------------|----------------------|--------------------------------|---------------------------|---------------------------|
| Spray-clearing of brush    |                      |                                |                           |                           |
| Cowell and Steinmetz, 1990 | 16                   | Urinalysis (5/16 participants) | 18.8 µg                   | 0.01 µg/mL                |
|                            |                      | Passive (patch)                | 274 µg                    | 0.1 µg/patch              |
| Jauhainen et al., 1991     | 5                    | Urinalysis                     | ND <sup>b</sup>           | 0.1 µg/mL <sup>c</sup>    |
|                            |                      | Passive (air)                  | ≤15.7 µg/m <sup>3</sup>   | 0.3 µg/m <sup>3c</sup>    |
| Tree nursery work          |                      |                                |                           |                           |
| Lavy et al., 1992, 1993    | 14                   | Urinalysis                     | ND                        | 0.01 µg/mL                |
| Farm and non-farm families |                      |                                |                           |                           |
| Acquavella et al., 2004    | 48 farmers           | Urinalysis                     | 4 µg/kg <sup>d</sup>      | 0.001 µg/mL <sup>c</sup>  |
|                            | 48 spouses           |                                | 0.04 µg/kg <sup>d</sup>   |                           |
|                            | 78 children          |                                | 0.8 µg/kg <sup>d</sup>    |                           |
| Curwin et al., 2007a,b     | 65 farm children     | Urinalysis                     | 0.11 µg/kg <sup>e</sup>   | 0.0009 µg/mL <sup>c</sup> |
|                            | 51 non-farm children |                                | 0.13 µg/kg <sup>e</sup>   |                           |

<sup>a</sup>LLOMV = Lower limit of method validation

<sup>b</sup>ND = Not detectable

<sup>c</sup>Assay detection limit

<sup>d</sup>Based on highest reading registered

<sup>e</sup>Based on maximum likelihood model

1 ppb = 1 µg/L = 1 µg/1000ml = 0.001 µg/mL

## 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  $\mu\text{m}$ ) and intensity (2.19%) for 580  $\mu\text{g}/\text{ml}$ , and increased tail intensity (1.88%) at 92.8  $\mu\text{g}/\text{ml}$ , compared to control values of 18.15  $\mu\text{m}$  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  $\mu\text{g}/\text{ml}$ . Using the hOGG1 comet assay, a significant increase in tail intensity was observed at 2.91  $\mu\text{g}/\text{ml}$  with S9 and 580  $\mu\text{g}/\text{ml}$  without S9. Without S9, the frequency of micronuclei, nuclear buds and nucleoplasmic bridges slightly increased at concentrations 3.5  $\mu\text{g}/\text{ml}$  and higher. The presence of S9 significantly elevated the frequency of nuclear instabilities only for 580  $\mu\text{g}/\text{ml}$ . FRAP values slightly increased only at 580  $\mu\text{g}/\text{ml}$  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. © 2009 Wiley-Liss, Inc.

**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-enolpyruvylshikimate-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, sternbra 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 Dickinson, 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  $\mu\text{g}/$

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<sub>50</sub> (Annex I, EU directive 91/414/EEC) and 1/16 of oral LD<sub>50</sub> 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 hr at 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  $\mu\text{l}$  of 1 mM H<sub>2</sub>O<sub>2</sub> 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  $\mu\text{g}/\text{ml}$  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  $\mu\text{g}/\text{ml}$  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 microliters of treated blood was mixed with 50  $\mu\text{l}$  of a solution of acridine orange (100  $\mu\text{g}/\text{ml}$ ) and ethidium bromide (100  $\mu\text{g}/\text{ml}$ , 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 $\times$  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  $\mu\text{l}$ ) 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 $\times$  Flare buffer (Trevigen) for 5 min at room temperature. Afterwards, hOGG1 was added to the gel in 100  $\mu\text{l}$  of Flare reaction buffer dilutions (1:4000), as described by Smith et al. [2006]. Simultaneously, for each concentration parallel slides were treated with 100  $\mu\text{l}$  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<sub>2</sub>EDTA, 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  $\mu\text{g}/\text{ml}$ ). Each slide was examined using a 250 $\times$  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 Surrallés 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 anti-fade 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-triazine (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-tetra-methoxypropane (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**

| Concentration<br>µg/ml | -S9                  |                     |                    | +S9                  |                     |                    |
|------------------------|----------------------|---------------------|--------------------|----------------------|---------------------|--------------------|
|                        | Early<br>apoptosis % | Late<br>apoptosis % | Necrosis %         | Early<br>apoptosis % | Late<br>apoptosis % | Necrosis %         |
| 0.50                   | 9.0                  | —                   | 0.5                | 10.5                 | 1.0                 | 2.5                |
| 2.91                   | 11.0                 | —                   | 2.0                | 13.5 <sup>*</sup>    | 0.5                 | 3.0                |
| 3.50                   | 13.0                 | —                   | 3.0                | 20.0 <sup>**</sup>   | 3.0                 | 2.5                |
| 92.8                   | 14.2                 | —                   | 3.4                | 23.1 <sup>**#</sup>  | 2.8                 | 2.7                |
| 580                    | 19.5 <sup>**</sup>   | —                   | 5.5 <sup>**</sup>  | 30.0 <sup>**#</sup>  | 2.0                 | 6.5 <sup>*</sup>   |
| Positive control       | 21.0 <sup>**</sup>   | 30.5 <sup>**</sup>  | 19.0 <sup>**</sup> | 17.5 <sup>**</sup>   | 36.0 <sup>**</sup>  | 14.5 <sup>**</sup> |
| 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<sub>2</sub>O<sub>2</sub> 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)**

| Concentration<br>µg/ml | Alkaline comet assay         |                                |                                |                                |
|------------------------|------------------------------|--------------------------------|--------------------------------|--------------------------------|
|                        | -S9                          |                                | +S9                            |                                |
|                        | Tail length<br>(µm) ± SD     | Tail intensity<br>(% DNA) ± SD | Tail length<br>(µm) ± SD       | Tail intensity<br>(% DNA) ± SD |
| 0.50                   | 18.3 ± 2.11                  | 1.32 ± 1.96                    | 20.03 ± 3.48                   | 3.11 ± 3.93 <sup>#</sup>       |
| 2.91                   | 18.2 ± 3.41                  | 1.57 ± 2.34                    | 20.32 ± 4.21                   | 3.27 ± 4.07 <sup>#</sup>       |
| 3.50                   | 17.11 ± 2.34                 | 1.80 ± 2.85 <sup>**</sup>      | 22.08 ± 5.83 <sup>**#</sup>    | 3.46 ± 4.55 <sup>#</sup>       |
| 92.8                   | 17.92 ± 2.56                 | 1.88 ± 2.71 <sup>**</sup>      | 23.44 ± 5.97 <sup>**#</sup>    | 3.59 ± 4.62 <sup>#</sup>       |
| 580                    | 20.39 ± 4.63 <sup>**++</sup> | 2.19 ± 3.88 <sup>**</sup>      | 26.12 ± 7.92 <sup>**++##</sup> | 4.69 ± 5.36 <sup>**++##</sup>  |
| Positive control       | 41.22 ± 25.73 <sup>**</sup>  | 29.80 ± 9.58 <sup>**</sup>     | 35.84 ± 17.32 <sup>**</sup>    | 31.15 ± 10.48 <sup>**</sup>    |
| 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<sub>2</sub>O<sub>2</sub> 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)**

| Concentration<br>µg/ml | hOGG1 Comet assay          |                                |                            |                                |
|------------------------|----------------------------|--------------------------------|----------------------------|--------------------------------|
|                        | -S9                        |                                | +S9                        |                                |
|                        | Tail length<br>(µm) ± SD   | Tail intensity<br>(% DNA) ± SD | Tail length<br>(µm) ± SD   | Tail intensity<br>(% 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 <sup>#</sup>       |
| 3.50                   | 1.0 ± 0.83                 | 1.15 ± 1.61 <sup>-</sup>       | 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 <sup>†</sup>       |
| 580                    | 0.6 ± 0.2                  | 0.41 ± 0.40                    | 1.9 ± 0.37 <sup>**++</sup> | 0.63 ± 0.94 <sup>#</sup>       |
| Positive control       | 10.93 ± 4.38 <sup>**</sup> | 12.57 ± 7.62 <sup>**</sup>     | 11.13 ± 3.78 <sup>**</sup> | 15.22 ± 8.10 <sup>**</sup>     |
| 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.

