1. Information on the study

Data point:	CA 5.4
Report author	Roustan A. *et al.*
Report year	2014
Report title	Genotoxicity of mixtures of glyphosate and atrazine and their environmental transformation products before and after photoactivation
Document No	Chemosphere (2014) Vol. 108, 93–100
Guidelines followed in study	OECD Test Guideline 487
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Abstract

The photo-inducible cytogenetic toxicity of glyphosate, aminomethyl phosphoric acid (AMPA), and their various mixtures was assessed by the in vitro micronucleus assay on CHO-K1 cells. Results demonstrated that the cytogenetic potentials of pesticides greatly depended on their physico-chemical environment. The mixture made with the four pesticides exhibited the most potent cytogenetic toxicity, which was 20-fold higher than those of the most active compound AMPA, and 100-fold increased after light-irradiation. Intracellular ROS assessment suggested the involvement of oxidative stress in the genotoxic impact of pesticides and pesticide mixtures.

Materials and methods

Chemicals - Glyphosate and AMPA were purchased from Sigma-Aldrich Chemical Company, St Quentin-Fallavier, France.

Cell culture - Experiments were performed in CHO-K1 cells (ATCC-LGC Standards Sarl, Molsheim, France). CHO-K1 cells were maintained in McCoy's 5A medium supplemented with 10% bovine calf serum, 1 mM glutamine, and 100 U/mL-10 µg/mL penicillin-streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Metabolic activation mixture (S9-mix) - The metabolic activation system used was a 9000g centrifuged supernatant (S9) of a 10% liver homogenate prepared from male Sprague-Dawley rats treated with a single injection of Aroclor 1254 (500 mg/kg bw), 5 days before sacrifice. The protein concentration in the S9 homogenate was 26 mg/mL. For the micronucleus assay, the S9-mix contained 10% S9, 5 mM glucose-6-phosphate, 4 mM NADP, 33 mM KCl and 8 mM $MgCl_2$ diluted in PBS.

Photoactivation - To reproduce photoinduction during exposure to glyphosate and AMPA in the environment, the micronucleus assay was performed with a photoactivation procedure. Irradiation of cell cultures was carried out 1 hour after the addition of test compound using a solar simulator Suntest CPS+ apparatus equipped with a xenon arc lamp (1,100 W), a special glass filter restricting transmission of light below 290 nm and a near IR-blocking filter. The irradiance for the photoactivation was fixed at 750 W/m^2 throughout testing. The combined light dose was 4.5 J/cm^2 for one minute irradiation (0.03 J/cm^2 of UVB, 0.41 J/cm^2 of UVA and 4.06 J/cm^2 of visible light). This irradiation dose is representative of a 1-3 minute period of solar exposure during a clear summer day in the United Kingdom. The temperature of the samples was kept at 4 °C. UVA-visible light (320-800 nm) was obtained using the solar ID65 filter plus a window glass filter.

Micronucleus assay - A total of 50,000 CHO-K1 cells was plated in chamber slides and incubated for 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2. Various concentrations of glyphosate (5, 10, 50, and 100 µg/mL) and AMPA (0.005, 0.01, 0.05, and 0.1 µg/mL without S9-mix; 0.1, 0.5, 1, and 5 µg/mL with S9-mix; 0.00005, 0.0001, 0.0005, and 0.001 µg/mL with light irradiation) were incorporated into duplicate CHO-K1 cell cultures. To determine the background DNA-damage levels in CHO cells in the dark and with light irradiation 2 negative controls were added for glyphosate and AMPA: culture medium and PBS. Mitomycine C (0.06 µg/mL) without S9-mix and benzo[a]pyrene (5 µg/mL) with S9-mix were selected as positive controls. After 3 hours of exposure, cells were rinsed with PBS and incubated in fresh medium containing cytochalasin B (3 µg/mL) for an additional 24 hours to stop cytokinesis. At the end of the incubation period, cells were rinsed twice with PBS and fixed with methanol. The slides were air dried and stained with 5% Giemsa stain in Milli-Q water for 15 minutes. The Cytokinesis Blocked Proliferation Index (CBPI) was used to select adequate concentrations for the assessment of micronuclei. CBPI was determined by scoring the number of mononucleated (M1), binucleated (M2), and trinucleated (M3) cells among 500 Giemsa-stained cells with well-preserved cytoplasm: CBPI = [(M1) + (2 x M2) + (3 x M3)]/500. When a cytotoxic effect was observed, micronucleated cell rates were determined for concentrations inducing less than a 50% decrease of CBPI. When no cytotoxic effect was observed, the maximal concentration assessed was 100 µg/mL. When a clastogenic/aneugenic activity was observed, 4 relevant concentrations were selected to obtain a dose-response relationship. A total of 2000 binucleated cells were examined for each concentration, and micronuclei were identified according to the morphological criteria previously defined.

Intracellular ROS analysis - Intracellular ROS was determined with the cell-based OxiSelect™ Intracellular ROS Assay Kit using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). After diffusion into the cell, this fluorescent probe is deacetylated by cellular esterases to non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by ROS. A total of 50,000 cells were seeded in a black 96-well cell culture plate and incubated at 37 °C for 24 hours. Cells were rinsed 3 times with PBS and incubated in 100 µL of DCFH-DA/media solution at 37 °C for 60 minutes. Cells were subsequently rinsed with PBS and treated with glyphosate or AMPA. Experiments were performed in triplicate. The concentrations tested were the same as those assessed for the micronucleus assay. H_2O_2 (100 µM) was used as the positive control. After a 30-minute incubation period, fluorescence was measured with a fluorometric plate reader at 480 nm and 530 nm.

Statistical analysis – Statistically significant differences between negative controls and treated samples were determined using the ^{2}test. The standard micronucleus assay was considered positive when a dose-response relationship could be established between the numbers of micronucleated cells and the concentrations of pesticide solutions, and when at least one concentration induced a significant increase of micronucleated cells as compared to the medium-only control culture. The dose-response relationships were calculated by nonlinear regression analysis with TableCurve2D®. Model significance was based on three criteria: (i) correlation coefficient r^2 being > 0.62; (ii) model probability (P) being < 0.05; (iii) error probability (PE) being > 0.05. The Minimal Clastogenic Concentration (MCC) was defined as the lowest concentration of test item (µg/mL) that induced a significant increase of micronucleated cells. The Cytogenetic Potency (CP) was defined as the slope of the dose–response curves. It was calculated by non-linear regression analysis with TableCurve2D®.
For the determination of ROS, the dose-response relationships were calculated by linear regression analysis with TableCurve2D®. Model significance was based on three criteria: (i) correlation coefficient r^2 being > 0.62; (ii) model probability (P) being < 0.05; (iii) error probability (PE) being > 0.05. The Oxidative Potency was defined as the slope of the dose-response curves and was calculated according to a standard curve obtained with various concentrations of fluorescent DCF.

Results

No statistically significant increase in the incidence of bi-micronucleated cells (BMC) was observed with glyphosate in the dark and without S9-mix at concentrations up to 100 ug/mL. In the presence of S9-mix a statistically significant and dose-related increase was noted from 10 µg/mL. The calculated

minimal clastogenic concentration (MCC) was 5.8 µg/mL. With light irradiation a statistically significant increase in BMC was noted at the highest concentration of glyphosate tested (100 µg/mL) with a MCC of 93.4 µg/mL. Without S9-mix a statistically significant and dose-related increase in BMC was produced by AMPA from a concentration of 0.01 µg/mL with a MCC of 0.006 ug/mL. In the presence of S9-mix a statistically significant increase in BMC was seen with AMPA from 1 µg/mL with a MCC of 0.78 µg/mL. With light irradiation, the lowest test concentration of AMPA with a statistically significant increase in BMC was 0.0005 µg/mL with a MCC of 0.0004 µg/mL. The oxidative potency of H_2O_2, used as a positive control, was 333.3 nM DCF/μM^{-1} (data not shown). Only AMPA exerted an elevated oxidative effect (5.9 nM DCF/$\mu g.mL^{-1}$), whereas the oxidative potency of glyphosate was very low.

Discussion

The results obtained in the present study confirmed the cytogenetic toxicity of glyphosate and AMPA *in vitro*. In the dark, glyphosate was not directly active in the absence of S9-mix, but it induced micronuclei in the presence of S9-mix. On the contrary, without S9-mix, AMPA displayed a direct cytogenetic effect which was 1,000 fold higher than that of its parent compound. The weak mutagenic activity of glyphosate with S9-mix could be explained by its weak metabolization yield *in vivo*. After sunlight irradiation, glyphosate was weakly active, whereas the cytogenetic effect of AMPA was about 20-fold increased. Since the photodegradation of glyphosate by UV light has been shown to produce AMPA, the photoinducible genotoxic potential of glyphosate could be partially explained by the formation of its metabolite AMPA in the intracellular environment. In the present study, cells were subjected to a very low irradiation that did not induce intrinsic DNA damage. The results of the present study implied more probably the involvement of a photoinduced oxidative stress, which transformed glyphosate and AMPA into photoactivated intermediates favoring interactions with cellular targets.

Conclusion

The results of this study demonstrated that light-irradiation, corresponding to a few minutes of solar exposure, greatly potentiated the cytogenetic impact of AMPA. *In vitro* experiments showed that the genotoxic impact of pesticides greatly depend on their physico-chemical environment,

3. Assessment and conclusion

Assessment and conclusion by applicant:

The cytogenetic effect of two herbicides (glyphosate and atrazine), their metabolites (AMPA and DEA), and mixtures thereof was investigated in CHO-K1 cells in the *in vitro* micronucleus test. Only the results of glyphosate and AMPA tested alone are reported and discussed in the summary. Glyphosate and AMPA were tested with and without metabolic activation and with light irradiation. Also the potency of glyphosate and AMPA to produce ROS was investigated. The concentrations tested ranged from 5 to 100 µg/mL for glyphosate and from 0.00005 to 5 µg/mL for AMPA. No statistically significant increase in the incidence of bi-micronucleated cells (BMC) was observed with glyphosate at concentrations up to 100 ug/mL in the dark and without metabolic activation. However, a statistically significant and dose-related increase in BMC was noted from 10 µg/mL in the presence of metabolic activation. With light irradiation a statistically significant increase in BMC was noted for glyphosate at a concentration of 100 µg/mL. AMPA produced a statistically significant and dose-related increase in BMC from a concentration of 0.01 µg/mL in the dark and without metabolic activation. With metabolic activation a statistically significant increase in BMC was seen with AMPA from 1 µg/mL. With light irradiation the lowest test concentration of AMPA with a statistically significant increase in BMC was 0.0005 µg/mL. Only AMPA was found to produce an elevated oxidative effect, whereas the oxidative potency of glyphosate was very low. The results of glyphosate in the *in vitro* micronucleus test with metabolic activation reported in this study are surprising since glyphosate is essentially unmetabolized *in vitro* in the presence of a rat liver S9 homogenate. Moreover, these results are not corroborated by regulatory *in vivo* micronucleus tests in the mouse dosed up to more than 2,000 mg/kg bw.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate and AMPA tested were not sufficiently characterized and no positive and control historical data were reported. The *in vitro* micronucleus test carried out was in compliance with OECD TG 487.

