Research Article

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

Marin Mladinic, Suzana Berend, Ana Lucic Vrdoljak, Nevenka Kopjar, Bozica Radic, and Davor Zeljezic*

Institute for Medical Research and Occupational Health, Zagreb 10000, Croatia

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

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

INTRODUCTION

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

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

Grant sponsor: Ministry of Science, Education and Sport of the Republic of Croatia; Grant Numbers: 022-0222148-2137, 022-0222148-2139.

*Correspondence to: Davor Zeljezic, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb 10000, Croatia. E-mail: dzeljezi@imi.hr

Received 30 September 2008; provisionally accepted 19 March 2009; and in final form 19 March 2009

DOI 10.1002/em.20495

Published online in Wiley InterScience (www.interscience.wiley.com).

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

MATERIALS AND METHODS

Blood Sampling

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

Treatment in vitro

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

Vital Staining Using Ethidium Bromide and Acridine Orange

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

Alkaline and hOGG1 Modified Comet Assay

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

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

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

Lymphocyte Cultures and FISH Analysis of Chromosomal and Nuclear Instability

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

Ferric-Reducing Ability of Plasma (FRAP) Assay

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

Lipid Peroxidation Thiobarbituric Acid Reactive Substances (TBARS)

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

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

Statistical Analysis

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

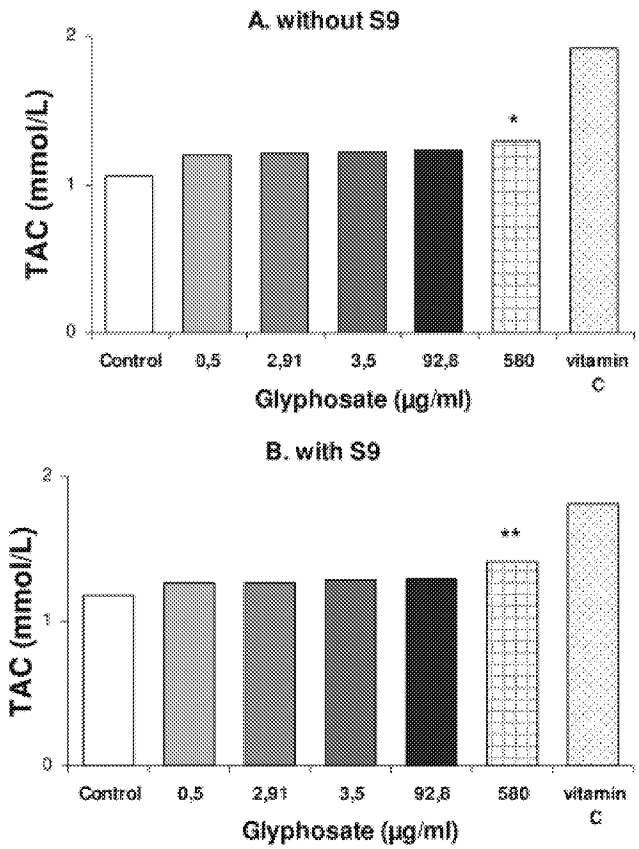

Fig. 1. Changes in plasma total antioxidant capacity (TAC) following treatment with glyphosate alone (A) or in combination with metabolic activation system S9 (B). Values are mean ± SD, $n = 3$. * Significantly different from control without S9 ($P < 0.05$). ** Significantly different from control with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