<sup>-</sup>*P* < 0.05; \*\**P* < 0.01 vs. control; <sup>†</sup>*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<sub>2</sub>O<sub>2</sub> 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

| Concentration<br>µg/ml | MN                |      |         | NB                |        | NPB               |        | CBPI  |
|------------------------|-------------------|------|---------|-------------------|--------|-------------------|--------|-------|
|                        | Mean/1,000<br>BNC | % C+ | % DAPI+ | Mean/1,000<br>BNC | % C+   | Mean/1,000<br>BNC | % C+   |       |
| 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<br>µg/ml | MN     |          |           | NB      |          | NPB    |          | CBPI  |
|------------------------|--------|----------|-----------|---------|----------|--------|----------|-------|
|                        | Σ      | % C+     | % DAPI+   | Σ       | % C+     | Σ      | % C+     |       |
| 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-



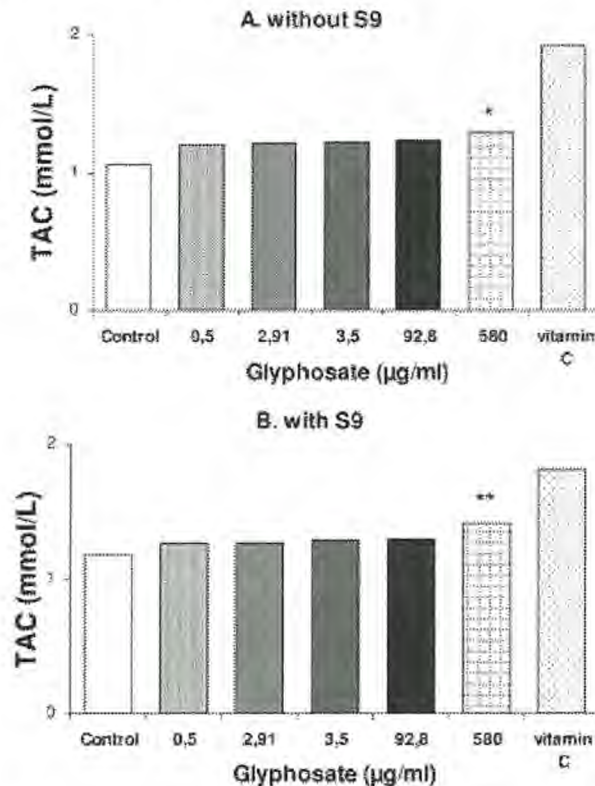


Fig. 1. Changes in plasma total antioxidant capacity (TAC) following treatment with glyphosate alone (A) or in combination with metabolic activation system S9 (B). Values are mean  $\pm$  SD,  $n = 3$ . \* Significantly different from control without S9 ( $P < 0.05$ ). \*\* Significantly different from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

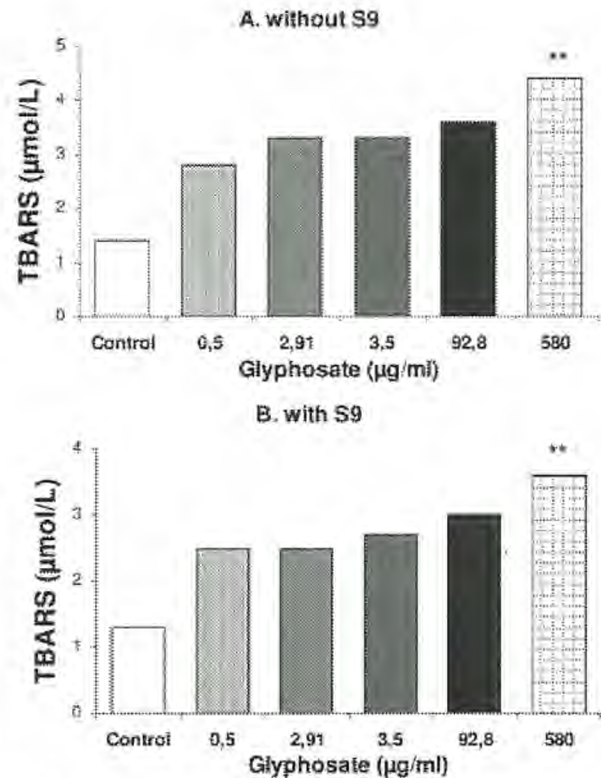


Fig. 2. Changes in plasma lipid peroxidation following treatment with glyphosate alone (A) or in combination with metabolic activation system S9 (B). Lipid peroxidation was determined by level of thiobarbituric reactive substances (TBARS). \*\* Significantly different from control with and without S9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

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                    |
|------------------------------|--------------------------------|-------------------------------|---------------|----------------------------|
| <i>E. coli</i>               | WP-2 reversion assays          | Active ingredient             | Negative      | Li and Long [1988]         |
| <i>Bacillus subtilis</i>     | 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,  $C_3H_5NO_5P$ ), 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.

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## 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

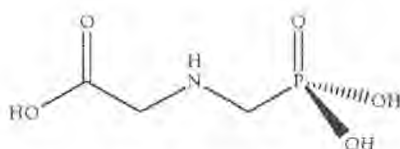


FIGURE 1: Chemical structure of glyphosate.

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* tadpoles 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,  $18 \pm 2$  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,  $25 \pm 2^\circ\text{C}$  temperature, and  $57 \pm 7\%$  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 intraperitoneally (*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 I 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  $37^\circ\text{C}$  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  $\pm$  SE) for all groups. Statistical comparisons were made using Student's *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.

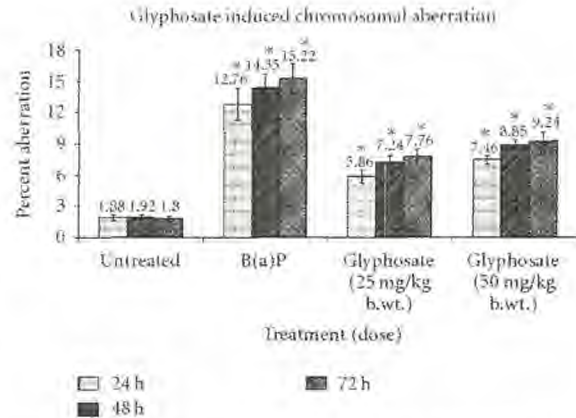


FIGURE 2: Mutagenic activity of glyphosate in Swiss albino mice showing incidence of aberrant cells at sampling time of 24, 48, and 72 hours. Values are expressed as mean  $\pm$  SE of 5 animals. \*Represent significant increase over untreated control group at their respective sampling time. Data are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

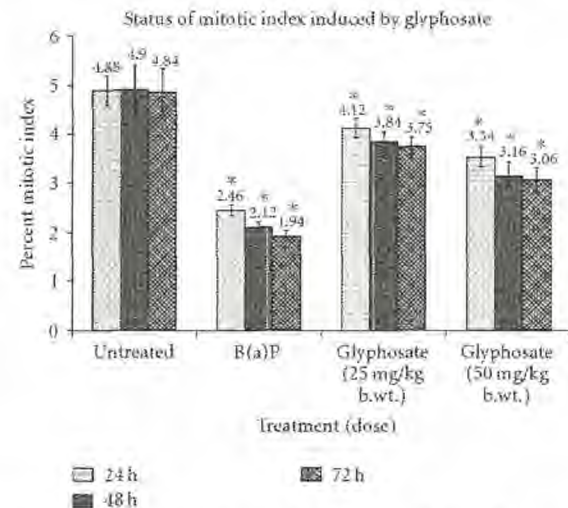


FIGURE 3: Cytotoxic effects of glyphosate in Swiss albino mice indicated by decrease in mitotic index (MI) at 24, 48, and 72 hours of sampling time. Values are expressed as mean  $\pm$  SE of 5 animals. \*Represent significant decrease over untreated control group at their respective sampling time. Data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

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

| Groups   |                                | Untreated   | B(a)P<br>(100 mg/kg b.wt) | Glyphosate<br>(25 mg/kg b.wt) | Glyphosate<br>(50 mg/kg b.wt) |
|--|--------------------------------|-------------|---------------------------|-------------------------------|-------------------------------|
| 24 hours<br>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<br>aberrant cells | 1.81 ± 0.03 | 12.76 ± 0.17*             | 5.86 ± 0.12*                  | 7.46 ± 0.14*                  |
| Number of<br>aberrant cells<br>(%) after<br>48 hours<br>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<br>aberrant cells | 1.92 ± 0.03 | 14.35 ± 1.27*             | 7.24 ± 0.15*                  | 8.85 ± 0.14*                  |
| 72 hours<br>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<br>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<br>(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<br>(100 mg/kg b.wt)           | 2.46 ± 0.09 <sup>#</sup>           | 2.12 ± 0.01 <sup>#</sup> | 1.94 ± 0.02 <sup>#</sup> | 15.46 ± 0.03*   | 17.50 ± 0.10* | 18.25 ± 0.12* |
| Group III (glyphosate<br>dose 25 mg/kg b.wt) | 4.12 ± .05 <sup>#</sup>            | 3.84 ± 0.04 <sup>#</sup> | 3.75 ± 0.03*             | 3.87 ± 0.02*  | 5.76 ± 0.08*  | 6.12 ± 0.07*  |
| Group IV (glyphosate<br>dose 50 mg/kg b.wt)  | 3.54 ± 0.01 <sup>#</sup>           | 3.16 ± 0.03 <sup>#</sup> | 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.

<sup>#</sup> $P < .05$  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

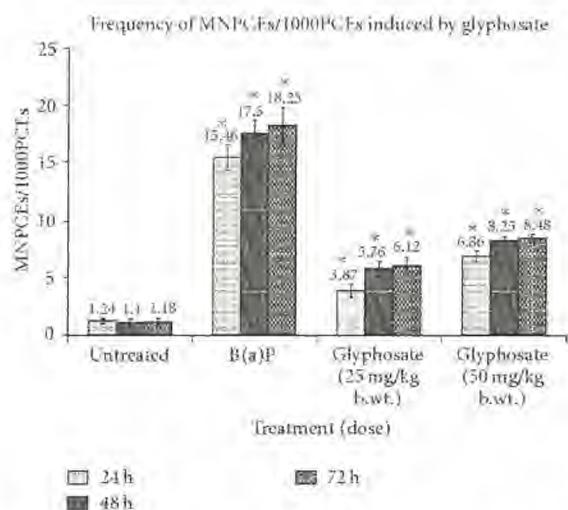


FIGURE 4: Mutagenic activity of glyphosate in Swiss albino mice showing increased micronuclei (MN) induction at sampling time of 24, 48, and 72 hours. Values are expressed as mean  $\pm$  SE of five animals. \*Represent significant increase over untreated control group at their respective sampling time. Data were significant as  $P < .05$ . MNPCs: 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-hydroxyguanine (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<sup>-1</sup> 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.