Reliability criteria for *in vitro* toxicology studies

Publication: Roustan *et al*., 2014	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	In vitro MN test compliant with OECD TG 487.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity not reported. Source: Sigma-Aldrich Chemical Company, St Quentin-Fallavier, France.
Only glyphosate acid or one of its salts is the tested substance	N	Other pesticides (atrazine, desethyl atrazine (DEA)) were tested and mixtures thereof.
AMPA is the tested substance	Y	Tested alone and as mixtures with glyphosate, atrazine and DEA.
Test system clearly and completely described	Y	CHO-K1 cells.
Test conditions clearly and completely described	Y	CHO-K1 cell MN test.
Metabolic activation system clearly and completely described	Y	S9-mix.
Test concentrations in physiologically acceptable range (< 1 mM)	Y	From 5 to 100 µg/mL for glyphosate and from 0.00005 to 5 µg/mL for AMPA.
Cytotoxicity tests reported	Y	Cytokinesis Blocked Proliferation Index (CBPI) and incidence of bi-micronucleated cells (BMC).
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate and AMPA tested were not sufficiently characterized and no positive and control historical data were reported. The *in vitro* micronucleus test carried out was in compliance with OECD TG 487.		

1. Information on the study

Data point:	CA 5.4
Report author	Santovino A. *et al.*
Report year	2018
Report title	In vitro evaluation of genomic damage induced by glyphosate on human lymphocytes
Document No	Environmental Science and Pollution Research (2018) 25:34693-34700s
Guidelines followed in study	Some compliance with OECD 473 and OECD 487
Deviations from current test guideline	Only continuous treatments in the absence of S9 were performed and these exceeded the 1.5 cell cycles recommended by both guidelines No historical control data are reported. Treatment commenced within 24 hours of PHA stimulation rather than 44-48 hours
Previous evaluation	No
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this study, its was analyzed the *in vitro* clastogenic and/or aneugenic effects of glyphosate by chromosomal aberrations and micronuclei assays. Human lymphocytes were exposed to five glyphosate concentrations: 0.500, 0.100, 0.050, 0.025, and 0.0125 μg/mL, where 0.500 μg/mL represents the established acceptable daily intake value, and the other concentrations were tested in order to establish the genotoxicity threshold for this compound.

Materials and Methods

Study population; Peripheral venous blood was collected from six healthy Italian subjects (two males and four females, mean age ± SD, 27.50 ± 12.55), non-smoking, not alcoholics, not under drug therapy, and with no recent history of exposure to mutagens. All subjects signed the informed consent. The study was approved by the University of Turin ethics committee and was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki.

Blood sample collection and lymphocyte cultures; Blood samples were obtained by venipuncture, collected in heparinized tubes, cooled (4 °C), and processed within 2 h after collection. Lymphocyte cultures, fixation, and staining procedures were performed as previously described. Total time of lymphocyte cultures was 52 and 72 h for CA and MNi assays, respectively. After 24 h of incubation, 8.6 μL of glyphosate stock solution (Sigma-Aldrich, Saint Louis, MO, USA, CAS n. 1071-83-6) at the final concentration of 0.5 mg/mL in dimethyl sulfoxide (DMSO) was added to the lymphocyte culture in order to reach a final glyphosate concentration of 0.500 μg/mL. Similarly, 8.6 μL of glyphosate stock solution diluted 5, 10, 20, and 40 times with DMSO were added to the lymphocyte cultures in order to reach the final glyphosate concentrations of 0.100 μg/mL, 0.050 μg/mL, 0.025 μg/mL, and 0.0125 μg/mL, respectively. In particular, 0.500 μg/mL represents the ADI concentration established by EFSA for this compound, whereas 0.100, 0.050, 0.025, and 0.0125 μg/mL concentrations were tested in order to evaluate the genotoxicity threshold. Three control cultures were assessed: (1) positive control, by adding only MMC (final concentration 0.1 μg/mL culture); (2) 0.1% DMSO solvent control, obtained by adding 8.6 μL of DMSO to the lymphocyte culture; and (3) negative control culture without both glyphosate and DMSO, obtained adding 8.6 μL of RPMI medium to the lymphocyte culture. Only for MNi assay, after 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 μg/mL to block cytokinesis. Similarly, only for CA assay, to arrest cells in mitosis, colchicine was added at the concentration of 0.06 μg/mL during the last 2 h of culture. After 52 h (for CAs assay) and 72 h

(for MNi assay) of incubation at 37°C, the cells were collected by centrifugation, treated for 10 min with a pre-warmed hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a solution of methanol/acetic acid (3:1 v/v). The treatment with the fixative was repeated three times. Finally, the supernatant was discarded, and the pellet, dissolved in a minimal volume of fixative, was seeded on the slides to detect CAs and MNi by conventional staining with 5% Giemsa (pH 6.8) prepared in Sörensen buffer.

Cytokinesis-block micronucleus assays; Microscope analysis was performed at ×400 magnification on a light microscope (Dialux 20, Leica, Germany). MNi, nucleoplasmic bridges (NPB), and nuclear buds (NBUD) were scored in 2000 binucleated lymphocytes with well-preserved cytoplasm per subject (total 12,000 binucleated cells per concentration). Cells containing one of more MNi were scored as "micronucleated cell" (MNC). A total of 2000 lymphocytes per donor per concentration were scored to evaluate the cytokinesis-block proliferation index (CBPI), according to the following formula: [1 × N1] + [2 × N2] + [3 × (N3 + N4)] / N, where N1–N4 represents the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

Chromosomal aberration assay; Microscope analysis was performed at ×1000 magnification on a light microscope (Dialux 20, Leica, Germany). For each subject and glyphosate concentration, 200 complete complete metaphases (for a total of 1200 metaphases for each dose) were analyzed. Cells containing one of more types of CAs were scored as "aberrant cell" (Ab.C). In order to determine cytotoxicity, the mitotic index (MI) was calculated from the number of metaphases in 1000 cells analyzed per subject per concentration (a total of 6000 cells per concentration).

Statistical analysis; Comparison of mean values of the percentage of cells with MNi, MNC, CBPI, NPBs, NBUDs, CAs, Ab.C, and MI between exposure levels and controls was assessed by the nonparametric Mann-Whitney test. Statistical calculations were carried out using the SPSS software package program (version 24.0, Inc., Chicago, IL, USA). All P values were two-tailed, and P values of 5% or less were considered statistically significant for all tests carried out.

Results

Effect of glyphosate on CA formation; Table 1 shows values of CAs found in the human peripheral lymphocytes cultured in the presence of different glyphosate concentrations. Glyphosate was found to induce the following structural CAs: gaps, chromatid and chromosome breaks, dicentric chromosomes, rings, tri-tetraradials, and acentric fragments. This last, together to chromatid breaks, represent the most frequent observed aberrations (Table 1Gaps were excluded from statistical analysis. Glyphosate was found to significantly increase the CA and Ab.C frequencies at all tested concentrations when compared with the solvent control, including the concentration of 0.025 μg/mL, but with the exception of 0.0125 μg/mL. A dose effect was also observed, since the regression analysis revealed a significant correlation between glyphosate concentrations and the CA and Ab.C frequencies (Table 2). Vice versa, no significant differences were found between the DMSO solvent-control and the negative control, whereas the cultures treated with the MMC showed a significant increase of the cytogenetic damage with respect to all concentrations of glyphosate. Finally, no significant differences were found in the MI values between solvent control and all tested concentrations of glyphosate, although at 0.500 μg/mL, the P value resulted to be borderline.

Table 1 Induction of chromosomal aberrations by Glyphosate in human lymphocytes in vitro Number of scored metaphases for each concentration = 1200

Test substance (μg/mL)	Structural CAs							Total CAs	Total CAs + Gaps	Total Ab.C	Total Ab.C + Gaps	(%) CAs/Cell ± S.E.	(%) Ab.C/Cell ± S.E.	(%) MI ± S.E.
	Gaps	B'	B"	DC	R	TR	AF							
NC	8	8	2	0	0	0	7	17	25	17	25	1.417±0.154	1.417±0.154	5.567±0.042
0.1% DMSO	10	9	4	1	0	6	7	27	37	27	37	2.250±0.335	2.250±0.250	5.433±0.056
MMC (0.100)	41	46	30	9	10	14	36	145	186	127	168	12.083±0.300[a]	10.583±0.473	4.200±0.058[a]
Gly (0.500)	17	41	12	12	3	0	28	96	113	95	112	8.000±0.428[a]	7.917±0.375	5.300±0.026
Gly (0.100)	23	31	10	7	2	2	23	75	98	75	98	6.250±0.359[a]	6.250±0.359	5.333±0.080
Gly (0.050)	9	21	6	7	0	0	16	50	59	50	59	4.167±0.167[a]	4.167±0.167	5.367±0.095
Gly (0.025)	10	15	4	4	3	0	20	46	56	46	56	3.833±0.211[b]	3.833±0.211[b]	5.383±0.040
Gly (0.0125)	8	14	5	1	0	0	14	34	42	34	42	2.833±0.211	2.833±0.211	5.400±0.037

CAs, chromosomal aberrations; *Ab.C*, aberrant cells (cells with 1 ore more aberrations); *MI*, Mitotic Index; *NC*, Negative Control; *MMC*, Mitomycin-C; *B'*, chromatid break; *B"*, chromosome break; *DC*, dicentric; *R*, ring; *TR*, tri-tetraradials; *AF*, acentric fragments; *S.E.*, standard error; *Gly*, Gliphosate

[a] $P = 0.004$; [b] $P < 0.006$ (significantly differs from the DMSO solvent control, Mann-Whitney test)

Table 2 Multiple regression analysis between Glyphosate concentrations

Biomarker	β-co	95% CI (Lower) – (Upper)	*P*-value
CAs	0.914	(2.112) – (2.988)	<0.001
Cells with CAs	0.919	(2.099) – (2.935)	<0.001
MI	-0.275	(-0.666) – (0.099)	0.141
MNi	0.908	(4.025) – (5.075)	<0.001
Cells with MNi	0.935	(3.639) – (4.527)	<0.001
CBPI	0.269	(-28.171) – (4.571)	0.151
NPBs	0.674	(0.268) – (0.665)	<0.001
NBUDs	0.395	(0.023) – (0.444)	0.031

CAs, Chromosomal Aberrations; *MI*, Mitotic Index; *MNi*, Micronuclei; *CBPI*, Cytokinesis-block Proliferation Index; *NPBs*, nucleoplasmic bridges; *NBUDs*, nuclear buds

Effect of glyphosate on MNi formation; Table 3 shows the frequencies of MNi found in the human peripheral lymphocytes cultured in the presence of different glyphosate concentrations. Glyphosate significantly increased the MNi frequency at all tested concentrations when compared to the solvent control, with exception of 0.0125 μg/mL (Table 3). Vice versa, no effect has been observed on the frequencies of NBUD and NPB, with the only exception of 0.500 μg/mL of glyphosate that was found to increase in a significant manner the frequency of NPBs with respect to the solvent control. Also in this case, a relationship between the frequency of MNi and the concentrations of glyphosate was observed (Table 2), as well as the DMSO solvent-control cultures did not show significant differences with respect to the negative controls. MMC was found to significantly increase the MNi, NPB, and NBUD formation compared with the negative control solvent controls and all tested concentrations of glyphosate, with exception of 0.500 μg/mL. After 48-h exposure, a significant reduction of the CBPI value in cultures treated with glyphosate was not observed, indicating that, at the tested concentrations, glyphosate does not seem to produce effects on the proliferation index. Finally, at 0.500 μg/mL, glyphosate significantly induced the NPB formation, whereas no differences were found in the frequency of NBUD between DMSO solvent control and all glyphosate concentrations.

