Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup

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Glyphosate (*N*-phosphonomethylglycine) is an effective herbicide acting on the synthesis of aromatic amino acids in plants. The genotoxic potential of this herbicide has been studied: the results available in the open literature reveal a weak activity of the technical formulation. In this study, the formulated commercial product, Roundup, and its active agent, glyphosate, were tested in the same battery of assays for the induction of DNA damage and chromosomal effects *in vivo* and *in vitro*. Swiss CD1 mice were treated intraperitoneally with test substances, and the DNA damage was evaluated by alkaline elution technique and 8-hydroxydeoxyguanosine (8-OHdG) quantification in liver and kidney. The chromosomal damage of the two pesticide preparations was also evaluated *in vivo* in bone marrow of mice as micronuclei frequency and *in vitro* in human lymphocyte culture as SCE frequency. A DNA-damaging activity as DNA single-strand breaks and 8-OHdG and a significant increase in chromosomal alterations were observed with both substances *in vivo* and *in vitro*. A weak increment of the genotoxic activity was evident using the technical formulation.

Keywords: *Pesticides; in vivo genotoxicity; in vitro genotoxicity; SCE; micronucleus test; alkaline elution; DNA oxidative damage*

INTRODUCTION

Roundup, an extremely effective nonselective postemergence herbicide, is a combination of an active ingredient, the isopropylamine salt of glyphosate, and a surface-active agent that enhances the spreading of spray droplets when they contact foliage. Glyphosate (GLYPH), *N*-(phosphonomethyl)glycine, is selectively toxic to plants and relatively nontoxic to animals. The mechanism of action of this herbicide is a competitive inhibition of the enolpyruvyl-shikimate-phosphate synthase (ESP-synthase), an enzyme absent in animals, essential to the synthesis of aromatic amino acids in plants (Amrhein et al., 1980; Hollander and Amrhein, 1980; Grossbard and Atkinson, 1985; Jaworski, 1972).

Glyphosate has been reported to have a low acute toxicity to different animal species with an oral LD_{50} ranging from 0.7 to 11.0 g/kg of body weight (Worthing and Hance, 1991; EPA, 1982; FAO, 1986). The results of a number of tests on a variety of species have shown that glyphosate has, at the range of concentration normally used in agriculture, no chronic or neurotoxic effects (Atkinson, 1985).

Chronic feeding studies did not evidence a carcinogenic activity of this herbicide in rats and dogs (FAO, 1986; IPCS, 1994). In these studies the active ingredient glyphosate demonstrated an organospecificity for the urinary system, inducing an increase in proximal renal tubule basophilia/hypertrophy and in hyperplasia of the urinary bladder (FAO, 1986).

No adverse effects on reproduction or fetal development have been observed in three-generation studies on rats and in a developmental toxicity study on rabbits (Atkinson, 1985).

Glyphosate is a mild skin and eye irritant (Maibach, 1986; California Department of Food and Agriculture, 1985), but Roundup has been identified as a cause of irritation phenomenon or contact dermatitis, reported in occupationally exposed agricultural workers (FAO, 1986).

The formulated commercial product, Roundup, seems to be rather more toxic than the parent compound. Clinical reports citing cases of acute poisoning indicate that the lung could be considered the target organ of Roundup toxicity. The pulmonary toxicity of Roundup has been demonstrated in rats, after a direct tracheal administration (Martinez et al., 1990; Martinez and Brown, 1991). This effect has been attributed to surface active ingredients (Sawada et al., 1988); nevertheless, a study on the interaction between glyphosate and surfactants revealed an antagonistic effect (Baba et al., 1989)

The genotoxicity of glyphosate and its technical formulations has been studied in different test systems. The results obtained indicate no genotoxic activity for glyphosate and a weak effect for technical formulation.

The aim of our investigation is the evaluation of the genotoxic potential of this herbicide, testing both Roundup and glyphosate in the same battery of assays.

Few data have been reported on the DNA-damaging activity of this herbicide *in vivo*. We have evaluated the DNA damage in terms of single-strand breaks and 8-hydroxydeoxyguanosine (8-OHdG).