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### 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<sub>50</sub>96 h) of glyphosate is very high, that is, 620 mg L<sup>-1</sup> [12]. On the contrary, the LC<sub>50</sub>96 h of the formulated product Roundup® was much lower, varying from 2 to 55 mg L<sup>-1</sup>, depending on the species of fish, life stage and conditions of the test [13]. The LC<sub>50</sub>96 h of Roundup® was determined as 13.7 mg L<sup>-1</sup> to juveniles of the Neotropical fish *Prochilodus lineatus* [14], a detritivorous 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 another 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  $9.6 \pm 3.4$  g and  $9.7 \pm 1.81$  cm (mean  $\pm$  S.D.,  $N=30$ ), 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 ( $T=25^\circ\text{C}$ ; pH = 7.0) and a 14/10 h light/dark photoperiod. During this period, fish were fed every 48 h with commercial peller food containing 36% of protein (Guabirú, 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  $10\text{ mg L}^{-1}$  of Roundup® ( $360\text{ g glyphosate L}^{-1}$  or 41% of glyphosate, Monsanto Brazil) LTDA) to *P. lineatus*. This Roundup® concentration corresponds to 75% of the  $\text{LC}_{50}$  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 ( $\pm$  S.D.) for NC and experimental groups were, respectively, temperature:  $25.7 \pm 0.5$  and  $25.0 \pm 0.0^\circ\text{C}$ ; pH:  $7.4 \pm 0.2$  and  $7.4 \pm 0.1$ ; dissolved oxygen:  $6.9 \pm 0.8$  and  $7.0 \pm 0.8\text{ mg O}_2\text{ L}^{-1}$ ; conductivity:  $53.7 \pm 10.4$  and  $62.8 \pm 4.4\ \mu\text{S cm}^{-1}$ . Positive control groups (PC), consisting of

fish injected with the clastogenic agent cyclophosphamide ( $40\text{ mg Kg}^{-1}$ , 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 ( $0.1\text{ g L}^{-1}$ ) 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 ( $10\ \mu\text{L}$ ) was diluted in  $700\ \mu\text{L}$  of phosphate-buffered saline (PBS:  $126.6\text{ mM NaCl}$ ,  $4.8\text{ mM KCl}$ ,  $1.5\text{ mM CaCl}_2$ ,  $3.7\text{ mM NaHCO}_3$ ,  $8.9\text{ mM Na}_2\text{HPO}_4$ ,  $2.9\text{ mM NaH}_2\text{PO}_4$ ) 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  $700\ \mu\text{L}$  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  $200\ \mu\text{L}$   $0.25\%$  trypsin-EDTA and homogenized by periodic manual inversion at room temperature for tissue dissociation. To halt the enzymatic digestion  $200\ \mu\text{L}$  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 Härtmann [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  $4^\circ\text{C}$ , protected from light, in a lysis buffer ( $2.5\text{ M NaCl}$ ,  $100\text{ mM EDTA}$ ,  $10\text{ mM Tris}$ ,  $10\% \text{ DMSO}$ ,  $1\text{ mL Triton X-100}$ , pH 10.0); (b) DNA unwinding: 30 min, in the dark, in an electrophoresis buffer ( $0.3\text{ N NaOH}$ ,  $1\text{ mM EDTA}$ , pH > 13); (c) electrophoresis: 20 min, 300 mA, 25 V,  $1\text{ V cm}^{-1}$ ; (d) neutralization: three washes for 5 min each in buffer ( $0.4\text{ M Tris}$ , 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 ( $20\ \mu\text{g mL}^{-1}$ ) were analyzed under a Nikon fluorescence microscope ( $1000\times$  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 ( $1000\times$  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  $\pm$  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's *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  $\pm$  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 | <i>N</i> | Comet Classes |       |       |       | Damaged nucleoids<br>(mean $\pm$ S.E.) |
|-----------------|------|--------|----------|---------------|-------|-------|-------|--|
|                 |      |        |          | 0 (%)         | 1 (%) | 2 (%) | 3 (%) |  |
| Erythrocytes    | 6 h  | NC     | 6        | 70.5          | 23.0  | 3.2   | 3.3   | 29.5 $\pm$ 2.4                         |
|                 |      | RDP    | 6        | 54.5          | 33.3  | 8.8   | 3.3   | 45.5 $\pm$ 6.1 <sup>*</sup>            |
|                 |      | PC     | 6        | 25.7          | 63.1  | 10.2  | 1.0   | 74.3 $\pm$ 1.5 <sup>*</sup>            |
|                 | 24 h | NC     | 5        | 77.8          | 17.6  | 2.8   | 1.7   | 22.2 $\pm$ 1.4                         |
|                 |      | RDP    | 6        | 78.5          | 18.7  | 2.2   | 0.7   | 21.5 $\pm$ 2.4                         |
|                 |      | PC     | 6        | 9.3           | 53.0  | 34.3  | 2.7   | 90.7 $\pm$ 1.6 <sup>*</sup>            |
|                 | 96 h | NC     | 9        | 78.4          | 20.9  | 0.7   | 0.0   | 20.3 $\pm$ 1.6                         |
|                 |      | RDP    | 12       | 76.7          | 21.3  | 1.6   | 0.3   | 23.3 $\pm$ 1.1                         |
|                 |      | PC     | 8        | 25.1          | 69.0  | 4.5   | 0.3   | 74.9 $\pm$ 0.9 <sup>*</sup>            |
| Branchial cells | 6 h  | NC     | 6        | 71.5          | 24.5  | 2.2   | 1.8   | 28.5 $\pm$ 1.5                         |
|                 |      | RDP    | 5        | 66.6          | 28.0  | 3.2   | 2.6   | 33.8 $\pm$ 1.1 <sup>*</sup>            |
|                 |      | PC     | 7        | 63.9          | 24.7  | 7.3   | 4.1   | 36.1 $\pm$ 2.7 <sup>*</sup>            |
|                 | 24 h | NC     | 6        | 71.2          | 23.4  | 3.0   | 2.4   | 28.8 $\pm$ 1.0                         |
|                 |      | RDP    | 6        | 61.2          | 29.2  | 4.8   | 4.5   | 38.5 $\pm$ 1.3 <sup>*</sup>            |
|                 |      | PC     | 4        | 65.0          | 23.3  | 6.8   | 5.0   | 35.0 $\pm$ 3.2                         |
|                 | 96 h | NC     | 4        | 76.7          | 13.2  | 5.8   | 4.3   | 23.5 $\pm$ 2.3                         |
|                 |      | RDP    | 3        | 74.3          | 11.7  | 9.3   | 4.7   | 25.7 $\pm$ 1.7                         |
|                 |      | PC     | 4        | 65.0          | 23.3  | 6.8   | 5.0   | 35.0 $\pm$ 3.2 <sup>*</sup>            |

One hundred nucleoids were analyzed per fish.

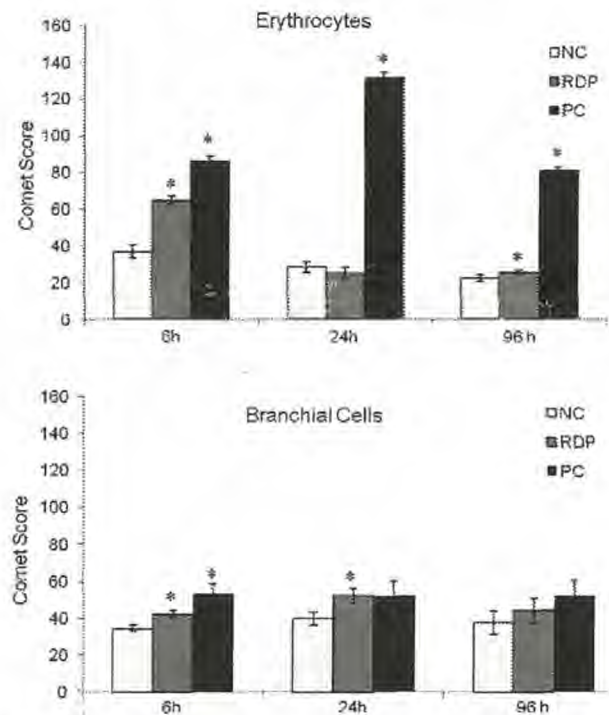
<sup>\*</sup> Different from respective negative controls ( $p < 0.05$ ).

### 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.



**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 ( $p < 0.05$ ).

**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 | <i>N</i> | MN frequency (%)             | ENA frequency (%) |
|------|-------|----------|------------------------------|-------------------|
| 6 h  | NC    | 8        | 0                            | 3.00 $\pm$ 0.34   |
|      | RDP   | 10       | 0                            | 2.37 $\pm$ 0.16   |
|      | PC    | 7        | 0.05 $\pm$ 0.05              | 2.33 $\pm$ 0.38   |
| 24 h | NC    | 9        | 0.07 $\pm$ 0.05              | 2.11 $\pm$ 0.30   |
|      | RDP   | 12       | 0.05 $\pm$ 0.05              | 2.33 $\pm$ 0.46   |
|      | PC    | 8        | 0.71 $\pm$ 0.22 <sup>*</sup> | 2.33 $\pm$ 0.35   |
| 96 h | NC    | 9        | 0.18 $\pm$ 0.11              | 4.11 $\pm$ 0.38   |
|      | RDP   | 12       | 0.11 $\pm$ 0.08              | 3.67 $\pm$ 0.36   |
|      | PC    | 8        | 0.54 $\pm$ 0.09 <sup>*</sup> | 4.00 $\pm$ 0.30   |

Three thousand erythrocytes were analyzed per fish and results are shown as mean  $\pm$  S.E.