Table 3 Induction of micronuclei by Glyphosate in human lymphocytes in vitro. Number of scored binucleated cells for each concentration of the test substance = 12,000

Test substance (μg/mL)	Distribution of BNCs according to the number of MNi				MNi	MNC	MNi/BNCs ± S.E. (%)	MNC/BNCs ± S.E. (%)	CBPI ± S.E	Frequency of BNCs with NPBs (‰)	Frequency of BNCs with NBUDs (‰)
	1	2	3	4							
NC	27	0	0	0	27	27	0.225±0.021	0.225±0.021	1.713±0.003	0.417±0.083	0.833±0.105
0.1% DMSO	33	1	0	0	35	34	0.292±0.024	0.283±0.025	1.589±0.076	0.500±0.129	1.083±0.154
MMC (0.100)	129	9	3	2	164	143	1.367±0.067	1.192±0.015[a]	1.366±0.019	2.333±0.357	3.083±0.473 [a]
Gly (0.500)	132	6	2	1	154	141	1.283±0.017	1.175±0.021[a]	1.545±0.054	1.667±0.211	1.666±0.247
Gly (0.100)	107	7	0	0	121	114	1.008±0.030	0.950±0.029[a]	1.556±0.017	0.883±0.105	1.333±0.167
Gly (0.050)	93	6	1	0	108	100	0.900±0.053	0.833±0.046[a]	1.576±0.015	0.750±0.111	1.250±0.1112
Gly (0.025)	68	5	0	0	78	73	0.650±0.048	0.608±0.035[a]	1.585±0.010	0.667±0.105	1.167±0.105
Gly (0.0125)	39	0	0	0	39	39	0.325±0.021	0.325±0.021	1.589±0.008	0.583±0.083	1.167±0.167

BNCs, Binucleated cells; *MNi*, micronuclei; *MNC*, cells with 1 or more micronuclei; *CBPI*, Cytokinesis-Block Proliferation Index; *NPBs*, nucleoplasmic bridges; *NBUDs*, nuclear buds; *S.E.*, Standard Error; *NC*, Negative Control; *MMC*, Mitomycin-C; *Gly*, Gliphosate

[a] $P = 0.004$ (significantly differs from the DMSO solvent control, Mann-Whitney test)

Discussion

Glyphosate is an active ingredient of most widely used herbicides. Although it is believed to be less toxic than other herbicides, data about its possible genotoxicity are controversial and IARC classified this compound as probably carcinogenic to human (IARC 2015). The genotoxic effects of high concentrations of glyphosate have been documented, although with contradictory results, in a great number of scientific papers, as well as in evaluation reports of different international agencies. On the other hand, the effects of low concentrations of this compound, likely to be encountered in everyday life, were poorly investigated. Results of our study provided information about in vitro clastogenic effects of glyphosate on human lymphocytes at the low ADI concentration of 0.500 μg/mL and its submultiples. Based on the obtained data, it can be concluded that glyphosate significantly increased the CA and MNi levels in human lymphocytes at the ADI concentration of 0.500 μg/mL established by EFSA and at its submultiple concentrations, up to 0.025 μg/mL. The mechanisms underlying genotoxic potential of glyphosate alone or in complex with other compounds are unknown, although the exposure to glyphosate was found to trigger oxidative processes involved in the increase of the genomic damage. NPB frequency was found to increase with increasing glyphosate concentrations, although a statistical significance was found only at the higher glyphosate concentration (Table 3). However, we obtained a significant linear regression (Table 2) due to a steady increase with the dose, indicating a possible effect of the compound inducing this kind of damage, which is consistent with the increased dicentric frequency observed in the chromosomal aberration test. Also, other authors analyzed in vitro the genotoxic potential of glyphosate in lymphocytes, but at exposure levels of higher magnitude orders. For example, in human lymphocytes cultured without S9 and in the presence of glyphosate at concentrations of 3.5, 92.8, and 580 μg/mL, the authors observed a slightly increased frequency of MNi and a significant tail length increase after a comet assay. Other authors evaluated the induction of CAs and MNi in blood cells of other animal models. Positive clastogenic and genotoxic effects of glyphosate on bovine peripheral lymphocytes cultured in vitro with herbicide concentrations ranging from 17 μM (2.874 μg/mL) to 170 μM (28.740 μg/mL) were reported, whereas another study reported no CAs effect of glyphosate at concentrations ranging from 28 (4.734 μg/mL) to 1120 μM (189 μg/mL). Contradictory results were obtained by a further study, in which observed, after 48 h of treatment without S9, a statistically significant increase in MNi frequency at 280 μM (47.34 μg/mL) but not at 560 μM (94.68 μg/mL) of glyphosate in one donor, and the opposite in a second donor (positive at 560 μM but not at 280 μM). Finally, another study in in vitro experiments based on comet assay, showed that 7 mM of glyphosate (1183 μg/mL) caused DNA damage in blood cells of Nile tilapia (Oreochromis niloticus). Concentrations of glyphosate similar to those evaluated in the present paper were tested in HepG2 cells by the MNi assay. Similarly to what we observed in human lymphocytes, these authors found a significantly higher number of MNi at the ADI value of 0.500 μg/mL, as well as at the residential exposure level of 2.91 μg/mL, after 4 h of treatment. Vice versa, negative results on Hep-2 cells were obtained with CA assay at glyphosate concentrations of 0.20 mM (33.8 μg/mL), 1.20 mM (203 μg/mL), and 6.00 mM (1014 μg/mL). Significant levels of DNA damage were also observed in human buccal epithelial cells exposed to glyphosate concentrations ranging between 10 and 20 mg/L, whereas another

study showed that, in peripheral blood mononuclear cells, glyphosate induces DNA damage in the concentration range from 0.5 mM (84.54 μg/mL) to 10 mM (1690 μg/mL), and a significant decrease of global DNA methylation at concentration of 0.25 mM (42.27 μg/mL). Interestingly, the same authors also observed a significantly increased methylation of p53 promoter at concentrations of 0.25 mM and 0.5 mM (42.27 and 84.54 μg/mL). This hypermethylation was found to be able to downregulate the p53 gene expression and to activate proto-oncogenes, with consequent genomic alterations and cancer risk. The possibility of glyphosate causing cancer promotion in skin cells and proliferation in breast cells has been also observed in vivo and in vitro studies by mouse and human models, respectively. In this scenario, the results obtained in the present study require attention. Indeed, increased CA and MNi frequencies in peripheral blood lymphocytes have been positively associated with increased cancer risk and early events in carcinogenesis, respectively. Moreover, it should be emphasized that, beyond the cases of intoxication where glyphosate content in blood was found to range from 0.6 to 150 μg/mL, in subjects who were indirectly exposed to this substance, glyphosate was found in blood at concentrations of 0.074 ± 0.028 μg/mL, a value about seven times lower with respect to the established ADI value, but in the range of concentrations we tested (from 0.5 to 0.0125 μg/mL). At the same time, the genotoxicity of a compound should not be evaluated only after single administrations in in vitro or in vivo systems, but also, and especially, after chronic administration of the same compound, even at lower quantities than those established by the competent agencies. In this sense, the clastogenicity we observed at concentrations of 0.100, 0.050, and 0.025 μg/mL represents an important signal, especially in view of a chronic exposure to these glyphosate concentration levels. Finally, no significant differences in CBPI and MI values were found between all tested concentrations and the solvent control, indicating that, at these concentrations, glyphosate does not influence in a significant manner the replicative capacity of the cells. These data differ from a study which observed a reduction of mitotic and proliferation indices in bovine lymphocytes, but at higher glyphosate concentrations (94.68 μg/mL and 189 μg/mL). Similarly, other authors described decreased levels of MI for other herbicides or insecticides, also in this case, at concentrations much higher than those tested in the present work.

Conclusion

In the present work, there was evidence for cytogenetic effects of glyphosate on cultured human lymphocytes. Despite the limitations of an in vitro study due to the reduced sample size, it is our opinion that the increased cytogenetic damage observed by our group at glyphosate concentrations equal and lower than the established ADI value requires further investigations in order to establish the effective genotoxicity threshold of this extensively used compound. Indeed, the glyphosate concentrations tested in the present work represent more realistic concentrations, likely to be encountered in everyday life, with respect to the higher doses evaluated in other published papers. In this scenario, in order to draw conclusions about the effects associated to the chronic exposure to low doses, in vitro studies are useful tools to investigate the dose response effects, the molecular mechanisms of action of different environmental xenobiotics, and their genotoxicity. This last, compared to other types of toxicity, may result in severe consequences that can be also inherited after long periods following exposure. The same DNA damage that occurs in a single cell, caused by low but chronic exposure to genotoxic compounds, can cause unexpected severe consequences in the long run.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes human lymphocyte chromosome aberration and micronucleus tests with glyphosate. Although broadly compliant with OECD 473 and 487 there are some critical deficiencies which will have adversely influenced the reliability of the findings. Treatment with glyphosate was initiated 24 hours after lymphocyte cultures were stimulated to divide, instead of the recommended 48 hours, consequently the cultures would not have been asynchronous. This could mean cells in some stages of the cell cycle may have been under-represented, whilst others over-represented. Exposure to glyphosate was continuous for 28 hours in the chromosome aberration assay or 48 hours in the micronucleus assay. In contrast OECD test guidelines recommend maximum exposure of 1.5 cell cycles, equivalent to approximately 24 hours for lymphocyte cultures. For both endpoints the

paper does not confirm if the slides were coded prior to analysis. The positive control has been compared statistically to the glyphosate treated cultures rather than the solvent controls.

The authors consider that glyphosate induces tri-tetraradial aberrations (amongst other aberration types) but fails to comment that the frequency of these aberrations observed at a single glyphosate concentration is 3-fold lower than the frequency observed in the solvent control cultures. Furthermore, it is unusual that the only multi-aberrant metaphases observed were a small number of positive control metaphases and gaps did not appear to increase with treatment but chromatid and chromosome breaks did.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and although the genotoxicity tests conducted were in general in accordance with the OECD test guidelines, significant deficiencies were noted.

Reliability criteria for *in vitro* toxicology studies

Publication: Santovito *et al*., 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	In accordance with OECD TG.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity is not reported. Source: Sigma-Aldrich, Saint Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Lymphocytes.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.500, 0.100, 0.050, 0.025 and 0.0125 µg/mL.
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and although the genotoxicity tests conducted were in general in accordance with the OECD test guidelines, significant deficiencies were noted.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Sierra-Diaz E. *et al.*
Report year	2019
Report title	Urinary pesticide levels in children and adolescents residing in two agricultural communities in Mexico
Document No	International Journal of Environmental Research and Public Health (2019), Volume 16, Number 4, 562 p.
Guidelines followed in study	None.
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The objective of this study was to measure the concentration and prevalence of pesticides in a cross-sectional study involving children and adolescents under 15 years of age in two small farming communities located in in State of Jalisco in Mexico, with both communities having very similar characteristics.

Urine samples (first-morning urine) were taken from children under 15 years of age in Agua Caliente located near the largest lake in Mexico (n = 192) and in Ahuacapán located in the south coast of the state (n = 89). A total of 281 urine samples obtained in both communities and processed for the determination of pesticides with high-performance liquid chromatography together with tandem mass spectrometry.
In 100% of the samples, at least two pesticides of the 17 reported in the total samples were detected. The presence of glyphosate was detected in more than 70% of the cases. The mean urinary level of glyphosate 0.363 ± 0.3210 ng/mL in Agua Caliente and 0.606 ± 0.5435 ng/mL in Ahuacapán, detected in 72.91% and 100% of their respective total samples. Substantial differences were detected regarding the other compounds.