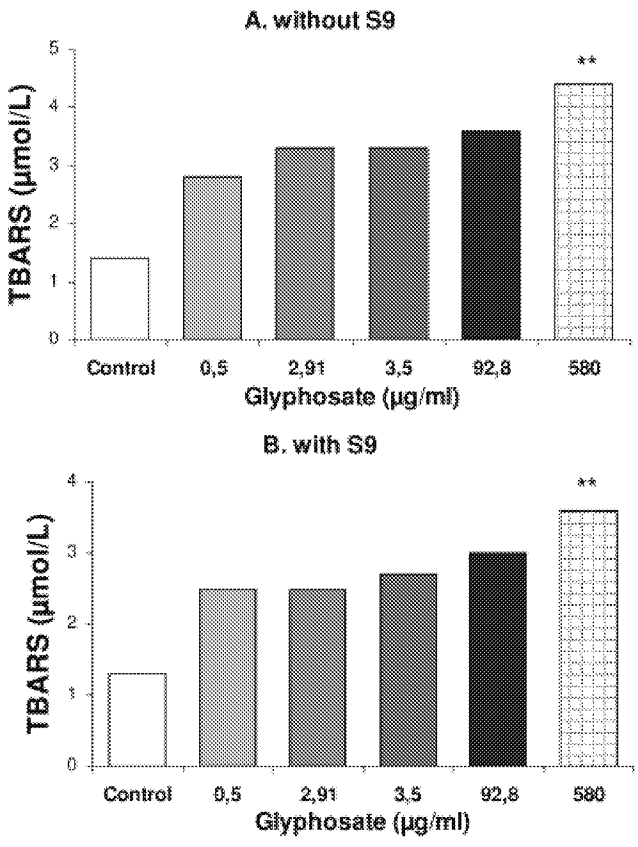

Fig. 2. Changes in plasma lipid peroxidation following treatment with glyphosate alone (A) or in combination with metabolic activation system S9 (B). Lipid peroxidation was determined by level of thiobarbituric reactive substances (TBARS). ** Significantly different from control with and without S9 ($P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

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

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

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

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

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

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

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

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

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

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

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

REFERENCES

Alibhai MF, Stallings WC. 2001. Closing down on glyphosate inhibition-with a new structure for drug discovery. PNAS 98:2944–2946.

Bellé R, Le Bouffant R, Morales J, Cosson B, Cormier P, Mulner-Lorillon O. 2007. Sea urchin embryo, DNA-damaged cell cycle checkpoint and the mechanisms initiating cancer development. J Soc Biol 201:317–327.

Benachour N, Sipahutar H, Moslemi S, Gasnier C, Travert C, Seralini GE. 2007. Time- and dose-dependent effects of roundup on

human embryonic and placental cells. Arch Environ Contam Toxicol 53:126–133.

Benzie IF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. Anal Biochem 239:70–76.

Bolognesi C, Bonatti S, Degan P, Gallerani E, Peluso M, Rabboni R, Roggieri P, Abbondandolo A. 1997. Genotoxic activity of glyphosate and its technical formulation roundup. J Agric Food Chem 45:1957–1962.

Bradberry SM, Proudfoot AT, Vale JA. 2004. Glyphosate poisoning. Toxicol Rev 23:159–167.

Brewster DW, Warren J, Hopkins WE. 1991. Metabolism of glyphosate in Sprague-Dawley rats: Tissue distribution, identification, and quantitation of glyphosate-derived materials following a single oral dose. Fundam Appl Toxicol 17:43–51.

Collins AR. 2004. The comet assay for DNA damage and repair (principles, application and limitations). Mol Biol 26:249–261.

Dallegrave E, DiGiorgio Mantese F, Coelho RS, Drawans Pereira J, Dalsenter PR, Langeloh A. 2003. The teratogenic potential of the herbicide glyphosate-roundup in Wistar rats. Tox Lett 142:5–52.

De Roos AJ, Zahm SH, Cantor KP, Weisenburger DD, Holmes FF, Burmeister LF, Blair A. 2003. Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men. Occup Environ Med 60:E11.

Dimitrov BD, Gadeva PG, Benova DK, Bineva MV. 2006. Comparative genotoxicity of the herbicides roundup, stomp, and reglone in plant and mammalian test systems. Mutagen 21:375–382.

Dipanker D, Tapas M. 2000. Study of Quinalphos formulation induced damage of testicular tissues and antioxidant. Appl Toxicol 20: 197–204.

Drury JA, Nycyk JA, Cooke RWI. 1997. Comparison of urinary and plasma malondialdehyde in preterm infants. Clin Chim Acta 263:177–185.

Duke RC, Cohen JJ. 1992.Morphological and biochemical assays of apoptosis. In: Coligan JE, Kruisbeal AM, editors. Current Protocols in Immunology. New York:Wiley. pp 1–3.

Fenech M. 2006. Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death. Mutat Res 600:58–66.

Guyton AC, Hall JE. 1996. Textbook of Medical Physiology, 4th ed. Philadelphia: W. B. Saunders Company. 1520 p.

Halliwell B. 1994. Free radical, antioxidants and human disease: Curiosity, causes or consequence. Lancet 344:721–724.