<sup>\*</sup> Different from respective negative controls ( $p < 0.05$ ).

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 isolated 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 gill 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 Cordelro [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<sup>-1</sup> 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<sup>-1</sup>) here employed, which corresponds to 4.1 mg L<sup>-1</sup> of glyphosate. This result agrees with Çavas and Koneň [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<sup>-1</sup>, 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 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. 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 pyrethroids) 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-naphthyl 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 (Mitchellmore 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 (Mitchellmore *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 Microfast/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-Kärber method (Hamilton *et al.* 1977) with statistical software provided by the U.S. Environmental Protection Agency (<http://www.epa.gov/nlercerd/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  $\mu\text{g/L}$ ) 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  $\mu\text{g/L}$  Cu and 0.5  $\mu\text{g/L}$  pesticides) (Marking 1977).

### Genotoxicity Tests

Glochidia were exposed to sublethal concentrations of lawn care chemicals equivalent to 1/4 and 1/2 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  $\mu\text{L}$  of an osmotically modified  $\text{Ca}^{2+}/\text{Mg}^{2+}$  free Hanks balanced salt solution (HBSS) containing 4.2 mM  $\text{NaHCO}_3$ , 26.2 mM NaCl, 1.3 mM KCl, 0.44 mM  $\text{KH}_2\text{PO}_4$ , 0.34 mM  $\text{Na}_2\text{HPO}_4$ , and 5.55 mM  $\text{D-glucose}$  (adjusted pH 7.3). Proteinase K was added (10  $\mu\text{L}$  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  $\mu\text{L}$  0.65% low-melting agarose (made with HBSS and melted at a constant temperature of 37°C). Samples (50  $\mu\text{L}$ ) were transferred to microscope slides precoated with 1% normal melting agarose (made with 40 mM Tris-acetate EDTA), solidified (5 min at 4°C) and top-coated with 50  $\mu\text{L}$  0.65% low-melting

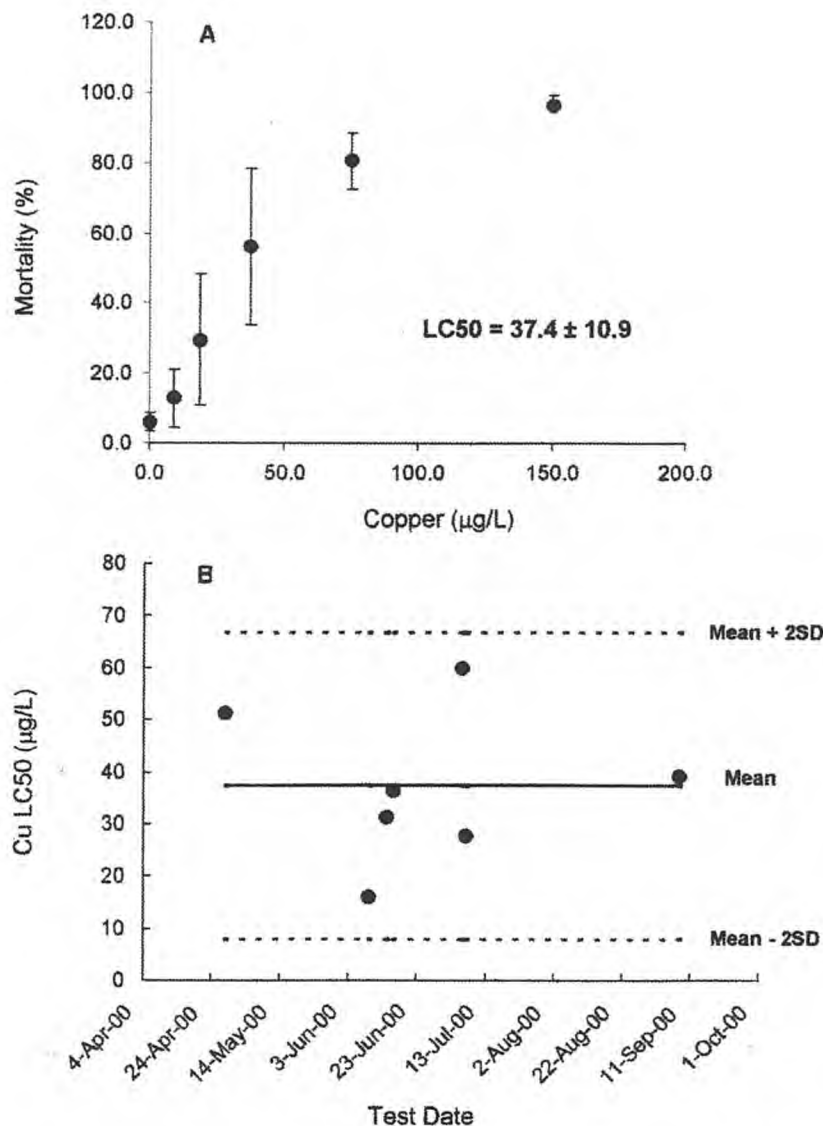


Fig. 1. Mortality of *U. imbecillis* glochidia exposed to copper (A). Data are means (SD) and LC50s are means  $\pm$  95% confidence intervals from seven replicate tests. Positive copper control chart (B)

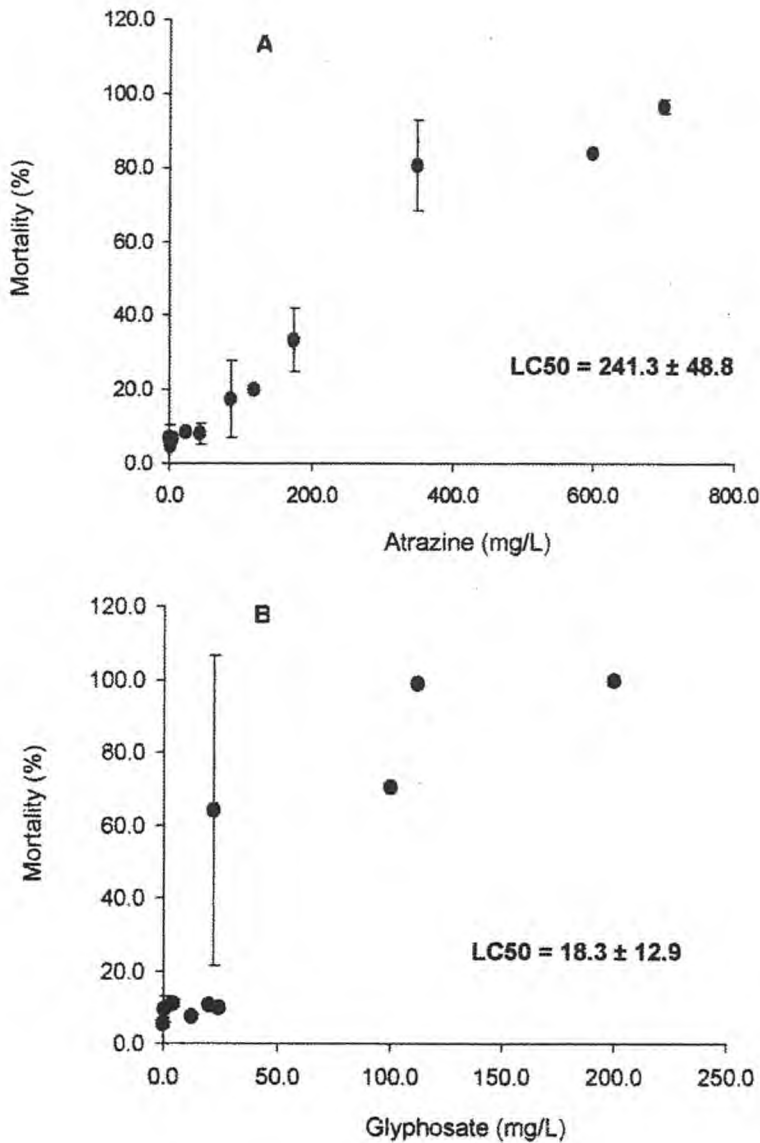
agarose. Once solidified, slides were transferred to light-protected coplin jars containing cold lysis buffer (10 mM Tris-HCl, 2.5 M NaCl, 100 mM EDTA, 10% DMSO, and 1% Triton X-100, pH 10.0), and incubated at 4°C overnight. To prevent confounding DNA damage from ultraviolet light, samples were light-protected 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 mM NaOH, 1 mM 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 mM Tris, three times for 2 min. Samples were then immersed in cold 100% ethanol for 5 min, air-dried, and stored in a desiccator until analyzed. For analyses, slides were stained with 50 µl ethidium bromide (20 µg/ml) and viewed under epifluorescent microscopy (200 $\times$  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