MATERIALS AND METHODS

Chemicals. Analytical grade glyphosate (CAS Registry No. 1071-83-6) was purchased from Società Italiana Chimici, Rome, Italy. Purity declared by the producer was 99.9%. Roundup formulate (30.4% glyphosate) was from Monsanto Italiana, Milan, Italy. Methyl methanesulfonate (MMS, CAS Registry No. 66-27-3) and benzo[*a*]pyrene (B[*a*]P, CAS Registry No. 50-32-8) were obtained from Sigma Chemical Co. St. Louis, MO. (CAS Registry No. were provided by the author.)

In Vitro. **SCE.** Heparinized venous blood samples were obtained from two healthy female donors. Whole blood (0.5

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mL) was added to 4.5 mL of RPMI 1640 medium (Gibco) supplemented with 17% fetal bovine serum. After addition of 50 μL of phytohemoagglutinin (PHA, Gibco) and 50 μL of 1 mM bromodeoxyuridine (BUdR), cultures were incubated in complete darkness at 37 °C.

Test chemicals were added 24 h after PHA stimulation. Two hours before determination, 75 μl of Colcemid (Gibco) was added.

At 72 h from the onset of culture, slides were prepared according to standard methods. After drying, the slides were stained with Hoechst 33258. Slides were scored by two observers: at least 50 metaphases were scored for each experimental point.

In Vivo. *Animals.* Swiss CD1 male mice, 8−10 weeks old and weighting 30−40 g, obtained from Charles River, Como, Italy, were used with all assays.

Alkaline Elution Assay. Groups of three male mice were treated by ip injection with a single dose of either glyphosate (300 mg/kg) or Roundup (900 mg/kg) (corresponding to 270 mg/kg of glyphosate). Glyphosate and Roundup were dissolved in physiologic saline; the pH of the solution was carefully checked and adjusted to pH 7.0 before treatment. The animals were sacrificed 4 and 24 h after the injection. Liver and kidneys were removed and processed to obtain crude nuclei free from adhering tissues (Bolognesi et al., 1992).

Crude nuclei were centrifuged at 50*g* for 2 min, and pelleted nuclei were resuspended in saline EDTA, pH 7.4. Aliquots containing (0.5−1.0) × 10^6 nuclei were applied to membrane filters (25 mm diameter, 5 μm pore size, Millipore Co., Milford, MA). Nuclei were lysed on the filters at room temperature. Single-stranded DNA fractions were eluted with a solution containing 0.04 M EDTA and 10% tetraethylammonium hydroxide, pH 12.3. Fractions were collected at 12 min intervals during 1 h at a flow rate of 0.17 mL/min. Fluorometric determination of DNA was performed with Hoechst 33258 reagent according to the method developed in our laboratory (Cesarone et al., 1979). The results were expressed as elution rate constant, *K*, according to the formula

$$K\ (\mathrm{mL}^{-1}) = \frac{-\ln \text{ fraction of DNA retained on the filter}}{\text{eluted volume}}$$

DNA Oxidative Damage. Livers and kidneys from animals treated as for the alkaline elution assay were homogenised in 5 mL of PBS. Nuclei were obtained after centrifugation and resuspended in 3 mL of SE buffer (75 mM NaCl, 25 mM EDTA pH 8.0), 50 μL of 10 mg/mL Proteinase K, 250 μL of 20% SDS, and 30 μL of 2 mg/mL RNase A and are then added to the tubes; the samples are incubated overnight at 37 °C. The next day 0.4 volume of 5 M NaCl is added, and the samples are vortexed for 20 s. Tubes are then centrifuged at 4000 rpm for 30 min. Supernatant is collected, and 1 volume of chloroform/isoamyl alcohol (24:1) is added. The tubes are left in slow orbital centrifugation for 30 min before a 4000 rpm centrifugation for 30 min. Supernatant is then collected, and DNA is precipitated by adding 2 volumes of ice-cold 100% ethanol. The pellet is then washed with 70% ethanol, and finally DNA is resuspended in 75 mM Tris, pH 7.4. Aliquots of DNA are then hydrolyzed with Nuclease P1 and alkaline phosphatase (Degan et al., 1995) and filtered through cellulose acetate (Centricon) filter units, 0.22 mm. Approximately 80 μg of DNA per sample is injected in HPLC.