Materials and methods

A cross-sectional study was carried out simultaneously in two communities in State of Jalisco, which is one of the three states in Mexico with the greatest index of people poisoned due to the application of pesticides. The first of these was, Agua Caliente, near Lake Chapala, the largest lake in Mexico, and the second was a community in the region of the south coast of the state (Ahuacapán).

Beginning hundreds of years ago, multiple autochthonous communities of people of Nahua native origin who were dedicated to fishing and agriculture settled on the bank of Lake Chapala. Since 2016, the Department of Public Health of the University of Guadalajara has carried out studies in the zone, specifically in the community of Agua Caliente, Poncitlán Municipality, State of Jalisco. In this community, health problems have been detected such as malnutrition and albuminuria, specifically in children and adolescents under the age of 17 years.

The community is inhabited by 998 persons, whose main activities are farming (37.9%), construction work (29.3%), and laboring 7.2% and who alternate with fishing as a means of subsistence. The most common local crops are corn, seasonal beans, and chayote (*Sechium edule*), which the inhabitants irrigate with lake water. The weekly average family income is approximately 52.63 USD.

The community of Ahuacapán is found toward the coast of the Pacific Ocean, in a 180 km straight line from Agua Caliente. A total of 950 inhabitants live there and their principal economic activity is

agriculture, producing sugar cane, corn, tomatoes, citrus, and horticultural products. The water necessary for agriculture derives from springs, deep wells, and the Ayuquila River. The weekly family income in this community is approximately 75 USD. In both localities, there is a total of 550 children and adolescents (58%) aged between less than 1 year and 15 years.

For the urine sampling, communities were invited to participate voluntarily, with the approval of the Department of Public Health of the University of Guadalajara and the local authorities of the two communities. The sample included only children and adolescents aged under 17 years in Agua Caliente and those under 12 years of age in Ahuacapán. Their parents were informed concerning the objective of the study and, after obtaining the parents' signed consent, the minors were asked for a urine sample (first-morning urine). In both communities, anthropometric measurements (weight and height) were performed on the minors.

Urine samples were transported to the laboratory and were processed for the determination of pesticides with the HPLC/MS/MS (high-performance liquid chromatography coupled with tandem mass spectrometry) method with Agilent Technologies® Model 1200 equipment for HPLC and Model 6430B for MS/MS spectrometry. The method for HPLC used a column Zorbax Eclipse XDB C18, Rapid Resolution, 2.1 × 50 mm, 3.5 µm. Mobile phases: A, 0.1% formic acid in water; B, acetonitrile (ACN); gradient of 40% to 100% B; injection volume, 5 µL; flow, 0.5 min; curve range for each pesticide, 0.01 to 1000 µg/mL. The latter was performed at the Laboratory of Applied Pharmacokinetics of the University Center of Exact and Engineering Sciences of the University of Guadalajara. With this method, it was possible to determine the presence of 16 pesticides, as presented in Table 1.

Table 1. Category of pesticides analyzed in urine samples from the two communities.

Name	IUPAC ID	PubChem CID	Agrochemical Category
Acetochlor	2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide	1988	Herbicide
Atrazine	6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine	2256	Herbicide
Carbendazim	methyl N-(1H-benzimidazol-2-yl)carbamate	25429	Fungicide
Carbofuran	(2,2-dimethyl-3H-1-benzofuran-7-yl) N-methylcarbamate	2566	Insecticide, Nematicide, Acaricide
Cyhalothrin	[cyano-(3-phenoxyphenyl)methyl] 3-[(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate	5281873	Insecticide
Diazinon	*O,O*-Diethyl O-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate	3017	Insecticide, Acaricide
Dimethoate	2-dimethoxyphosphinothioylsulfanyl-N-methylacetamide	3082	Insecticide, Acaricide
Emamectin	4″-Deoxy-4″-epi-methylamino-avermectin B1; Epi-methylamino-4″-deoxy-avermectin	11549937	Insecticide
Enilconazole (imazalil)	1-[2-(2,4-dichlorophenyl)-2-prop-2-enoxyethyl]imidazole	37175	Fungicide
Glyphosate	2-(phosphonomethylamino)acetic acid	3496	Herbicide
Malathion	diethyl 2-dimethoxyphosphinothioylsulfanylbutanedioate	4004	Insecticide, Acaricide
Methomyl	methyl (1E)-N-(methylcarbamoyloxy)ethanimidothioate	5353758	Insecticide
Metoxuron	3-(3-chloro-4-methoxyphenyl)-1,1-dimethylurea	29863	Herbicide
Molinate	S-ethyl azepane-1-carbothioate	16653	Herbicide
Pyraclostrobin	Methyl N-[2-[[1-(4-chlorophenyl) pyrazol-3-yl]oxymethyl]phenyl]-N-methoxycarbamate	6422843	Fungicide, plant growth regulator
Thiabendazole	4-(1H-benzimidazol-2-yl)-1,3-thiazole	5430	Fungicide

Conditions for MS/MS spectrometry are described in Table 2 and Figure 1 shows a chromatogram urine sample.

Table 2. Mass spectrometer conditions for pesticide determination.

Mass spectrometer conditions				
Electrospray Interface Condition				
Gas emperature	350 °C			
Gas flow	12 L/min			
Nebulizer	25 psi			
Capillary	+4000	−4000		
Compound name	**Precursor Ion**	**Product Ion**	**Fragmentor**	**Polarity**
L-Cyhalotrin (225.1)	467.1	225.1	80	Positive
Meclizina (201.1)	391.2	201.1	90	Positive
Pyraclostrobin (163)	388	163	120	Positive
Malation (99)	331	99	80	Positive
Clorpyrifos (200)	325	200	30	Positive
Oxandrolona (289.2)	307.2	289.2	100	Positive
Oxandrolona (271.2)	307.2	271.2	100	Positive
Oxandrolona (229.1)	307.2	229.1	100	Positive
Diazinon (153)	305	153	160	Positive
Imazalil (159)	297	159	160	Positive
Paration (264)	292	264	90	Positive
Paration (236)	292	236	90	Positive
Acetoclor (224.2)	270.1	224.2	60	Positive
Acetoclor (148.4)	270.1	148.4	60	Positive
Picloram (222.9)	240.9	222.9	90	Positive
Picloram (194.9)	240.9	194.9	90	Positive
Dimethoate (171)	230	171	80	Positive
Metoxuron (72.1)	229.1	72.1	93	Positive
Ametryn (186)	228.1	186	120	Positive
Ametryn (96)	228.1	96	120	Positive
Carbofuran (123)	222	123	120	Positive
Atrazine (132)	216	132	120	Positive
Thiabendazole (131)	202	131	120	Positive
Carbendazim (160)	192.1	160	110	Positive
Molinate (55.1)	188.1	55.1	78	Positive
Methomyl (106)	163.1	106	30	Positive
Methomyl (88.1)	163.1	88.1	30	Positive
Methomyl (65)	163.1	65	30	Positive
Emamectina (158.1)	887.1	158.1	60	Positive
Glyphosate (149.9)	168	149.9	80	Negative
Glyphosate (124.2)	168	124.2	80	Negative
2,4-D (161.1)	219	161.1	50	Negative

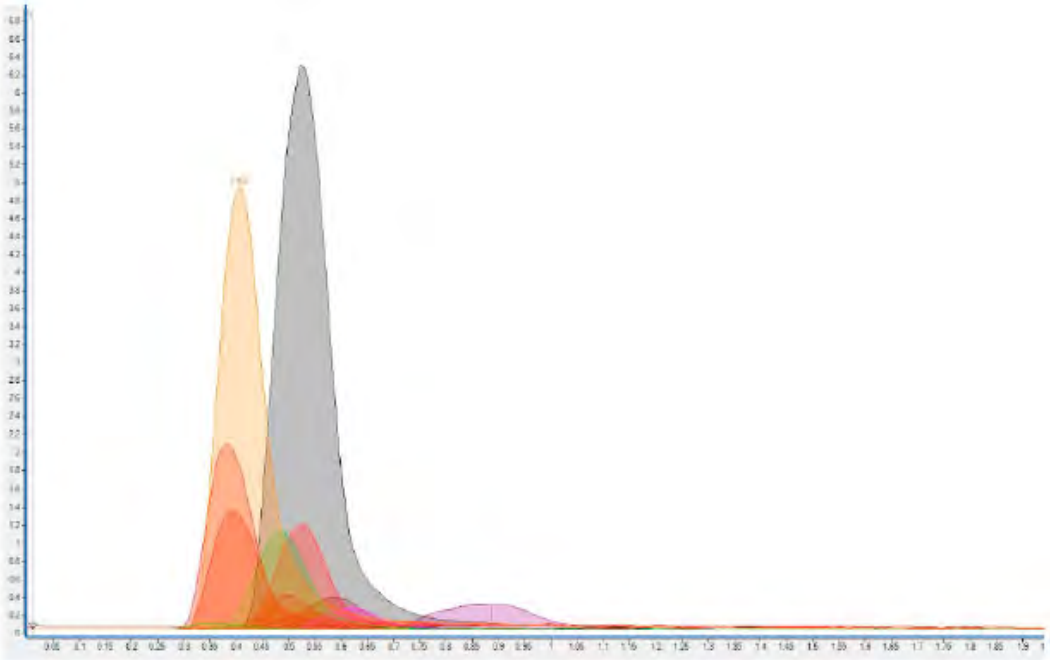

Figure 1. Shows a chromatogram of a urine sample.

Statistical analysis

For the statistical description, absolute frequencies, percentages, means, and standard deviations (SD) were used. Statistical significance was evaluated by means of the Mann–Whitney U, the Chi-squared, and the Fisher exact tests. To compare the two populations and evaluate the differences in the urine pesticide levels, the Mann–Whitney U test was used. Similarly, to compare the frequency of detection rate, the Fisher test was used. Statistical significance was considered with a p of ≤ 0.05. For data processing, Excel® (Microsoft, Redmond, WA, USA) and Epi Info ver. 7.2 (Centers for Disease Control and Prevention (CDC) Atlanta, GA, USA) statistical software were used.

This research was carried out with the authorization of the ethics committee of the Department of Public Health of the University of Guadalajara (registration number DCSP/CEI/2016/260618/038).

Results

A total of 281 children participated, of whom 192 (68.3%) corresponded to the community of Agua Caliente with an average age of 9.4 years (range, 5–15 years). In the community of Ahuacapán, 89 (31.7%) samples were collected, with an average age of 8.31 years (range, 5–13 years; Table 3).

Table 3. Demographic and anthropometric data of the children in both communities.

Variable	Agua Caliente (n = 192)	Ahuacapán (n = 89)
Gender		
Female	84 (43.8%)	40 (44.9%)
Male	108 (56.3%)	49 (55.1%)
Age (years)	9.40 (SD 2.52)	9.31 (SD 2.05)
Age groups		
5–8 y	78 (40.6%)	49 (55.1%)
9–11 y	67 (34.9%)	34 (38.21%)
12–15 y	47 (24.5%)	6 (6.7%)
Weight (kilograms)	29.39 (SD 10.06)	32.27 (SD 11.73)
Height (centimeters)	131.58 (SD 14.12)	132.94 (SD12.97)
Body mass index (k/m^2)	16.46 (SD 2.44)	17.72 (SD 3.66)

Detection of the pesticides was frequent in both communities. Substantial differences in exposure and detection rate were also identified (Table 4). Glyphosate was detected with a higher frequency in Ahuacapán than in Agua Caliente, which presented in 100% of the minors studied in Ahuacapán vs. 73% in Agua Caliente ($p > 0.001$), with minimal values of 0.0020 ng/mL and maximal values of 2.63 ng/mL. Glyphosate is currently one of the most utilised herbicides in Mexico. The differences between the two localities were considered to be associated with the agro-industrial activity and the practices that have become generalised among the small producers of basic crops.