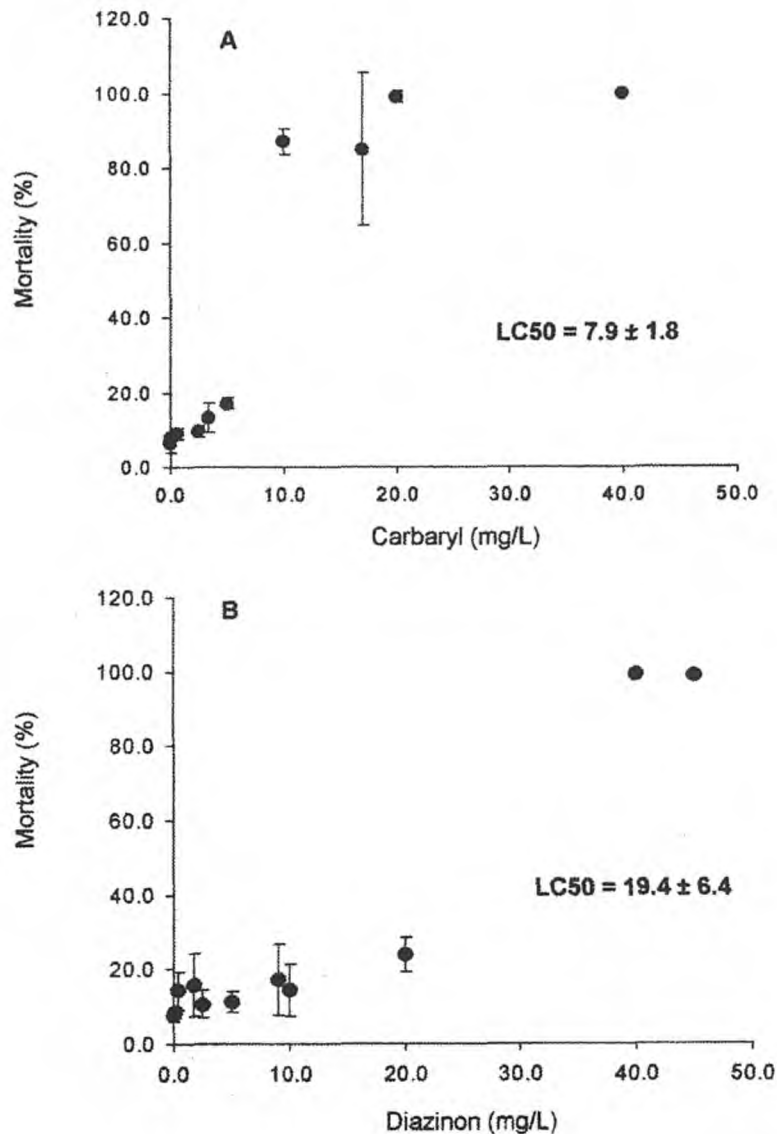


**Fig. 2.** Mortality of *U. imbecillis* glochidia exposed to the herbicides atrazine (A) and glyphosate (B). Data are means (SD) and LC50s are means  $\pm$  95% confidence intervals from three and four replicate tests, respectively

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  $\frac{1}{4}$  and  $\frac{1}{2}$  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 =  $0.44 \pm 0.13$ , DMSO =  $1.36 \pm 1.15$ , and 4-nitroquinoline =  $11.38 \pm 3.5$ ) ( $p < 0.05$ ). 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  $\pm$  95% confidence intervals from four replicate tests

observed at the highest concentration of diazinon tested. The genotoxicity of atrazine at levels equal to  $\frac{1}{4}$  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 <sup>a</sup>     | Amphipod   | <i>Gammarus</i> sp.                        | 1.2              | Technical       | Rehwoldt <i>et al.</i> (1973)              |
|                         |            | <i>Chironomus</i> sp.                      | 0.65             | Technical       | Rehwoldt <i>et al.</i> (1973)              |
|                         | Cladoceran | <i>Polypedium nubifer</i>                  | 2.05             | Technical       | Hatakeyama (1988)                          |
|                         |            | <i>Ceriodaphnia dubia</i>                  | 0.0201           | Technical       | Kim <i>et al.</i> (1999)                   |
|                         |            | <i>Ceriodaphnia dubia</i>                  | 0.0196           | Technical       | Nelson and Roline (1998)                   |
|                         | Bivalve    | <i>Pyganodon grandis</i> <sup>b</sup>      | 0.046            | Technical       | Jacobson <i>et al.</i> (1997)              |
|                         |            | <i>Lampsilis fasciola</i> <sup>b</sup>     | 0.046            | Technical       | Jacobson <i>et al.</i> (1997)              |
|                         |            | <i>Villosa iris</i> <sup>b</sup>           | 0.0546           | Technical       | Jacobson <i>et al.</i> (1997)              |
|                         |            | <i>Anodonta anatina</i> <sup>b</sup>       | 0.0418           | Technical       | Hanstén <i>et al.</i> (1996)               |
| Atrazine <sup>c</sup>   | Bivalve    | <i>Utterbackia imbecillis</i> <sup>b</sup> | 0.0374           | Technical       | This study                                 |
| Glyphosate <sup>d</sup> | Bivalve    | <i>Utterbackia imbecillis</i> <sup>b</sup> | 241.3            | Atrazine 4L     | This study                                 |
|                         | Amphipod   | <i>Gammarus pseudolimnaeus</i>             | 100.0            | Roundup         | Folmar <i>et al.</i> (1979)                |
| Carbaryl                | Cladoceran | <i>Daphnia magna</i>                       | 24.0 to 37.0     | Roundup         | Material Data Safety Sheet                 |
|                         | Bivalve    | <i>Utterbackia imbecillis</i> <sup>b</sup> | 18.3             | Roundup         | This study                                 |
|                         | Amphipod   | <i>Gammarus lacustris</i>                  | 0.04             | Technical       | Sanders (1969)                             |
|                         | Chironomid | <i>Chironomus thummi</i>                   | 0.127            | Technical       | Fisher and Lohner (1986)                   |
|                         | Cladoceran | <i>Daphnia magna</i>                       | 22.9             | Technical       | Lejczak (1977)                             |
| Diazinon                | Bivalve    | <i>Utterbackia imbecillis</i> <sup>b</sup> | 30.1             | Technical       | Johnson <i>et al.</i> (1993)               |
|                         | Bivalve    | <i>Utterbackia imbecillis</i> <sup>b</sup> | 7.9              | Sevin           | This study                                 |
|                         | Amphipod   | <i>Hyalella azteca</i>                     | 0.03             | Technical       | Werner and Nagel (1997)                    |
|                         | Amphipod   | <i>Gammarus lacustris</i>                  | 0.8              | Technical       | Sanders (1969)                             |
|                         | Chironomid | <i>Chironomus tepperi</i>                  | 0.036            | Gesapon         | Stevens (1992)                             |
|                         | Cladoceran | <i>Moina marcopa</i>                       | <0.010           | NS <sup>e</sup> | Wong (1997)                                |
|                         | Bivalve    | <i>Ceriodaphnia dubia</i>                  | 0.0006           | Technical       | Bailey <i>et al.</i> (1997)                |
|                         |            | <i>Daphnia magna</i>                       | 0.0009           | Technical       | Fernández-Casalderrey <i>et al.</i> (1994) |
|                         | Bivalve    | <i>Utterbackia imbecillis</i> <sup>b</sup> | 19.4             | Diazinon        | This study                                 |

<sup>a</sup> Hardness ranged from 30 to 85 mg/L in all studies evaluated.

<sup>b</sup> Mussels used in toxicity tests were glochidia.

<sup>c</sup> No comparative toxicity data were found for commercial formulations of atrazine, which substantially alter the solubility of the active ingredient.

<sup>d</sup> Review data for glyphosate were restricted to Roundup because different formulations are known to vary in toxicity (Solomons and Thompson 2003).

<sup>e</sup> 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 (Baturu and Lagadic 1996; Livingstone 1998), and detoxification is often reduced or absent in juvenile aquatic organisms (Andersson and Forlin 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 (chlor-dane and toxaphene), as these chemicals do not require bioac-

tivation and target GABA receptors, which are highly reduced in bivalves (Karlunen *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;

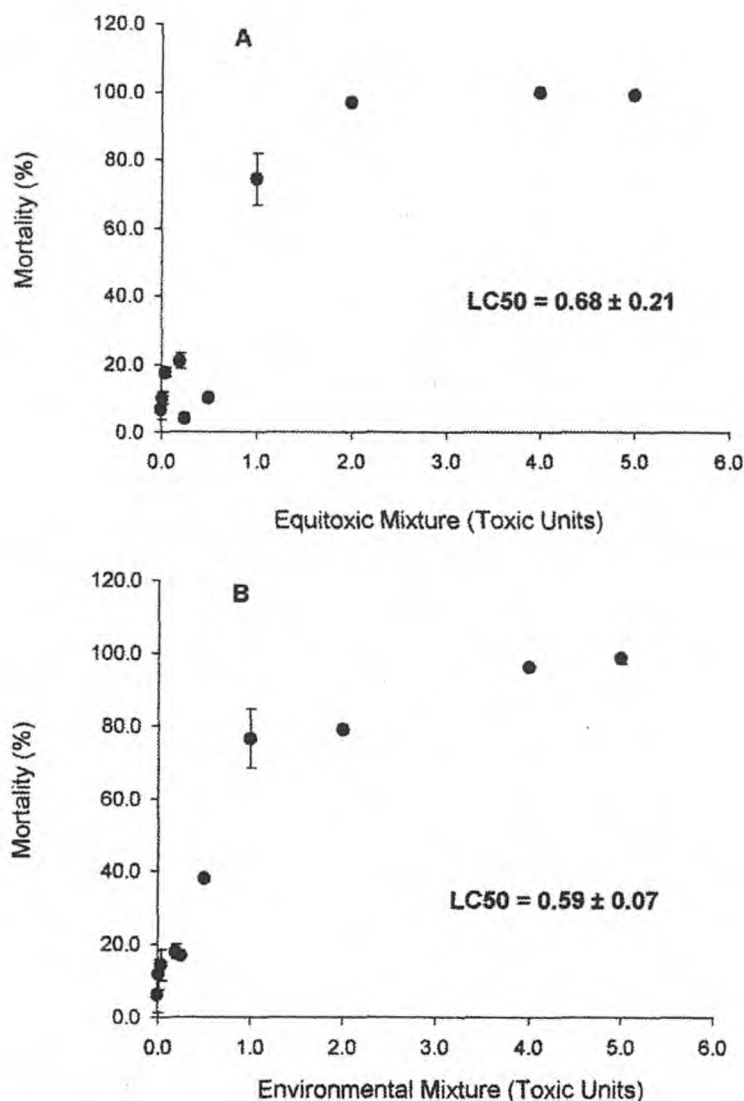


Fig. 4. Mortality of *U. imbecillis* glochidia exposed to an equitoxic (A) and an environmentally realistic (B) mixture of lawn care chemicals. Data are means (SD) and LC50s are means  $\pm$  95% confidence intervals from three replicate tests

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.