The separation of 8-OHdG and normal deoxynucleosides is performed in a LC-18-DB column (Supelco, 75 × 4.6 mm) equipped with an LC-18-DB guard column cartridge. The solvent system consists of an isocratic mixture of 90% 50 mM potassium phosphate, pH 5.5, and 10% methanol at 1 mL/min flow rate. UV detection is accomplished at 254 nm, and electrochemical analysis is carried out by a pulsed electrochemical detector (PED; Dionex, Sunnyvale, CA) in the amperometric mode, with a glass−carbon electrode using an Ag/AgCl reference electrode. The levels of 8-OHdG are referred to the amount of deoxyguanosine (dG) detected by UV absorbance at 254 nm. The amount of DNA is determined by a calibration curve *vs* known amounts of calf thymus DNA. The levels of 8-OHdG are expressed as the number of 8-OHdG adducts per 10^5 dG bases (8-OHdG/10^5 dG).

Micronucleus Assay. Three male mice were treated by two ip injections of half of the chosen dosage of 300 mg/kg for glyphosate and 450 mg/kg Roundup (corresponding to 135 mg/kg glyphosate) with an interval of 24 h. MMS, chosen as a positive control, was administrated by single ip injection. Animals were killed by cervical dislocation 6 and 24 h after the last injection of the test chemicals. Cells were obtained and processed following the method described by Schmid (1975) with minor modifications. The bone marrow from both femora was flushed into a test tube containing calf serum. Bone marrow smears were prepared, and the percentage of polychromatic erythrocytes (PCE) among 1000 erythrocytes was determined for each mouse. The results were expressed as the average number of micronucleated PCEs (MN PCEs ± SD) following scoring of 1000 PCEs on each of three separate slides.

Statistical Analysis. The standard deviation and the nonparametric test of Mann−Whitney were used for the statistical analysis (Siegel, 1956).

RESULTS

DNA Damage. *DNA Breaks and Alkali Labile Sites.* Figure 1 shows the results obtained with the alkaline elution assay in liver and kidney of Swiss CD1 mice treated with glyphosate or Roundup. A significant increase in the elution rate constant was observed 4 h after treatment with the technical formulation or its active ingredient, glyphosate. The increase in the elution rate was consistently higher in kidney of mice treated with glyphosate or Roundup.

After 24 h, the elution rate constant returned to control values (data not shown). This transient effect could be attributed to the rapid elimination of this compound from the body and/or to repair of the DNA damage. No significant difference was observed in the extent of damage for the two compounds.

DNA Oxidative Damage. Figure 2 reports the results obtained for the quantification of 8-OHdG from liver and kidney DNA of the animals treated with Roundup or glyphosate. Treatment with glyphosate does not result in any increase of 8-OHdG in kidney but does induce a large and significant increase in liver at 24 h. Treatment with Roundup results in a significant, although small, increase of 8-OHdG content over the control in kidney and in a nonsignificant increase in liver. In both tissues the largest effect is found at 24 h after the treatment of the animals.

Overall, either the pure compound or the technical formulation is able to stimulate the oxidative metabolism in the liver and in the kidney.

Cytogenetic Damage. *In Vitro. SCE Analysis.* Glyphosate and Roundup were tested for the induction of SCE in human lymphocytes *in vitro.* The results are shown in Figure 3. A dose-dependent increase of SCE frequencies was observed in glyphosate-treated cells. A significant increment of the cytogenetic damage was evident in Roundup-treated lymphocytes when compared to the active ingredient. For Roundup the concentrations tested were lower due to the high toxicity of the technical formulation, resulting in the absence of mitotic cells above 0.33 mg/mL. At the highest concentration of Roundup tested, the SCE/cell ratio was comparable with that obtained at a dose of glyphosate 10 times higher.