In general terms, 100% of the study subjects were exposed to at least two of the compounds identified in urine. Positive results are noteworthy of six of the compounds in more than 70% of the subjects studied in both communities: malathion, metoxuron, glyphosate, dimethoate, enilconazole, and acetochlor. The greatest prevalence was for the herbicides (60.49%), in second place, fungicides (39.05%), and lastly, the insecticides (20.92%).

The urinary samples in this study was collected during winter, during which there is a significant diminution in the agricultural use of pesticides. However, the authors emphasised that consumption of a conventional diet with seasonal products could play a very important role regarding the presence of these pesticides in urine.

Table 4. Frequencies, percentages, means, and standard deviations (SD) of pesticides in urine.

Pesticide	Agua Caliente	Ahuacapán	
	n (%)	*n* (%)	*p* (Fisher Test)
	Mean ng/mL (SD)	Mean ng/mL (SD)	*p* (Mann–Whitney)
Acetochlor	161 (83.85) 0.008 (0.0867)	44 (49.43) 0.001 (0.0017)	<0.01 0.04
Atrazine	22 (11.45) 0.016 (0.0486)	22 (24.71) 0.043 (0.0930)	<0.01 0.06
Carbendazim	29 (15.10) 0.141 (0.4192)	52 (41.57) 0.330 (0.5040)	<0.01 <0.01
Carbofuran	1 (0.52) 0.246	0	NA NA
Cyhalothrin	138 (71.87) 0.083 (0.0823)	45 (50.56) 0.080 (0.0855)	<0.01 0.52
Diazinon	29 (15.10) 0.007 (0.0199)	20 (22.47) 0.008 ± 0.0180	0.09 0.41
Dimethoate	179 (93.22) 0.146 (0.1834)	44 (49.43) 0.169 (0.2299)	<0.01 0.03
Emamectin	6 (3.12) 0.006 (0.0339)	9 (10.11) 0.019 (0.0582)	0.02 0.34
Enilconazole	177 (92.18) 1.582 (5.6623)	29 (32.58) 0.069 (0.1023)	<0.01 <0.01
Glyphosate	140 (72.91) 0.363 (0.3210)	89 (100) 0.6060 (0.5435)	<0.01 <0.01
Malathion	191 (99.47) 0.681 (0.6431)	55 (61.79) 0.177 (0.1730)	<0.01 <0.01
Methomyl	46 (23.95) 0.016 (0.0292)	0	<0.01 <0.01
Metoxuron	188 (97.91) 0.038 (0.0403)	50 (56.17) 0.037 (0.0414)	<0.01 0.10
Molinate	79 (41.14) 0.191 (0.3698)	55 (61.79) 0.273 (0.4240)	<0.01 <0.01
Pyraclostrobin	62 (32.29) 0.049 (0.2331)	37 (41.57) 0.042 (0.0509)	0.08 0.18
Thiabendazole	29 (15.10) 0.007 (0.0511)	24 (26.96) 0.002 (0.0046)	<0.01 0.12

Conclusion

In a comparative cross-sectional study in two agricultural communities with very similar characteristics in Mexico (a total of 281 children participated in the study), glyphosate was detected in more than 70% of the cases in both communities, having a higher prevalence rate along with malathion, metoxuron, compared to other pesticides analysed in the study. The mean urinary levels of glyphosate were 0.363 ± 0.3210 ng/mL in Agua Caliente and 0.606 ± 0.5435 ng/mL in Ahuacapán. In general, substantial differences in exposure and detection rate were also identified between the communities.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In a comparative cross-sectional study using the urine of children living in two agricultural communities with very similar characteristics in Mexico (a total of 281 children participated in the study), glyphosate was detected in more than 70% of the cases in both communities. The mean urinary levels of glyphosate were 0.363 ± 0.3210 ng/mL in Agua Caliente and 0.606 ± 0.5435 ng/mL in Ahuacapán.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no validation data were presented for the analytical method employed.

Reliability criteria of exposure studies

Publication: Sierra-Diaz *et al*., 2019.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	
Study		
Study design clearly described	Y	Survey of glyphosate concentrations in children.
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	?	No pesticide exposures reported.
Sampling scheme sufficiently documented	Y	Production of one early morning urine sample.
Analytical method described in detail	Y	
Validation of analytical method reported	N	
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no validation data were presented for the analytical method employed.		

1. Information on the study

Data point:	CA 5.5
Report author	Sorahan T.
Report year	2015
Report title	Multiple Myeloma and Glyphosate Use: A Re-Analysis of US Agricultural Health Study (AHS) Data
Document No	International Journal of Environmental Research and Public Health (2015) Vol. 12, 1548-1559
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

RRs for exposed and non-exposed subjects were calculated using Poisson regression; subjects with missing data were not excluded from the main analyses. Using the full dataset adjusted for age and gender the analysis produced a RR of 1.12 (95% CI 0.50 to 2.49) for ever-use of glyphosate. Additional adjustment for lifestyle factors and use of ten other pesticides had little effect (RR 1.24, 95% CI 0.52 to 2.94). There were no statistically significant trends for multiple myeloma risks in relation to reported cumulative days (or intensity weighted days) of glyphosate use. The doubling of risk reported previously arose from the use of an unrepresentative restricted dataset and analyses of the full dataset provides no convincing evidence in the AHS for a link between multiple myeloma risk and glyphosate use.

Materials and Methods

The secondary data file which served as a basis for this study was provided by researchers of the Agricultural Health Study (AHS) taking care of the privacy of the participants. AHS researchers supplied an informative description of the file and the file was found to be internally consistent as well as consistent with data descriptions supplied earlier. All subjects gave their informed consent for inclusion before they participated in the AHS study and ethics approval for the original data collection by AHS researchers was obtained from the Institutional Review Board of the National Institutes of Health. This secondary analysis was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the University of Birmingham Science, Technology, Engineering and Mathematics Ethical Review Committee.

Data on lifestyle factors and use of pesticides were collected from 57,311 private and commercial pesticide applicators from Iowa and North Carolina. Previous analyses were carried out on three subsets of data: Set 1 comprised 54,315 applicators excluding those with any cancers diagnosed before enrolment, applicators lost to follow-up, who had missing data for age at enrolment, or who provided no information on whether they had ever used glyphosate. Set 2 comprised 49,211 applicators and further excludes applicators with missing data on level of education, smoking history, or use of alcohol. Set 3 comprised 40,719 applicators and further excludes applicators with missing data on either use or estimated cumulative exposure days for 2,4-D (2,4-dichlorophenoxy acetic acid), alachlor, atrazine, metolachlor, or trifluralin, and missing data on ever-use of benomyl, maneb, paraquat, carbaryl, or diazinon. The objective of this analysis was to examine findings in an as full a dataset as possible and some analyses have also been carried out on a larger fourth set of 55,934 applicators, a category that does not exclude applicators with missing data on ever-use of glyphosate but only applicators with cancers diagnosed before enrolment, applicators lost to follow-up, or who had missing data for age at enrolment.

Poisson regression was used to estimate RRs and 95% CIs associated with glyphosate exposure metrics, with and without adjustment for other variables. Each variable under analysis was classified into levels

or categories. The analytical approach for the full dataset was to have a “not known/missing” category for each variable so that analyses of all available cases could be maintained. However, it was necessary to ensure that there was at least one case of multiple myeloma in each level of each variable for the regression to successfully calculate RRs. There were no cases of multiple myeloma in those applicators with “unknown use of 2,4-D”, such applicators were combined with those reporting “no use” to create a new category of “no claim of use”. There were no cases of multiple myeloma in applicators with an “unknown level of education”. These applicators were combined with those reporting no education beyond high school. All significance tests were two-tailed and tests for trend (where applicable) were calculated by scoring the levels of a variable and treating the variable as unfactored. All analyses were performed with the EPICURE statistical software, using the double precision DOS version 2.12 of DATAB and AMFIT, dated March 2002.

Results

There were 32 cases of multiple myeloma in Set 1, 26 cases in Set 2, and 22 in Set 3. For the calculation of the RRs there was no adjustment for gender. None of the RRs calculated (RR including statistical adjustment for age at enrolment and RR with additional adjustments) for the three sets is statistically significant and in Set 1, the largest data set, the RR for ever-use of glyphosate is close to unity (RR 1.08, 95% CI 0.48 to 2.41). The point estimates of risk for the smaller datasets show an approximate doubling of risk irrespective of whether adjustment for other variables is carried out. The largest RR is shown for the fully adjusted model of the smallest dataset (RR 2.79, 95% CI 0.78 to 9.96).

Estimated risks of multiple myeloma for reported ever-use for the 54,315 applicators in Set 1 (total of 32 cases) were calculated in relation to use of pesticides and other variables (smoking, alcohol consumption, family history of cancer and education). The RR for ever-use of glyphosate, with adjustment for age at enrolment and gender only, is close to unity (RR 1.12, 95% CI 0.50 to 2.49). The RR for ever-use of glyphosate is little changed with additional adjustment for all 14 other variables (RR 1.24, 95% CI 0.52 to 2.94).

RR estimates were calculated for multiple myeloma in terms of levels of reported cumulative days of glyphosate use and levels of estimated intensity-weighted exposure days for the 54,315 applicators in Set 1. For each exposure metric three sets of RRs have been calculated: firstly, adjusting for age at enrolment and gender; secondly, with additional adjustment for cigarette smoking, use of alcohol, family history of cancer, and level of education; and thirdly, with additional adjustment for use of ten other pesticides. Two tests for trend were applied to each of these analyses, the first scored the levels of cumulative exposure as 1-4, the second scored the level by mean values. There were no statistically significant trends, but a not statistically significantly elevated RR was shown for the highest category of intensity-weighted exposure days in the fully adjusted model (RR 1.87, 95% CI 0.67 to 5.27). There were no cases of multiple myeloma in glyphosate users with unknown extent of use.

RR estimates were calculated for multiple myeloma in terms of levels of ever-use of glyphosate, reported cumulative days of glyphosate use, and estimated intensity-weighted exposure days for the 55,934 applicators in Set 4 with a total of 34 cases of multiple myeloma. The risk of multiple myeloma in ever-users of glyphosate was close to unity (RR 1.18, 95% CI 0.36 to 8.20) and there were no significant trends with either of the two cumulative exposure metrics.

Discussion

This study found no significant trends of multiple myeloma risk with reported cumulative days of glyphosate use and unexceptional point estimates of risk for ever-use of glyphosate. This was irrespective of whether the analyses had adjustment for a few basic variables (age and gender) or adjustment for many other lifestyle factors or pesticide exposures, as long as data on all available pesticide applicators were used. The suspiciously elevated RRs reported previously arose from the use of restricted data sets that, probably by chance, turned out to be unrepresentative. These restrictions would seem to be unnecessary because there is no technical problem in dealing with missing data in Poisson regression. The practice of restricting analyses to subjects with complete data for all variables is, perhaps, a procedure to be carried out with caution as it is clear from this example that such restrictions can lead to misleading findings. It also ignores the fact that findings for missing categories

can often be interesting in their own right.