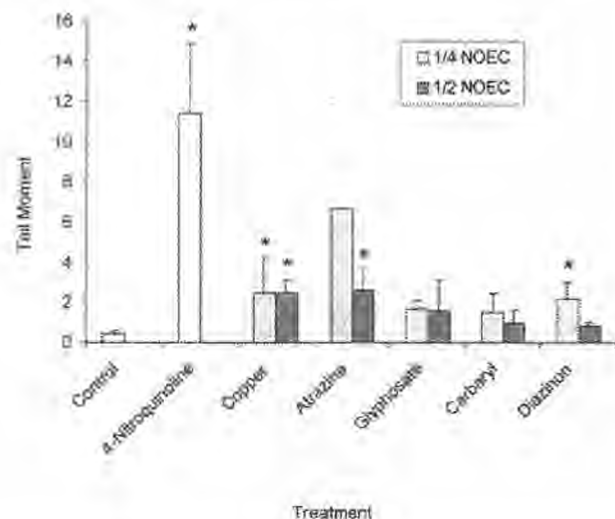
In the environment, non-target organisms are exposed to mixtures of chemicals, and we have observed that equitoxic and environmentally realistic mixtures of copper, atrazine, glyphosate, carbaryl, and diazinon were adequately described by an additive model in producing lethal effects in *U. imbecillis* 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

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

| Chemical <sup>a</sup>      | NOEC  | Genotoxicity test concentration |        |
|----------------------------|-------|---------------------------------|--------|
|                            |       | ¼ NOEC                          | ½ NOEC |
| Copper ( $\mu\text{g/L}$ ) | 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   |

<sup>a</sup>Pesticides used were commercial formulations, and NOECs represent amount of active ingredient.

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



**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  $1/4$  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  $\mu\text{g/L}$ ) were not substantially different from copper concentrations producing 50% mortality singly (19.7 to 48.3  $\mu\text{g/L}$ ). 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  $\beta\beta$  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 carboaryl 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  $1/4$  NOECs was comparable to or higher than that in glochidia exposed to  $1/2$  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  $\mu\text{mol/L}$  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  $\mu\text{mol/L}$ . 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 weak 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 isopropylamine 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, Wellcome, 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)<br>24h | Number<br>of cells analysed | Chromosome 1<br>aneuploidy (2n±1) | Polyploidy<br>(4n)         | Acentric fragments<br>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 <sup>a</sup>   | 0.8 ± 0.088                           |
| 56                   | 900                         | 1.0 ± 0.090                       | 1.2 ± 0.110 <sup>a</sup>   | 0.6 ± 0.074                           |
| 140                  | 650                         | 0.9 ± 0.095                       | 1.1 ± 0.103 <sup>a,b</sup> | 0.5 ± 0.074                           |
| 280                  | 610                         | 0.8 ± 0.090                       | 0.8 ± 0.090 <sup>a,b</sup> | 0.3 ± 0.057                           |
| 560                  | 400                         | 1.0 ± 0.099                       | 1.0 ± 0.099 <sup>a,b</sup> | 1.2 ± 0.111                           |
| 1120                 | 350                         | 1.1 ± 0.106                       | 0.3 ± 0.053 <sup>a,b</sup> | 0.8 ± 0.092                           |

<sup>a</sup>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  $\mu\text{mol/L}$ , 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  $\mu\text{mol/L}$ .

### 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  $\mu\text{mol/L}$ ) 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  $\mu\text{mol/L}$ . 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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*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  $\mu\text{M}$  and 560  $\mu\text{M}$ , 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 chorismate,

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<sub>50</sub> 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 monooxygenase 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  $\mu\text{M}$ . 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  $\mu\text{M}$ ), cyclophosphamide (CP, Jenapharm, Ankerwerk, Rudolstadt, Germany, 0.1 mM) and ethylmethanesulphonate (EMS, Sigma, St. Louis, MO, USA, 250  $\mu\text{g/ml}$ ) 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  $\mu\text{M}$  HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250  $\mu\text{g/ml}$ ) and phytohaemagglutinin (PHA, 180  $\mu\text{g/ml}$ , Wellcome, 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  $\mu\text{g/ml}$  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 $\mu\text{M}$ | 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 $\mu\text{M}$ )<br>2h (-S9)            | Control                     | 1.52 | 55***      |
|   | 28                          | 1.54 | 21         |
|   | 56                          | 1.48 | 17         |
|   | 140                         | 1.48 | 14         |
|   | 280                         | 1.53 | 19         |
|   | 560                         | 1.57 | 20         |
| EMS (250 $\mu\text{g}/\text{ml}$ )<br>2 h (+S9) | Control                     | 1.48 | 48***      |
|   | 28                          | 1.52 | 20         |
|   | 56                          | 1.44 | 23         |
|   | 140                         | 1.65 | 20         |
|   | 280                         | 1.72 | 21         |
|   | 560                         | 1.71 | 19         |
| CP (0.1 mM)                                     |                             | 1.58 | 28         |
|   |                             | 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 $\mu\text{M}$ | 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 $\mu\text{M}$ )<br>2h (-S9)    |                             | 1.48 | 36***      |
|   | 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 $\mu\text{g/ml}$ )<br>2 h (+S9) |                             | 1.44 | 35***      |
|   | 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  $\mu\text{M}$ ) in the experiments without microsomal activation, CP (0.1 mM) in the experiments with S9 and EMS (250  $\mu\text{g/ml}$ ) 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  $\mu\text{M}$  and 560  $\mu\text{M}$ , 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  $\mu\text{M}$  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 responsible 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 genotoksične 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  $\mu\text{M}$  i 560  $\mu\text{M}$ . Pri ovom tretmanu govedih 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.

## 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 ( $\geq 35$  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) (Martínez 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 Fundación 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% phytohemagglutinin (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 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:

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

### 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  $30.4 \pm 7.8$  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 |
|--------------------|-------------|-------------|-------------|-------------|-----------------|
| <b>Phase 1</b>     |             |             |             |             |                 |
| 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)     |
| <b>Phase 2</b>     |             |             |             |             |                 |
| 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)     |
| <b>Phase 3</b>     |             |             |             |             |                 |
| 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)     |

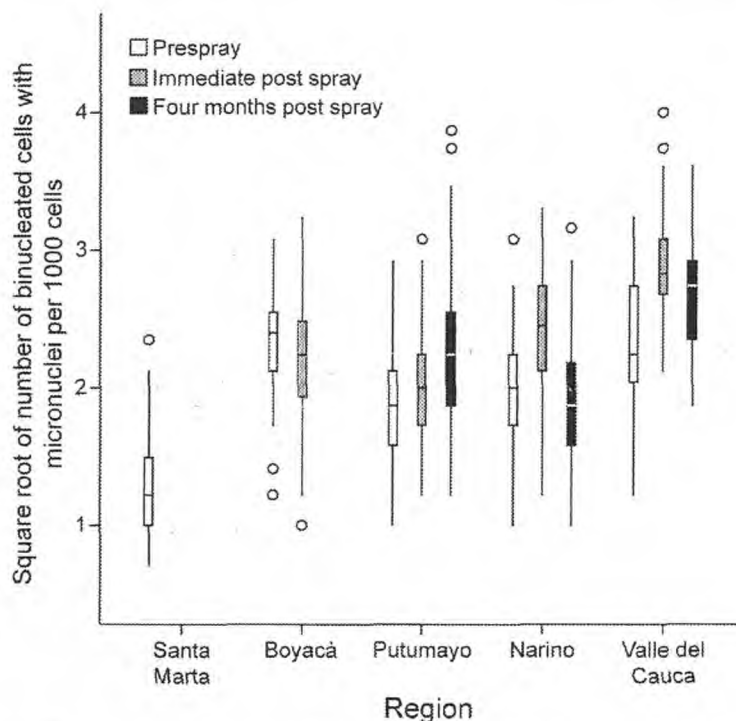


FIG. 1. Box plot of frequency of BNMN in the five study regions with samples taken prespray, 4–5 d post-spray, and 4 mo post-spray. Box plots: The center horizontal line marks the median of the sample. The length of each box shows the range within which the central 50% of the values fall, with the top 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  $\circ$  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  $4.43 \pm 2.36$  vs.  $3.61 \pm 1.82$ , 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 ( $\geq 35$  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 BNMN 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       |
|--------------------------------------|-------------|-------------|--------------|-------------|-----------------|-------------|
| <b>Sex</b>                           |             |             |              |             |                 |             |
| 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) |
| <i>p</i>                             | .236        | .007        | .131         | .028        | .138            | .002        |
| <b>Age</b>                           |             |             |              |             |                 |             |
| 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) |
| <i>p</i>                             | .438        | .929        | .574         | .564        | .313            | .093        |
| <b>Ethnicity</b>                     |             |             |              |             |                 |             |
| 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)  |
| <i>p</i>                             |             |             | .162         | .588        | .850            | .368        |
| <b>Smoking</b>                       |             |             |              |             |                 |             |
| 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) |
| <i>p</i>                             | .693        | .756        | .395         | .233        | .459            | .592        |
| <b>Folic acid intake (quartiles)</b> |             |             |              |             |                 |             |
| 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) |
| <i>p</i>                             | .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