In Vivo. Micronucleus Test. Table 1 shows the incidence of micronuclei observed in mouse bone marrow after treatment with glyphosate and its technical formulation, Roundup. A low, but statistically significant,

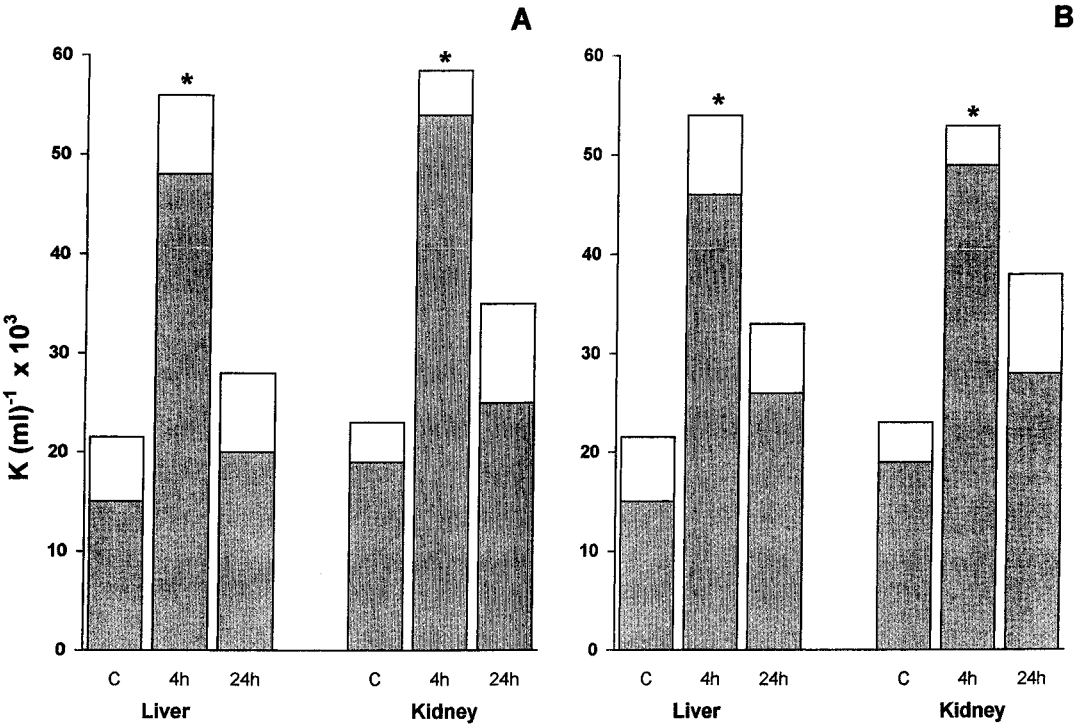

Figure 1. Induction of DNA single-strand breaks and alkali labile sites by glyphosate (A) and Roundup (B). Dose was 300 mg/kg expressed as glyphosate. Mean data with SD of at least four independent experiments (8 animals) in treated animals and at least six experiments in controls (12 animals) are shown. * indicates $p < 0.05$.

Table 1. Frequencies of Micronuclei in PCEs of Bone Marrow of Mice Treated with Glyphosate and Roundup

compound	no. of animals	treatment (mg/kg of bw)	time of death (h)	PCEs/NCEs $x \pm$ SD	MN/1000 PCEs[a] $x \pm$ SD
glyphosate	4	300 (2 × 150)	6	0.6 ± 0.05	1.4 ± 0.9
	4	300 (2 × 150)	24	0.5 ± 0.2	2.4 ± 1.5*
Roundup[b]	3	450 (2 × 225)	6	0.3 ± 0.09	2.2 ± 0.4*
	3	450 (2 × 225)	24	0.4 ± 0.1	3.1 ± 1.2*
MMS	6	80	24	0.75 ± 0.01	80.0 ± 8.5*
controls	6			0.73 ± 0.06	0.75 ± 0.46

[a] A minimun of 3000 PCEs were scored for animal. *Statistically significant [$p < 0.05$, Mann−Whitney test (Siegel, 1956)]. [b] Glyphosate 30.4%.

increase in MN frequencies was seen in all groups of treated mice.

The values were generally higher in mice sacrificed 24 h after the last treatment, the increase being more pronounced in the Roundup-treated mice.