Conclusion
This secondary analysis of AHS data does not support the hypothesis that glyphosate use is a risk factor for multiple myeloma, and suggests that the practice of restricting analyses to subjects with complete data for all variables is perhaps not to be recommended.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study the relative risk estimates for exposed and non-exposed applicators were calculated using Poisson regression and subjects with missing data were not excluded from the main analyses. When using the full dataset adjusted for age and gender the analysis produced a RR close to unity for ever-use of glyphosate. Additional adjustment for lifestyle factors and use of ten other pesticides had little effect. This study found no statistically significant trends of multiple myeloma risk with reported cumulative days of glyphosate use and unexceptional point estimates of risk for ever-use of glyphosate. This was irrespective of whether the analyses had adjustment for a few basic variables (age and gender) or adjustment for many other lifestyle factors or pesticide exposures, as long as data on all available pesticide applicators were used. The suspiciously elevated RRs reported previously arose from the use of restricted data sets that, probably by chance, turned out to be unrepresentative.

This publication concerns a secondary analysis of the data from the Agricultural Health Study (AHS) and is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the reliability criteria of a well conducted epidemiology study.

Reliability criteria for epidemiology studies

Publication: Sorahan T., 2015	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.		
Study completely described and conducted following scientifically acceptable standards	Y	Secondary analysis of the AHS data
Test substance		
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	
Study		
Study design – epidemiological method followed	Y	
Description of population investigated	Y	
Description of exposure circumstances	Y	
Description of results	Y	
Statistical analysis	Y	
Overall assessment		
Reliable withoiut restrictions	Y	
Reliable with restrictions		
Not reliable		

Publication: Sorahan T., 2015	**Criteria met? Y/N/?**	**Comments**
This publication concerns a secondary analysis of the data from the agricultural health study and is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the reliability criteria of a well conducted epidemiology study.		

1. Information on the study

Data point	CA 5.9 Exposure study
Report author	Steinborn A. *et al.*
Report year	2016
Report title	Determination of Glyphosate Levels in Breast Milk Samples from Germany by LC-MS/MS and GC-MS/MS
Document No.	J Agric Food Chem (2016), Vol. 64, 1414−1421
Guidelines followed in study	Guidance document on analytical quality control and validation procedures for pesticide residues in food and feed, SANCO/12571/2013
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No/Not stated
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study describes the validation and application of two independent analytical methods for the determination of glyphosate in breast milk. They are based on liquid chromatography − tandem mass spectrometry (LC-MS/MS) and gas chromatography − tandem mass spectrometry (GC-MS/MS), respectively. For LC-MS/MS, sample preparation involved an ultra filtration followed by chromatography on an anion exchange column. The analysis by GC-MS/MS involved an extraction step, clean-up on a cation exchange column, and derivatization with heptafluorobutanol and trifluoroacetic acid anhydride. Both methods were newly developed for breast milk and are able to quantify glyphosate residues at concentrations as low as 1 ng/mL. The methods were applied to quantify glyphosate levels in 114 breast milk samples, which had been collected from August to September of 2015 in Germany. The mothers participated at their own request and thus do not form a representative sample. In none of the investigated samples were glyphosate residues above the limit of detection found.

Materials and Methods

Chemicals – Glyphosate standard solution (10 μg/mL), reference glyphosate and internal standard ($^{13}C_2{}^{15}N$ labeled glyphosate) were purchased from LGC Standards, Wesel, Germany and from Dr. Ehrenstorfer, Augsburg, Germany.

Collection of breast milk samples - Breast milk samples were collected in August and September 2015 by the Governmental Institute of Public Health of Lower Saxony, Germany and by the Bavarian Authority for Health and Food Safety Germany for the analysis of glyphosate. All participants signed a declaration of consent concerning the use of their samples for scientific purposes. Participating mothers had not been selected by random sampling and thus do not form a representative sample. There were no restrictions relating to, for example, age and point of sampling during the lactation period for participating in the monitoring program. The milk samples for this study were collected and stored in polypropylene tubes which remained frozen during storage and shipment. In total, 114 milk samples were analyzed and the participants completed a self-administered questionnaire. Information on sample collection, biometric data and self-reported pesticide exposure of the participants is given in Table 1.

Table 1: Biometric data of study participants

parameter	samples from Bavaria, Germany	samples from Lower Saxony, Germany
number of samples	17	97
age of mother (years)		
median	32.1	32.0
range	26–39	22–39
body weight of mother (kg)		
median	63.0	67.0
range	54–90	48–102
duration of lactating period (weeks)		
median	11.0	18
range	3–80	4–52
self-reported exposure to pesticides	6 participants	32 participants

The questionnaire also asked for the place of residence and the jobs practiced in the last 10 years. Thirty-eight participants declared the use of chemical insecticides, herbicides or wood preservatives. At least one participant has worked in a residue analytical laboratory and used pesticide standards regularly. Twenty of the 114 breast milk samples were divided each into two subsamples to allow the parallel analysis by LC-MS/MS and GC-MS/MS.

Fortification of breast milk samples for performance tests - A homogeneous sample of breast milk was prepared and spiked with different volumes of a glyphosate standard solution in water at 10 µg/mL. Twenty-eight stored breast milk samples from a previous study of the Governmental Institute of Public Health of Lower Saxony were pooled and 4 aliquots of 100 mL were spiked with glyphosate resulting in concentrations of 0.5, 1, 3, and 5 ng/mL. An additional aliquot of the pooled sample served as the control. All performance samples were divided into two subsamples and analyzed in parallel with LC-MS/MS and GC-MS/MS. These samples served as independent quality control samples.

Sample Preparation for LC-MS/MS analysis - Removal of fat by centrifugation and removal of proteins by ultra-filtration in one step through centrifugal filtration using a molecular weight cutoff filter of 30 kDa was found to be suitable. To 3 mL of sample, 30 µL of internal standard solution containing 1000 ng $^{13}C_2{}^{15}N$ glyphosate/mL was added to obtain a concentration of 10 ng/mL. After mixing, the sample was transferred to the top part of the cutoff filter tube. The filter was centrifuged at 3500 rpm for 20 minutes and 500 µL of the filtrate was then transferred to the LC filter vial, the solution was filtered and the vial used for measurement. After this procedure, one mL of final extract contained the glyphosate residue of one mL breast milk.

LC-MS/MS analysis - The LC-MS/MS system used consisted of a Nexera UHPLC system from Shimadzu equipped with a 5500 Qtrap system (Sciex) in triple-quad mode. The LC system consisted of an anion exchange LC column (Dionex Ionpac AS 11 (2 × 250 mm) and a AG-11 guard column (2 × 50 mm) from Thermo Fischer Scientific). Twenty-five µL of standard solution or filtrate were injected into the LC system and glyphosate was eluted from the column using a gradient of water (A) and 1 mM citric acid solution brought to a pH of 11 by addition of a dimethyl amine solution (B). Gradient elution consisted of following steps: 100% A from 0 to 2 minutes, linear to 25% B in 5.5 minutes, and linear to 50% B in 2.5 minutes which was held for 4 minutes. After returning to 100% A in 0.5 minutes the system was re-equilibrated for 7.5 minutes before the next injection. The total run time (including injection) was 22.5 minutes with a flow rate of 0.4 mL/min. A column temperature of 40°C was maintained while the temperature of the samples in the autosampler was 12°C. All transitions were measured using a declustering potential of -75 V, an entrance potential of -10 V and a dwell time of 50 ms. The Turbospray source was used in negative electrospray mode using the following parameters: Curtain gas 20 arbitrary units, collision gas medium, ion spray - 4000 V, temperature 400 °C, ion spray gas 1: 40 and ion spray gas 2: 50 arbitrary units. The mass transitions for evaluation [m/z] were 168.2 → 62.8 for quantification, 168.2 → 79.0 for confirmation and, 171.2 → 62.8 for $^{13}C_2{}^{15}N$ glyphosate.

Sample Preparation for GC-MS/MS analysis - A 2 mL milk sample was extracted with 3.75 mL of 0.6% acetic acid and centrifuged for 5 minutes at 4000 rpm. Two mL of the supernatant liquid was extracted with 2 mL dichloromethane for 2 minutes and the two phases separated by centrifugation at 4000 rpm for 5 minutes. One mL of the supernatant liquid was filtered using a 0.45 µm Nylon filter and a cation exchange clean-up was performed using disposable Bio-Rad Poly-Prep columns filled with 1.72 g (equivalent to 2 mL filling volume) of AG 50W-X8 resin (H^+-form). A 0.55 mL aliquot of the filtered extract (corresponding to 0.2 mL breast milk) and 0.100 mL of internal standard solution (20 ng/mL) were added onto the cation exchange column and were eluted followed by 2.0 mL of CAX solution (800 mL HPLC grade water, 13.5 mL 10 N HCl solution and 200 mL methanol). Both eluates were discarded. Glyphosate residues were eluted from the column with 12.5 mL of CAX solution and evaporated to dryness. The residues were dissolved in 1.0 mL of CAX solution. Derivatization reagent (2,2,3,3,4,4,4-hepta fluoro-1-butanol and trifluoroacetic acid anhydride 1:1) was cooled to a temperature of - 20°C. 0.05 mL of the dissolved eluate (corresponding to 0.01 mL breast milk) was added to 1.5 mL of the chilled reagent and after 5 minutes derivatization is started by heating to 92-97°C for 1 hour. After cooling, the excess of derivatization reagent was removed by evaporation. The dry residue was dissolved in 0.2 mL of ethyl acetate containing 0.2 mL/L citral and then concentrated to a final volume of 20 µL. Citral was used to prevent adsorption of the analytes in the inlet and the GC column. Following this procedure, one mL of final extract contained the glyphosate residue of 0.5 mL breast milk.

GC-MS/MS analysis - The GC-MS/MS system consisted of a Thermo Trace GC Ultra equipped with a TriPlus liquid autosampler, split/splitless injector and MS detector TSQ Quantum with triple quadrupole (Thermo Fisher Scientific). The GC column was a Optima 5HT of 30 m length and 0.25 mm internal diameter coated with a 0.25 µm film (Macherey-Nagel). Four µL of the extracts were injected splitless with the injector temperature at 280 °C. The oven temperature was held at 80°C for 1.5 minutes, ramped up at 10 °C/minute to 180 °C, then ramped up at 30°C/minute to 300 °C and held at 300 °C for 2.8 minutes. The carrier gas was helium and the flow rate 1 mL/minute. The expected retention time for the glyphosate derivative was 9.1 minutes. The temperature of the ion source was 280 °C and the electron impact (EI) energy was 70 eV with an emission current of 50 µA. Mass transition for evaluation [m/z] was 612 → 213 for quantification, 611 → 261 for confirmation, and 615 → 213 for $^{13}C_2\ ^{15}N$ glyphosate. Calculations were performed using the ratio of the peak areas of the quantifier transition of glyphosate derivative and the internal standard derivative. Calibration solutions were prepared by volumetric dilution of a glyphosate stock solution in a solution containing 20 ng/mL internal standard. The dilutions were made in CAX solution. Aliquots of 0.05 mL of these calibration solutions were derivatized as described for the breast milk extracts. The concentration of the derivatized calibration solutions ranged from 0.01 to 10 ng/mL. The concentration of the internal standard in the final extract was always 5 ng/mL.