| Route of exposure                             | Nariño ( <i>n</i> = 55) |                | Putumayo ( <i>n</i> = 53) |                | Valle del Cauca ( <i>n</i> = 26) |                |
|---|-------------------------|----------------|---------------------------|----------------|----------------------------------|----------------|
|   | <i>n</i>                | Mean BNMN (SD) | <i>n</i>                  | Mean BNMN (SD) | <i>n</i>                         | 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)    |                                  |                |
| <i>p</i> Value (ANOVA)                        |                         | 0.472          |                           | 0.612          |                                  | 0.760          |
| Any exposure                                  | 27                      | 6.16 (2.22)    | 40                        | 4.90 (2.69)    | 1                                | 9.50 (0)       |
| <i>p</i> 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 | <i>p</i> | 95% CI      |
|-----------------|-------------|----------|-------------|
| <b>Region</b>   |             |          |             |
| 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  |
| <b>Age (yr)</b> |             |          |             |
| 25–34           | 0.28        | .250     | –0.20, 0.76 |
| 35 and older    | 0.75        | .008     | 0.20, 1.31  |
| <b>Gender</b>   |             |          |             |
| Females         | 1.00        | ≤.0001   | 0.60, 1.40  |

start of cultivation, made it possible to distinguish between non-dividing 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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## Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate

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### Abstract

We analyzed the consequences of aerial spraying with glyphosate added to a surfactant solution in the northern part of Ecuador. A total of 24 exposed and 21 unexposed control individuals were investigated using the comet assay. The results showed a higher degree of DNA damage in the exposed group (comet length = 35.5  $\mu\text{m}$ ) compared to the control group (comet length = 25.94  $\mu\text{m}$ ). These results suggest that in the formulation used during aerial spraying glyphosate had a genotoxic effect on the exposed individuals.

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

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Glyphosate is a non-selective herbicide which is the main chemical component in many systemic herbicides used to control most annual and perennial plants. It controls weeds by inhibiting the synthesis of aromatic amino acids necessary for protein formation, which link primary and secondary metabolism in susceptible plants (Carlisle and Trevors, 1988; U.S. Forest Service, 1997).

According to some reports glyphosate shows no adverse effects on soil microorganisms, it is relatively non-toxic to fish (U.S. Forest Service, 1997) and is of relatively low toxicity to birds and mammals, including humans (Batt *et al.*, 1980; Evans and Batty, Williams *et al.*, 2000; Goldstein *et al.*, 2002). However, Lioi *et al.*, (1998) reported de induction of oxidative stress and mutagenic effects for some pesticides, including glyphosate, in bovines and Paz-y-Miño *et al.*, (2002a) reported that some pesticides were associated with genetic damage in human populations subjected to high pesticide exposure levels due intensive use, misuse or failure of control measures.

Since January 2001, the northern area of Ecuador (mainly Sucumbios district) has been subjected to aerial spraying by the Colombian Government with Roundup-Ultra, a herbicide formulation containing glyphosate, poly-

ethoxylated tallowamine surfactant (POEA) and the adjuvant Cosmoflux 41 IF which is a propriety Colombian component probably included to aid the adherence or absorption of the herbicide (Ministerio de Relaciones Exteriores, Ecuador (MREE), 2003). According to the National Narcotic Council for air spraying of illicit cultures the load of the airplane was 1137 to 1705 liters and the effective unloading with Roundup Ultra (43.9% of glyphosate) was 23.4 liters  $\text{ha}^{-1}$  equivalent to 10.3 L  $\text{ha}^{-1}$  of glyphosate (Acción Ecológica, 2003, Nivia, 2001). The main purpose of spraying glyphosate in this formulation is to eradicate illicit crops grown in this area, and several research projects have been carried out to investigate the consequences of the use of this formulation in Ecuador (MRE, Ecuador, 2003; Acción Ecológica, 2003).

The comet assay can be used to evaluate DNA damage and provides a useful tool for estimating the genetic risk from exposure to complex mixtures of chemicals (Paz-y-Miño *et al.*, 2002b), this assay having been widely applied in genotoxicity studies of factors such as X-rays and pesticides (Singh *et al.*, 1988; Tice *et al.*, 1990; Scarpato *et al.*, 1996; Slamenová *et al.*, 1999; Błasiak *et al.*, 1999; Garaj-Vrhovac and Zeljezic, 2000; Paz-y-Miño *et al.*, 2002a; Paz-y-Miño *et al.*, 2002b; Acción Ecológica, 2003).

The aim of the study described in this paper was to determine the possible influence of the formulation of

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glyphosate used during aerial spraying in northern Ecuador on the genetic material of exposed individuals.

The exposed (E) group consisted of 24 randomly selected individuals (Table 1) who lived 3 km or less from an area on the border between Ecuador and Colombia where aerial spraying with a glyphosate-based herbicide had occurred continuously during three days between December 2000 and March 2001, sporadic aerial spraying continuing for three weeks following continuous spraying (MREE, 2003, Acción Ecológica 2004). Exposed group individuals manifested symptoms of toxicity after several exposures to aerial spraying, with half of the individuals in this group having received spraying directly over their houses and the other half living within 200m to 3 km from the sprayed areas.

A clinical history was completed for each of the exposed individuals and a wide-range of reactions were noted, including intestinal pain and vomiting, diarrhea, fever, heart palpitations, headaches, dizziness, numbness, insomnia, sadness, burning of eyes or skin, blurred vision, difficulty in breathing and blisters or rash (MREE, 2003; Acción Ecológica 2003).

Venous blood (5 mL) was taken from the exposed individuals between two weeks and two months after their exposure to aerial spraying and processed immediately after collection.

The blood samples analyzed in this study were provided by Dr. Adolfo Maldonado, a specialist in tropical medicine and a member of the Ecological Action foundation and part of the group of investigators of the International Commission on the Impact on Ecuadorian Territory of Aerial Fumigations in Colombia. This study was approved by the Bioethics Committee of the Pontifical Catholic University of Ecuador, according to the international guidelines. Each individual completed a personal and biomedical survey and gave their informed consent to be part of this study. In the case of the adolescents involved in the study (14-17 year-olds) their legal guardians, as well as themselves, gave their informed consent.

All of the individuals included in this study combine their activities mainly in the house and sometimes cultivating and harvesting. This persons neither used herbicides, pesticides nor similar substances in the named activities (Acción Ecológica, 2004).

**Table 1** - DNA damage assessed by the comet assay in individuals exposed (E) to glyphosate and unexposed (U) control individuals. Note that the same numbers (1, 2, 3 etc.) for the individuals does not indicate that the exposed and control individuals were matched.