In addition, a significant reduction in the PCE/NCE ratio is evident in Roundup-treated animals, showing a target organ toxicity of the formulation.

DISCUSSION

Glyphosate has a broad spectrum herbicidal activity against a wide range of annual and perennial weeds. The lack of phytotoxicity after it contacts soil and its low mammalian toxicity have increased its popularity and use for weed control.

Roundup herbicide is tightly bound to soil and then rapidly and completely degraded by microorganisms. A low metabolism of the compound by plants has been described; however, it is readily degraded in soil, where it is metabolized to aminomethylphosphonic acid (AMPA) and CO_2 (Mueller et al., 1984).

This herbicide is practically nontoxic to non-plant-life forms such as aquatic and avian species, animals, and man (IPCS,1994).

No effects have been observed on various ecosystems (Malik et al., 1989; Wan et al., 1989).

The combined results from numerous toxicological studies using various animal species demonstrate that glyphosate does not cause relevant adverse effects in mammals. Glyphosate is rapidly eliminated from the body as unmetabolized parent compound. Little evidence of metabolism was observed in mammal gut content after an oral administration of this pesticide, presumably produced by the intestinal microbial action (Brewster et al., 1991).

Despite the many positive aspects of glyphosate use, there are some data that indicate its technical formulation, Roundup, is a potential toxic agent.

Glyphosate and its technical formulations have been studied for genotoxicity in different test systems (Table 2). The active ingredient, glyphosate, was consistently without mutagenic effect in a range of short-term tests.

Negative results were obtained in gene mutation test with *Salmonella typhimurium* (in the presence or absence of S9 fraction) and mammalian cells in culture (Wildeman and Nazar, 1982).

Glyphosate (98%) was negative in chromosomal mutation and DNA damage assays using different test systems *in vivo* and *in vitro*.

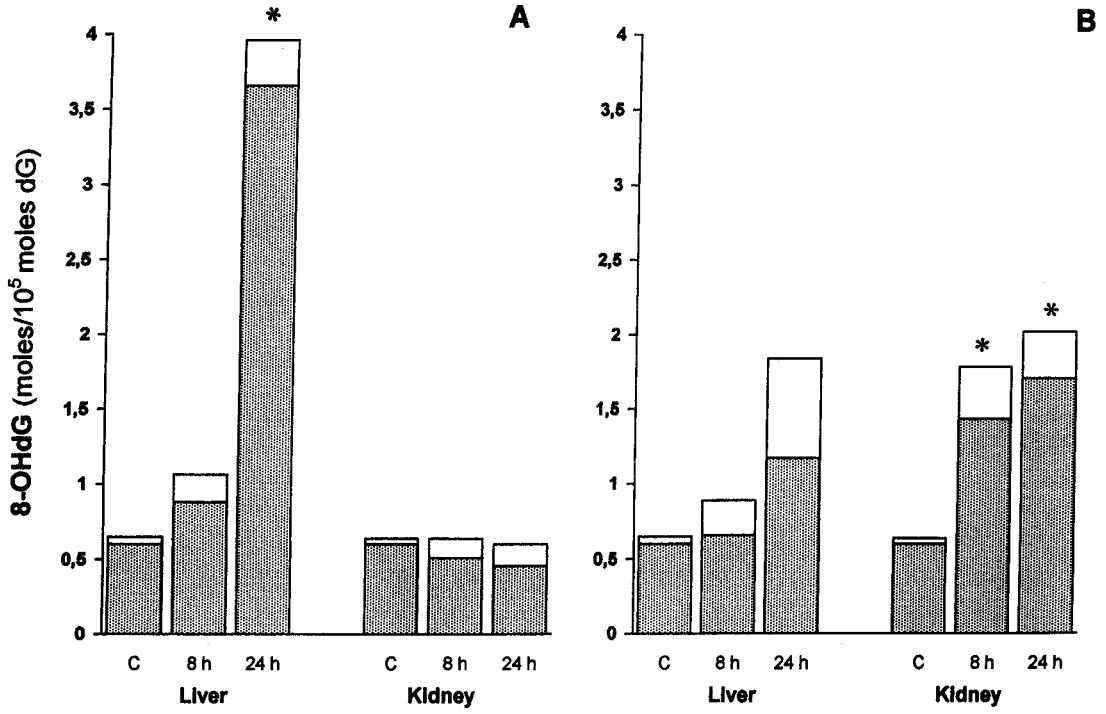

Figure 2. Induction of 8-OHdG in DNA from livers and kidneys of Swiss CD1 mice 8 and 24 h after treatment with glyphosate (A) and Roundup (B). Data are from at least three experiments (six animals). * indicates $p < 0.05$.