Results and Discussion

Since a very low transfer was observed of glyphosate into muscle, milk and fat in farm animal metabolism studies the LOQ should be as low as possible. The LC-MS/MS method was validated for glyphosate in accordance with the requirements of the EU guidance document for quality control and validation procedure. Recovery and precision of glyphosate were determined for 6 or 7 replicates at two fortification levels. The linearity of the system was tested by injecting 8 standards in water in a concentration range from 0 to 50 ng/mL. A linear relationship between concentration and the ratio of the peak area of glyphosate and its internal standard was observed with a coefficient of determination greater than 0.99. All calibration points were within 20% of the theoretical value. Quantification was performed using single-point calibration which is acceptable if the response of the analyte in the samples is close to the response in the standard. The average recovery of the LC-MS/MS method was 99% at 1 ng/mL and 91% at 5 ng/mL with a relative standard deviation of 16% and 7%, respectively. The LOQ of the LC-MS/MS method of 1 ng/mL demonstrated sufficient recovery and precision. Possible matrix effects were corrected using the stable isotope labeled internal standard $^{13}C_2{}^{15}N$ glyphosate. At a concentration of 0.5 ng/mL, a signal-to-noise ratio of approximately 4 was obtained. This concentration is considered the LOD of the LC-MS/MS method.

For the analysis of glyphosate by GC-MS/MS, extraction with acidified water was combined with clean-up on a cation exchange column to remove interfering substances present in breast milk. To enable analysis by gas chromatography glyphosate was derivatized using heptafluoro-1-butanol and trifluoroacetic acid anhydride. Calibration was performed with freshly prepared derivatives of 8 glyphosate standard solutions in the concentration range from 0.01 to 10 ng/mL. All standard solutions contained the internal standard at 5 ng/mL and the coefficient of determination was always equal to or greater than 0.9980. The average recovery of the GC-MS/MS method was 84% at 1 ng/mL and 83% at 10 ng/mL with a relative standard deviation of 13% and 8%, respectively. All results obtained by GC-MS/MS had to be corrected for (derivatization reagent) blank interferences. A set of at least 4 reagent blanks were analyzed within each set of breast milk samples. The average measured blank values ranged from 0.2 to 0.6 ng/mL. The relative standard deviations of blank values in the sample sets ranged from 19% to 33%. Considering the blank values from the derivatization reagent, the LOQ of the GC-MS/MS method was 1 ng/mL. Notwithstanding the interference problem of the GC-MS/MS method, both analytical methods were able to measure glyphosate residues in breast milk with an LOQ of 1 ng/mL.

In total, 114 different breast milk samples were analyzed for glyphosate. Seventy-five samples were analyzed by LC-MS/MS only. Because of the lower performance, only 19 samples were analyzed exclusively by GC-MS/MS. Twenty milk samples were analyzed by both methods. In addition to the 114 samples, 5 samples for performance testing were analyzed by both LC-MS/MS and GC-MS/MS, 4 breast milk samples which were spiked in advance with glyphosate and one control sample. Glyphosate was identified by LC-MS/MS in all samples spiked with glyphosate. The recoveries for the LC-MS/MS method were 110%, 97% and 102% for the spiking levels of 1, 3, and 5 ng/mL, respectively. In the sample spiked at 0.5 ng/mL, glyphosate could still be detected by the LC-MS/MS method. Due to the interference problem in GC-MS/MS method, no clear detection of glyphosate was possible at this level. The recoveries for the GC-MS/MS method were 70%, 70%, and 54% for the spiking levels of 1, 3, and 5 ng/mL, respectively. Generally, the GC-MS/MS method tended to result in lower concentrations, probably due to the correction for derivatization reagent blank values. The higher bias of the GC-MS/MS method might be due to dilution steps using very small volumes. The concentration step to yield the final volume might have resulted in a partial loss of the glyphosate derivative. Nevertheless, both methods are able to quantify glyphosate residues in breast milk at or above a concentration of 1 ng/mL. Because of the lack of significant blank values in the LC-MS/MS method, residues of glyphosate higher than 0.5 ng/mL are still detectable by this method. In none of the 114 breast milk samples obtained from German women glyphosate was detected.

Conclusion

A LC-MS/MS and a GC-MS/MS method were developed for the detection and quantification of glyphosate in human breast milk. Both methods have been fully validated and are suitable for the determination of glyphosate with an LOQ of 1 ng/mL. The LC-MS/MS method allows the detection of glyphosate at or above a level of 0.5 ng/mL. The LC-MS/MS method is much faster than the GC-MS/MS method, thus making it suitable for higher sample throughput. The positive findings of glyphosate in breast milk of American women could not be confirmed by the results of this study. In none of the 114 breast milk samples collected from German women in August and September 2015 glyphosate was found within the detection limitations of the analytical methods. Available data from farm animal studies on glyphosate with non-labeled material support these results. They provide no indication of a significant carry-over into fatty tissues or milk even at high dosing levels.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Two analytical methods were developed for the determination of glyphosate in human breast milk. In the first method fat was removed by centrifugation and the proteins by ultra-filtration using a molecular weight cutoff filter of 30 kDa. The final extract was then analyzed by LC-MS/MS. In the second method the milk sample was acidified with acetic acid, centrifuged and the supernatant extracted with dichloromethane. The aqueous phase was filtered and cleaned-up using a cation exchange resin. The final extract was then analyzed by GC-MS/MS after derivatization with

heptafluoro-1-butanol and trifluoroacetic acid anhydride. $^{13}C_2{}^{15}N$ glyphosate was used as the internal standard in both methods. Both analytical methods were validated according to the EU guidance document on analytical quality control and validation procedures for pesticide residues in food and feed (SANCO/12571/2013) and were found suitable for the determination of glyphosate in human breast milk with an LOQ of 1 ng/mL. In August and September 2015, 114 breast milk samples were collected from German women and were analyzed for glyphosate. In none of the samples analyzed glyphosate concentrations were found at or beyond the LOQ.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the breast milk samples analyzed were collected on a voluntary basis and there were no restrictions for participating in the monitoring program. As a consequence the samples cannot be considered representative of the German population. Both analytical methods developed were validated in accordance with the EU guidance on the procedures for the analysis of pesticide residues in food and feed.

Reliability criteria of exposure studies

Publication: Steinborn et al., 2016.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	Y	Guidance document on analytical quality control and validation procedures for pesticide residues in food and feed, SANCO/12571/2013.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.		Self-reported exposure to pesticides.
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides		
Study		
Study design clearly described	Y?	Mainly the development of methods for the analysis of glyphosate in human breast milk and applied to a set of 114 samples from German women.
Population investigated sufficiently described	Y?	Biometric data of the study population were rather limited. The samples were collected on a voluntary basis and without restrictions.
Exposure circumstances sufficiently described	Y?	No detail was provided on self-reported exposure to pesticides.
Sampling scheme sufficiently documented	Y?	More detail could have been provided.
Analytical method described in detail	Y	

Publication: Steinborn et al., 2016.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Validation of analytical method reported	Y	
Monitoring results reported	Y	None of the 114 samples analyzed was positive for glyphosate.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the breast milk samples analyzed were collected on a voluntary basis and there were no restrictions for participating in the monitoring program. As a consequence the samples cannot be considered representative of the German population. Both analytical methods developed were validated in accordance with the EU guidance on the procedures for the analysis of pesticide residues in food and feed.		

1. Information on the study

Data point:	CA 5.4
Report author	Suárez-Larios K. *et al.*
Report year	2017
Report title	Screening of Pesticides with the Potential of Inducing DSB and Successive Recombinational Repair
Document No	Journal of Toxicology (2017), Article ID 3574840,
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

A study was realized to ascertain whether glyphosate and other selected pesticides would induce double strand breaks (DSB) in lymphocyte cultures and whether this damage would induce greater levels of proteins Rad51 participating in homologous recombination or of p-Ku80 participating in nonhomologous end joining. Only five pesticides were found to induce DSB of which only glyphosate and another one pesticide induced a significant increase of p-Ku80 protein, indicating that nonhomologous end joining recombinational DNA repair system would be activated. The type of gamma-H2AX foci observed was comparable to that induced by etoposide at similar concentrations. These results are of importance since these effects occurred at low concentrations in the micromolar range, in acute treatments to the cells. Effects over longer exposures in actual environmental settings are expected to produce cumulative damage if repeated events of recombination take place over time.

Materials and Methods

Chemicals - Endosulfan, glyphosate, pentachlorophenol, permethrin, propoxur, paraoxon, AMPA (glyphosate metabolite), endosulfan lactone (endosulfan metabolite), and etoposide (positive control) were obtained from Sigma-Aldrich, Mexico. Purity was not reported.

Evaluation of DNA double strand breaks (DSB)- Three mL of blood were obtained from fully informed healthy young male donors of 21 to 35 years old. The volunteers were nonsmokers, did not consume alcohol and had not taken medication or were not subjected to radiation for medical purposes. The concentrations of glyphosate tested were 0, 0.4, 2, 10, and 50 μM, and those of AMPA were 0, 40, 200, 1,000 and 5,000 μM. Whole blood was diluted in RPMI-1640 medium and treated with the test compound 1.5 hours at 37 ºC, after which 0.075M KCl was added and the incubation continued for 30 minutes. Lymphocytes were recovered by centrifugation and formaldehyde added to reach a final concentration of 4%. Then PBS and Triton X-100 were added followed by an incubation of 30 minutes. Thereafter the cells were washed, supplemented with 4% fetal bovine serum and isolated by centrifugation and 1mL of cold 50% methanol added. After one night at −20 ºC the samples were centrifuged at 0 ºC, and cold methanol added to the cells which were kept at −20ºC until analysis. Treatments were done in duplicate. DNA double strand breaks were detected by means of immunofluorescence of phosphorylated histone H2AX foci. Staining of lymphocyte nuclei was done as follows: the slides were washed and blocked with KCMT buffer for 1 hour at room temperature. Primary antiphospho-histone H2AX (Ser139) in blocking solution was added and left to incubate overnight followed by washes with KCMT buffer. Then the cells were incubated with the secondary antibody Alexa-Fluor 555 goat anti-mouse for 1 hour at room temperature. The slides rinsed in deionized water before mounting in DAPI mounting medium and subsequently analyzed for γ-H2AX foci under a fluorescence microscope. Evaluation of foci was done in 2 slides per concentration and 50 cells were

evaluated in 3 different regions per slide. In total, 300 cells were evaluated per treatment. When a nucleus presented one or more foci, it was considered positive. The extent of DNA damage was classified in 3 categories: percentage of cells without γ-H2AX foci (no DNA damage), with less than 10 γ-H2AX foci (moderate DNA damage) and with more than 10 gamma-H2AX foci (severe DNA damage). Additionally, the damage was expressed as mean percentage of γ-H2AX positive nuclei.

Cytotoxicity - Cytotoxicity was tested using The CellTiter 96 AQueous One Solution Reagent from PROMEGA and following the manufacturer's instructions. Mononuclear cells were isolated from blood using Histopaque-1077. The cells were plated in well plates at 100,000 cells per well, and treated with the test compounds in triplicate for each concentration. Absorbance at 490 nm was recorded using a 96-well plate reader. The percentage of survival was calculated as (Absorbance at 490 nm of treatment/Absorbance at 490 nm of the negative control) × 100.