| Individual (gender, age) <sup>a</sup> | Exposed to glyphosate                |     |     |    |   | DNA migration (µm)      |                       | Unexposed controls                    |                                      |    |    |   | DNA migration (µm) |                          |                       |
|---------------------------------------|--------------------------------------|-----|-----|----|---|-------------------------|-----------------------|---------------------------------------|--------------------------------------|----|----|---|--------------------|--------------------------|-----------------------|
|                                       | Number of cells scored in each group |     |     |    |   | Mean                    | Median                | Individual (gender, age) <sup>a</sup> | Number of cells scored in each group |    |    |   |                    | Mean                     | Median                |
|                                       | A                                    | B   | C   | D  | E |                         |                       |                                       | A                                    | B  | C  | D | E                  |                          |                       |
| 1E (F, 53)                            | 2                                    | 120 | 76  | 5  | 3 | 39.5                    | 32.5                  | 1U (F, 17)                            | 150                                  | 59 | 3  | 0 | 0                  | 26.2                     | 25.0                  |
| 2E (F, 37)                            | 13                                   | 92  | 82  | 14 | 0 | 44.1                    | 32.5                  | 2U (F, 40)                            | 164                                  | 43 | 4  | 0 | 0                  | 25.4                     | 25.0                  |
| 3E (F, 40)                            | 2                                    | 64  | 62  | 77 | 4 | 56.6                    | 52.5                  | 3U (F, 26)                            | 165                                  | 40 | 2  | 0 | 0                  | 25.7                     | 25.0                  |
| 4E (M, 27)                            | 8                                    | 75  | 64  | 47 | 8 | 49.2                    | 37.5                  | 4U (M, 14)                            | 111                                  | 96 | 6  | 0 | 0                  | 27.3                     | 26.5                  |
| 5E (F, 44)                            | 9                                    | 138 | 63  | 3  | 0 | 34.6                    | 30.0                  | 5U (M, 32)                            | 165                                  | 38 | 3  | 0 | 0                  | 25.9                     | 25.0                  |
| 6E (F, 50)                            | 51                                   | 113 | 30  | 3  | 0 | 30.8                    | 27.5                  | 6U (M, 21)                            | 171                                  | 35 | 1  | 0 | 0                  | 25.7                     | 25.0                  |
| 7E (F, 38)                            | 21                                   | 139 | 48  | 3  | 0 | 33.2                    | 30.0                  | 7U (M, 16)                            | 177                                  | 25 | 6  | 0 | 0                  | 25.8                     | 25.0                  |
| 8E (F, 46)                            | 21                                   | 116 | 72  | 4  | 0 | 35.2                    | 30.0                  | 8U (F, 47)                            | 176                                  | 25 | 3  | 0 | 0                  | 25.7                     | 25.0                  |
| 9E (F, 55)                            | 26                                   | 100 | 84  | 1  | 0 | 32.8                    | 30.0                  | 9U (F, 15)                            | 190                                  | 14 | 1  | 0 | 0                  | 25.2                     | 25.0                  |
| 10E (F, 50)                           | 26                                   | 100 | 84  | 1  | 0 | 34.2                    | 30.0                  | 10U (F, 36)                           | 179                                  | 25 | 1  | 0 | 0                  | 25.4                     | 25.0                  |
| 11E (F, 22)                           | 28                                   | 123 | 60  | 0  | 0 | 32.0                    | 27.5                  | 11U (F, 21)                           | 150                                  | 46 | 9  | 0 | 0                  | 26.3                     | 25.0                  |
| 12E (F, 27)                           | 11                                   | 130 | 63  | 6  | 0 | 33.7                    | 30.0                  | 12U (F, 43)                           | 148                                  | 49 | 15 | 0 | 0                  | 26.8                     | 25.0                  |
| 13E (F, 28)                           | 40                                   | 132 | 40  | 2  | 0 | 31.0                    | 30.0                  | 13U (F, 53)                           | 161                                  | 27 | 0  | 0 | 0                  | 26.1                     | 25.0                  |
| 14E (F, 59)                           | 10                                   | 96  | 99  | 1  | 0 | 36.4                    | 32.5                  | 14U (F, 35)                           | 164                                  | 23 | 21 | 0 | 0                  | 27.0                     | 25.0                  |
| 15E (F, 55)                           | 35                                   | 110 | 62  | 1  | 0 | 32.7                    | 30.0                  | 15U (F, 38)                           | 169                                  | 28 | 11 | 0 | 0                  | 26.4                     | 25.0                  |
| 16E (F, 17)                           | 60                                   | 101 | 44  | 1  | 0 | 31.3                    | 37.5                  | 16U (F, 22)                           | 183                                  | 15 | 8  | 0 | 0                  | 25.1                     | 25.0                  |
| 17E (F, 34)                           | 7                                    | 114 | 57  | 2  | 0 | 33.4                    | 30.0                  | 17U (F, 71)                           | 191                                  | 8  | 5  | 0 | 0                  | 25.0                     | 25.0                  |
| 18E (F, 45)                           | 10                                   | 150 | 50  | 4  | 0 | 33.0                    | 30.0                  | 18U (F, 39)                           | 195                                  | 13 | 6  | 0 | 0                  | 25.5                     | 25.0                  |
| 19E (F, 28)                           | 13                                   | 160 | 44  | 0  | 0 | 31.1                    | 27.5                  | 19U (F, 21)                           | 179                                  | 20 | 8  | 0 | 0                  | 25.9                     | 25.0                  |
| 20E (F, 21)                           | 1                                    | 153 | 47  | 3  | 0 | 33.2                    | 30.0                  | 20U (F, 50)                           | 190                                  | 14 | 2  | 0 | 0                  | 25.3                     | 25.0                  |
| 21E (F, 34)                           | 2                                    | 130 | 25  | 1  | 0 | 31.8                    | 30.0                  | 21U (F, 45)                           | 150                                  | 56 | 9  | 0 | 0                  | 26.4                     | 25.0                  |
| 22E (F, 23)                           | 0                                    | 29  | 173 | 2  | 0 | 39.3                    | 37.5                  |                                       |                                      |    |    |   |                    |                          |                       |
| 23E (F, 34)                           | 2                                    | 88  | 115 | 1  | 0 | 35.5                    | 37.5                  |                                       |                                      |    |    |   |                    |                          |                       |
| 24E (F, 42)                           | 93                                   | 103 | 9   | 0  | 0 | 27.6                    | 27.5                  |                                       |                                      |    |    |   |                    |                          |                       |
| Mean age = 38 ± 12.2 <sup>b</sup>     |                                      |     |     |    |   | 35.5 ± 6.4 <sup>c</sup> | 30 ± 5.4 <sup>d</sup> | Mean age = 33 ± 15 <sup>b</sup>       |                                      |    |    |   |                    | 25.94 ± 0.6 <sup>c</sup> | 25 ± 0.3 <sup>d</sup> |

<sup>a</sup>F = female; M = male. <sup>b</sup>Mean ± standard deviation (SD). <sup>c</sup>Mean median value ± SD.

The unexposed (U) control group consisted of 21 unrelated healthy individuals living 80 km away from the spraying area. They were similar to the exposed group regarding their demographic characteristics and occupation but were not matched controls. Blood samples were collected and processed as for the exposed group, but not concomitantly.

None of the individuals analyzed in this study (neither the exposed group nor the control group) smoked tobacco, drank alcohol, took non-prescription drugs or had been exposed to pesticides during the course of their normal daily lives. All of the individuals included in this study mainly worked at home, sometimes cultivating and harvesting crops without the use of herbicides, pesticides or similar substances in the named activities and their windowed houses did not contain asbestos in the ceilings or roofs (Acción Ecológica, 2004).

The Comet assay is a rapid and sensitive method for the detection of DNA damage induced *in vivo* (Singh *et al.*, 1988, McKelvey-Martin *et al.*, 1993, Monroy *et al.*, 2005) or after environmental and occupational exposures (Albertini *et al.*, 1996, Leroy *et al.*, 1996).

The blood samples were assayed using the alkaline comet assay as described by Singh *et al.*, (Singh *et al.*, 1988) with the modifications implemented in our laboratory (Paz-y-Miño *et al.*, 2002). The comet assay slides were analyzed at 400x magnification using a Zeiss fluorescence microscope equipped with a calibrated ocular micrometer and a 50 W mercury lamp with an excitation filter of 515-560nm and a 590nm barrier filter.

Cells were visually allocated to classified one of five predefined categories (A-E) according to the amount of DNA in the comet's tail, tail and a rank-number of from 0 (A) to 400 (E) was assigned to quantify the damage in each cell and calculate a mean of the amount of DNA damage (Anderson *et al.*, 1994).

To measure the head-to-tail comet length randomly-selected cells from the center of the gel were measured using a calibrated scale and DNA migration was determined by measuring the nuclear DNA and the migrating DNA (Singh *et al.*, 1988).

An average of 200 cells per individual was scored and the mean and median comet length from each individual was used for statistical analysis by the Mann-Whitney U test, which was applied to determine the differences between exposed and control group in the comet assay.

We found that individuals in the group which had been exposed to spraying with the glyphosate-containing herbicide showed higher DNA migration levels than controls ( $p < 0.001$ ), the exposed group having a mean total migration level of 35.50  $\mu\text{m}$  as compared with 25.94  $\mu\text{m}$  for the control group (Table 1). Comet types D and E were not observed in the control group (Table 1).

This work reports the results of the cytogenetic monitoring and DNA damage assessment of individuals exposed

to aerial spraying of glyphosate in the northern part of Ecuador. A study of the genotoxicity of chemicals, such as glyphosate is important because of their possible consequences on human health and their association with cancer, as has been published in similar studies with pesticides (Paz-y-Miño *et al.*, 2002a). The Alaska Community Action on Toxics (ACAT, 1998) factsheet, other studies like Arbuckle *et al.*, (2001) and Richard *et al.*, (2005) reported that when people ingest or absorb glyphosate through their skin or bathe or drink in water contaminated with this herbicide a wide range of symptoms can occur, such as headaches or reactions which affect the eyes, skin, lungs, heart, blood cells and genitals and gonads. Ecuadorian governmental data confirms the existence of health problems associate with such symptoms in the spraying zone (MREE, 2003).

Published data showed that chromosomal damage induced by pesticides appears to be transient in acute or discontinuous exposure but cumulative in continuous exposure to complex agrochemical mixtures (Bolognesi, 2003).

Formulated herbicides containing glyphosate are more potent mutagens to animals and humans than pure glyphosate, most probably due to the concomitant effects of additional toxic components, such as surfactants (ACAT, 1998). The aerial spraying on the border between Ecuador and Colombia used 44% of Roundup-Ultra (see above) but the recommended application rate of this formulation in the USA is 1.6% to 7.7% up to a maximum concentration of 29% (MREE, 2003) and according to Acción Ecológica (2003) the application rate of the formulated product must not exceed 0.95 L ha<sup>-1</sup>. In the area of our study the application rate was 23.4 L ha<sup>-1</sup> (10.3 L ha<sup>-1</sup> with respect to glyphosate) and therefore more than 20 times the maximum recommended application rate for the formulated product, which may explain our comet assay results (Table 1) (Acción Ecológica, 2003, Nivia, 2001).

The analysis of genes implicated in the process of DNA detoxification, would be useful to understand the genetic influence of some chemicals like glyphosate. In our study factors such as age and DNA damage were not found to be related and because most members of the exposed and control groups were female we cannot conclude anything regarding the influence of sex on the results of the comet assay. Similar results have been reported in other investigations, which report that in general terms sex and age seem to have little, if any, effect in pesticide exposed populations (Carbonell *et al.*, 1993, Steenland *et al.*, 1986).

However, we did find a higher degree of DNA damage in the exposed group compared to the control group (Table 1). The significant increase in DNA damage levels observed seem to reflect a general response to the formulation used during aerial spraying, since none of the individuals in the exposed group smoked tobacco or drank alcohol

or had been exposed to other pesticides when the samples were taken.

Our findings suggest the existence of a genotoxic risk for glyphosate exposure in the formulation used during the aerial sprayings and indicate the need for further studies on individuals exposed to glyphosate to determine its possible influence on genetic material.

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