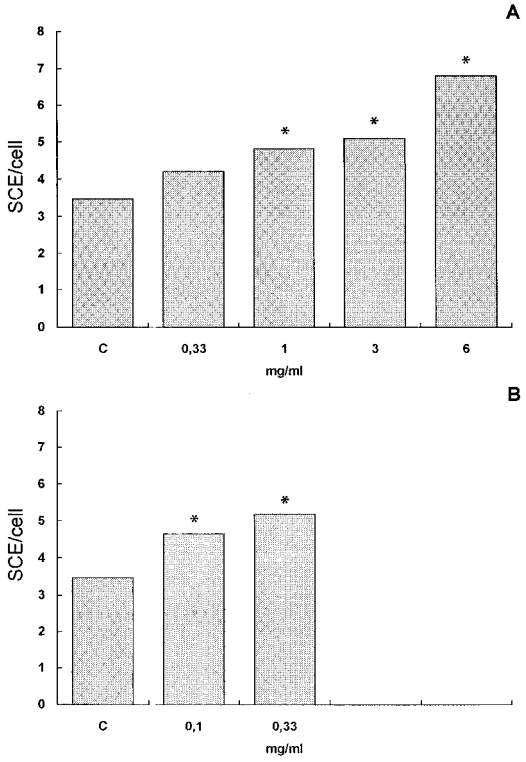

Figure 3. Induction of sister chromatid exchanges in human lymphocytes by glyphosate (A) and Roundup (B) (72 h of treatment). Pooled data from two independent experiments are reported. * indicates $p < 0.05$.

The technical formulation, Roundup, has demonstrated some genotoxic activity inducing gene mutations in *S. typhimurium* TA98 and TA100 and chromosomal damage in *Allium cepa* meristem root cells at concentrations close to the level of toxicity (Rank et al., 1993).

In addition, an increase in SCE frequency was observed in human lymphocytes treated with a Roundup of unidentified purity *in vitro.* Our results, using the same experimental system, confirm these data and show a dose-response effect with glyphosate. The higher toxicity of Roundup compared to glyphosate prevents the use of high dosages of the formulation.

Cytogenetic damage, more pronounced for Roundup formulation, was also evident in mouse bone marrow as an increase in micronucleus frequency.

A DNA-damaging activity of glyphosate and Roundup was observed in liver and kidney of mice, in terms of DNA breaks and alkali labile sites. No significant difference has been demonstrated between active ingredient and its technical formulation.

The two compounds show some degrees of oxidative damage in liver DNA, with glyphosate giving the largest damaging effect at 24 h after the treatment. In kidney, glyphosate does not increase the 8-OHdG level, while Roundup treatment results in a significant increase of DNA oxidative damage. The kinetic of the induction of DNA oxidative damage is apparently similar to that observed with another pesticide formulation in a previous study (Bolognesi et al., 1994).

The activity of these two compounds toward the stimulation of the oxidative metabolism is not surprising since the majority of the processes involved in the metabolism of pollutants and xenobiotics result in the production of highly reactive intermediate side products that are often in radical form. These reactive metabolites *per se* or the oxygen radicals generated in these processes are electrophilic compounds that have a strong affinity for the interaction with DNA and other cellular components, finally resulting in stable and covalent modifications to their structure.

So far, the measure of 8-OHdG may be seen as an integrated quantification of a stress at which a cell is exposed in the short-term range and also as a process that takes into account the balance between the processes of DNA damage and repair.

On the whole, available data on the scientific literature show a low acute and chronic toxicity of glyphosate at the concentration normally used in agriculture or found in treated products.