Hardell L, Eriksson M, Nordstrom M. 2002. Exposure to pesticides as risk factor for non-Hodgkin's lymphoma and hairy cell leukemia: Pooled analysis of two Swedish case-control studies. Leuk Lymp 43:1043–1049.

Heydens WF, Healy CE, Hotz KJ, Kier LD, Martens MA, Wilson AGE, Farmer DR. 2008. Genotoxic potential of glyphosate formulations: Mode-of -action ivestigations. J Agric Food Chem 56:1571–1523.

Kiely T, Donaldson D, Grube A. 2004. Pesticides Industry Sales and Usage 2000 and 2001 Market Estimates. Washington, DC: USEPA.

Kier LD, Stegeman SD. 1993. Final report. Mouse micronucleus study of AMPA. Unpublished report from Environmental Health Laboratory submitted to WHO by Monsanto. St. Louis Missouri, USA.

Li AP, Long TJ. 1988. An evaluation of the genotoxic potential of glyphosate. Fundam Appl Toxicol 10:537–546.

Lioi MB, Scarfi MR, Santoro A, Barbieri R, Zeni O, Salvemini F, Di Berardino D, Ursini MV. 1998. Cytogenetic damage and induction of pro-oxidant state in human lymphocytes exposed in vitro to gliphosate, vinclozolin, atrazine and DPX-E9636. Environ Mol Mutagen 32:39–46.

Lueken A, Juhl-Straus U, Krieger G, Witte I. 2004. Synergisitic DNA damage by oxidative stress (induced by H_2O_2) and nongenotoxic environmetal chemicals in human fibroblasts. Toxicol Lett 147: 35–43.

Marc J, Mulner-Lorillon O, Belle R. 2004. Glyphosate-based pesticides affect cell cycle regulation. Biol Cell 96:245–249.

Marzin D. 1999. New approaches to estimating the mutagenic potential of chemicals. Cell Biol Toxicol 15:359–365.

Monroy CM, Cortés AC, Sicard DM, de Restrepo HG. 2005. Cytotoxicity and genotoxicity of human cells exposed in vitro to glyphosate. Biomedica 25:335–345.

Norppa H, Falck GC-M. 2003. What do human micronuclei contain? Mutagen 18:221–233.

Ranjbar A, Solhi H, Mashayekhi FJ, Susanabdi A, Rezaie A, Abdollahi M. 2005. Oxidative stress in acute human poisoning with organophosphorous insecticides; a case control study. Environ Toxicol Pharmacol 20:88–91.

Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE. 2005. Differential effects of glyphosate and Roundup on human placental cells and aromatase. Environ Health Perspect 116:716–720.

Singh NP, McCoy MT, Tice RR, Schneider EL. 1988. A simple technique for quantitation of low levels of damage in individual cells. Exp Cell Res 175:184–191.

Sivikova K, Dianovsky J. 2006. Cytogenetic effect of technical gylphosate on cultivated bovine peripheral lymphocytes. Int J Hyg Environ Health 209:15–20.

Smith EA, Oehme FW. 1992. The biological activity of glyphosate to plants and animals: A literature review. Vet Hum Tox 34:531–543.

Smith CC, O'Donovan MR, Martin EA. 2006. hOGG1 recognizes oxidative damage using comet assay with greater specificity than FPG or ENDOIII. Mutagen 21:185–190.

Surralles J, Xamena N, Creus A, Catalan J, Norppa H, Marcos R. 1995. Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. Mutat Res 341:169–184.

U. S. Environmental Protection Agency. 1993. Glyphosate (CASRN 1071-83-6). Washington, DC: USEPA.

U. S. Environmental Protection Agency. 2004. Glyphosate: Pesticide Tolerance, Vol. 69. Number 217. Washington, DC: USEPA.

Vandana S, Poovolla V. 1999. Role of reactive oxygen metabolites in organophosphate Bidrine induced renal tubular cytotoxicity. J Am Soc Nephrol 10:1746–1752.

Vigfusson NV, Vyse ER. 1980. The effect of the pesticides, dexon, captan and roundup on sister chromatid exchanges in human lymphocytes in vitro. Mutat Res 79:53–57.

World Health Organization (WHO). 2003. Glyphosate and AMPA in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality. Geneva. (WHO/SDE/WSH/03.04/97).

Accepted by—
P. Schmezer