Table 2. Summary of Results on the Genotoxicity of Glyphosate

test organisms	genetic effect	compound (purity)	results[a] without exogenous metabolic system	results[a] with exogenous metabolic system	dose[b] LED/HID	references
		Gene Mutations				
S. typhimurium TA98, TA100, TA1535, TA1537, TA1538	reverse mutation	glyphosate (not specified)	−	−	2500	Shirasu et al. (1982)
S. typhimurium TA98, TA100	reverse mutation	glyphosate (not specified)	−	− (S9 plant)	25	Wildeman and Nazar (1982)
S. typhimurium TA98, TA100, TA1535, TA1537, TA1538	reverse mutation	glyphosate (98%)	−	−	2500	Li and Long (1988)
S. typhimurium TA98	reverse mutation	Roundup (glyphosate 48%)	+	−	180	Rank et al. (1993)
S. typhimurium TA100	reverse mutation	Roundup (glyphosate 48%)	−	+	360	Rank et al. (1993)
E. coli WP2 her	reverse mutation	glyphosate (not specified)	−	−	2500	Shirasu et al. (1982)
E. coli WP2	reverse mutation	glyphosate (98%)	−	−	2500	Li and Long (1988)
Chinese hamster ovary cells	reverse mutation	glyphosate (98%)	−	−	22.5	Li and Long (1988)
D. melanogaster	sex-linked recessive lethals	Roundup (not specified)	0	−		Gopalan and Njagi (1981)
		Chromosomal Mutations				
A. cepa	chromosomal aberrations	Roundup (glyphosate 48%)	+	0	1440	Rank et al. (1993)
rat bone marrow (*in vivo*)	chromosomal aberration	glyphosate (98%)	−	0	1000	Li and Long (1988)
human peripheral blood *in vitro*	SCE	Roundup (not specified)	+	0	2500	Vigfusson and Vyse (1980)
human peripheral blood *in vitro*	SCE	Roundup (glyphosate 30.4%)	+	0	100	this paper
human peripheral blood *in vitro*	SCE	glyphosate (99.9%)	+	0	1000	this paper
V. faba (root tips)	micronucleus test	Solado (glyphosate 21%)	−	0	1400[c]	De Marco et al. (1992)
mouse bone marrow (*in vivo*)	micronucleus test	glyphosate (not specified)	−	0	200	Rank et al. (1993)
mouse bone marrow (*in vivo*)	micronucleus test	Roundup (glyphosate 48%)	−	0	200	Rank et al. (1993)
mouse bone marrow (*in vivo*)	micronucleus test	glyphosate (99.9%)	+	0	300	this paper
mouse bone marrow (*in vivo*)	micronucleus test	Roundup (glyphosate 30.4%)	+	0	135	this paper
		DNA Damage				
B. subtilis	rec-assay	glyphosate (98%)	−	−	1000	Li and Long (1988)
rat epatocytes	UDS	glyphosate (98%)	−	−	125	Li and Long (1988)
mouse (*in vivo*)	DNA single-strand breaks	glyphosate (99.9%)	+	0	300	this paper
mouse (*in vivo*)	DNA single-strand breaks	Roundup (glyphosate 30.4%)	+	0	270	this paper
mouse (*in vivo*)	8-OHdG	glyphosate (99.9%)	±[d]	0	300	this paper
mouse (*in vivo*)	8-OHdG	Roundup (glyphosate 30.4%)	±[e]	0	270	this paper

[a] +, positive; −, negative; 0, not tested. [b] *In vitro* tests, μg/mL; *in vivo* tests, mg/kg of bw. LED, lowest effective dose; HID, highest ineffective dose. [c] Expressed as μg/g of soil. [d] Positive results have been obtained in liver cells. [e] Positive results have been obtained in kidney cells

The higher activity of technical formulations in inducing toxic and genotoxic damage in different experimental systems suggests a role of the surface active agents and/or coformulants in the potentiation of the effects of the active ingredient.

Considering the wide use of this herbicide for agricultural and nonagricultural uses, such as weed killing in water systems, parks, and gardens, the risk assessment process of commercial technical formulations has to be considered of primary importance.

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