Western blot analysis of proteins participating in DNA recombination - Mononuclear cells were isolated with Histopaque-1077 and treatments were applied to cells resuspended in RPMI-1640 at 500,000 cells per tube. Glyphosate was tested in duplicate at 1.25, 2.5, and 5 μM. The cells were then centrifuged, the supernatant discarded and 0.5 M sodium azide added. After centrifugation, the cell pellets were kept at −70 °C until used for Western blot analysis. Two separate experiments were performed per test compound, with two donors each time. RIPA lysis buffer solution containing phosphatase and protease inhibitors was added to each cell pellet. The samples were then sonicated, incubated in ice, and centrifuged. The supernatant was recovered and 5 μL of each sample was placed in a 96-well plate for protein quantification. The Lowry assay was performed with the DC Protein Assay kit and the concentration determined in a plate reader at 750 nm. The samples were then stored at −70∘C until use. For the determination of phosphorylated Ku80 (phospho-T714) and Rad51 by Western blotting 35 μg of total protein was separated in a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane with a Trans-Blot SD Semi-Dry Transfer Cell. After incubation with blocking solution and rinsing the appropriate membrane zones were isolated and incubated with rabbit anti-Rad51 polyclonal antibody or rabbit anti- phosphorylated Ku80 (phosphoT714) polyclonal antibody (p-Ku80). Incubation with primary goat anti-Actin polyclonal antibody was done for the determination of β-actin as the internal control. The membranes were then rinsed and incubated with secondary antibody goat anti-rabbit IgG-HRP for Rad51 and p-Ku80 detection and donkey anti-goat IgG-HRP for actin detection. After rinsing the protein fractions were quantified with a luminescence kit. Optical densities were measured with Quantity One software, version 4.1.1. The values obtained for each protein (Rad51 or p-Ku80) were normalized with respect to β-actin and the mean of the normalized negative controls. Results are presented as the % with respect to normalized negative controls. Two membranes were prepared per separate experiment with each test compound .

Statistical Analysis - Statistical calculations were made with the GraphPad Prism 6 software package and the results for γ-H2AX foci and optical density from Western blot analysis were evaluated with the Kruskal-Wallis test and Dunn's multiple comparison as a post hoc test. The value of etoposide as a positive control at the concentration of 50 μM was analyzed with the Mann-Whitney *U* test with respect to the negative control. Cytotoxicity was analyzed with linear regressions. The results were considered statistically significant if $p<0.05$.

Results

Identification of DNA double strand breaks(DSB) - For glyphosate, the mean of the % cells with more than 10 γ-H2AX foci was 0.33, 1.67, 9.33, 8.83, and 3.17 for 0, 0.4, 2, 10, and 50 μM (linear regression, $R^2 = 0.2$, $p = 0.02$). No effect was seen with AMPA. The positive control, etoposide, showed a significant correlation with dose (, $R^2 = 0.82$, $p < 0.0001$) for cells with more than 10 foci.

Cytotoxicity - The concentrations used for the determination of DSB were also used to assess the cytotoxicity of the test compounds that produced DSB. The survival range for glyphosate was 100% to 70% viability for concentrations ranging from 1.25 to 5 μM.

Quantification of p-Ku80 and Rad51 Proteins
The test compounds that showed positive for DSB were further tested to determine whether DNA recombination would be induced. Glyphosate was found to induce statistically significantly p-Ku80 protein in a dose-dependent manner, whereas Rad51 was not significantly affected. Etoposide consistently induced p-Ku80, although with a wide variation between tests at 10 µM.

Discussion and Conclusions

Pesticides amongst which glyphosate were evaluated for their capacity to induce DNA double strand breaks, a lesion related to the formation of chromosomal rearrangements and leukemia risk. Glyphosate exhibited an ability to induce this kind of DNA damage in the form of phosphorylated γ-H2AX foci in the nuclei of human lymphocytes in at least two of the concentrations tested. AMPA tested negative. It is noticeable that the positive control, etoposide, induced more than 85% of cells with more than 10 foci at 50 μM although it induced foci in a manner comparable to glyphosate at lower concentrations. This extent of DNA damage seems to be of relevance to the increase of repair proteins like p-Ku80, since glyphosate also produced a significant increase in this protein, whereas the rest of the test compounds that induced a lower percentage of cells with γ-H2AX foci did not. To evaluate proteins participating in DNA recombination, the highest concentration of glyphosate used was 5 µM to avoid cytotoxicity. Exposure of cells in a non-proliferative state to glyphosate not only induced the breakage of DNA, but also the phosphorylation of Ku80, a protein that participates in the c-NHEJ repair pathway. This pathway is known for being prone to error, introducing micro deletions or micro insertions, which could be mutagenic and alter cell behavior if they occur in coding or regulatory sequences. This is one possible outcome of the DSB in non-proliferating cells. Etoposide, the positive control, is well known as a topo II inhibitor capable of producing complex DSB. The DNA damage induced by etoposide in this study was comparable to the damage induced by paraoxon and glyphosate (DSB and pKu80 induction), so the question emerging from these results is whether the outcome for cells damaged by glyphosate would be similar to the outcome of cells damaged by etoposide and whether they would also induce chromosomal rearrangements.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to assess whether glyphosate and its metabolite AMPA produced DNA double strand breaks in human peripheral lymphocytes and whether proteins involved in DNA repair were induced. The results show that glyphosate, but not AMPA, increased the mean of the percent cells with more than 10 γ-H2AX foci, however, without a clear dose-effect relationship. Glyphosate was found to induce statistically significantly a protein involved in DNA repair, p-Ku80, at 5 µM without a dose-effect relationship (when measured as median OD).

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because glyphosate as a test chemical was not sufficiently characterized and the effect found on an indicator of DNA double strand breaks was not concentration related and occurred at concentrations that were much lower than the systemic concentrations (approx. 300 µM) of regulatory *in vivo* MN tests at 2000 mg/kg bw which were negative.

Reliability criteria for *in vitro* toxicology studies

Publication: Suarez-Larios *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	No guideline study for genotoxicity
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Only source reported: Sigma-Aldrich Mexico.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	Y	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	Not applied
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Up to 50 µM
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	SS increase at 2 concentrations but no dose effect relationship for γ-H2AX foci. SS increase of P-Ku80 at 5 µM but no dose-effect relationship in treated cultures (measured as median OD)
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	Results of this study are not corroborated by in vivo MN studies with much higher blood concentrations (approx. 300µM).
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because glyphosate as a test chemical was not sufficiently characterized and the effect found on an indicator of DNA double strand breaks was not concentration related and occurred at concentrations that were much lower than the systemic concentrations (approx. 300 µM) of regulatory *in vivo* MN tests at 2000 mg/kg bw which were negative.		

1. Information on the study

Data point:	CA 5.3
Report author	Tang J. *et al.*
Report year	2017
Report title	Ion imbalance Is Involved in the Mechanisms of Liver Oxidative Damage in Rats Exposed to Glyphosate
Document No	Frontiers in Physiology (2017) Vol. 8, Article 1083
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study aimed to investigate the effects of glyphosate on rats' liver function and induction of pathological changes in ion levels and oxidative stress in hepatic tissue. Sprague-Dawley rats were treated orally with 0, 5, 50, and 500 mg/kg body weight of the GLP. After 5 weeks of treatment, blood and liver samples were analyzed for biochemical and histomorphological parameters. The various mineral elements content in the organs of the rats were also measured. Significant decreases were shown in the weights of body, liver, kidney and spleen between the control and treatment groups. Changes also happened in the histomorphology of the liver and kidney tissue of GLP-treated rats. The GLP resulted in an elevated level of glutamic-oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and IL-1b in the serum. Besides, decreased total superoxide dismutase (T-SOD) activity and increased malondialdehyde (MDA) contents in the serum, liver, and kidney indicated the presence of oxidative stress. Moreover, increase of hydrogen peroxide (H_2O_2) level and catalase (CAT) activity in the serum and liver and decrease of glutathione (GSH) and lutathione peroxidase (GSH-Px) activity in the kidney tissue further confirmed the occurrence of oxidative stress. The results of RT-PCR showed that the mRNA expressions of IL-1α, IL-1β, IL-6, MAPK3, NF-κB, SIRT1, TNF-α, Keap1, GPX2, and Caspase-3 were significantly increased in the GLP-treated groups compared to the control group. Furthermore, PPARα, DGAT, SREBP1c, and SCD1 mRNA expressions were also remarkably increased in the GLP-treated groups compared to the control group

Materials and methods

Chemicals - Glyphosate was obtained from Shanghai Ryon Biological Technology Co. Ltd (Shanghai, China). Purity was not reported.

Animals - Male Sprague-Dawley rats of 8 weeks old and weighing 180–220 g were purchased and were allowed to acclimate for at least one week prior to testing. All rats were housed in separate cages and had unrestricted access to food and water throughout the study.

Animal treatment and sample collection - Rats were randomly assigned to 4 groups (n = 8/group) and were orally administered glyphosate by gavage at 5, 50, and 500 mg/kg bw/day for 35 days. Distilled water was used as the negative control. Twenty-four hours after the last gavage, rats were weighed and sacrificed. Blood samples were collected from the jugular vein and placed at 37°C for one hour before being centrifuged for biochemical assays. Liver, kidney, spleen, heart, lungs, brain, adrenal glands, muscle and fat tissue were collected, rinsed with PBS, dried and weighed. A piece of liver and right kidney was used for morphometric analysis and another piece was used to prepare homogenates for the analysis of parameters of oxidative stress or frozen in liquid nitrogen for subsequent qualitative reverse transcription polymerase chain reaction (RT-PCR).

Histological preparation - Samples of liver and kidney were fixed in 4% formaldehyde solution for 24 hours, dehydrated in alcohol, clarified with xylene, and embedded in paraffin. Paraffin blocks were sectioned into 5μm slices and stained with hematoxylin-eosin (HE) for microscopic examination.

Biochemical evaluation - Liver and kidney homogenate and serum were used for the assessment of liver function (serum GOT and GPT) and oxidative stress (total SOD, MDA, H_2O_2, CAT, GSH, and GSH-peroxidase or GSH-px). The activity of serum GOT and GPT was assayed according to the method that is normally applied in clinical biology. The analysis of total SOD activity was based on SOD-mediated inhibition of nitrite formation from hydroxyammonium in the presence of O^{2-}generators (xanthine/xanthine oxidase). Total SOD activity was expressed in U/mg protein. MDA was evaluated by the thiobarbituric acid reactive substances method (TBARS) and the results were expressed in nmol/mg protein. GSH-px activity was estimated by the determination of reduced GSH in the enzymatic reaction. GSH-px activity was expressed in U/mg protein. CAT activity was assayed by the method developed by Aebi, and calculated as nM H_2O_2 consumed/min/mg of protein. Protein concentrations in the supernatant were measured according to the Coomassie Brilliant Blue method.

Serum Cytokine Measures - Serum levels of IL-1β and IL-6 were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit.

Quantitative RT-PCR (qRT-PCR) analysis - Total RNA was extracted from tissue using the reagent box of Total RNA Kit, according to the manufacturer's instructions. The concentration of RNA was measured by spectrophotometry and the purity was ascertained by the A 260/A 280 ratio. Total RNA from each sample was reverse transcribed to cDNA with an Omniscript® Reverse Transcription kit with Oligo-dT primers according to the manufacturer's instructions and used for RT-PCR. The target fragments were quantified by real-time PCR with 100 ng of the cDNA template. Each sample was tested in duplicate. The gene expression data were normalized to β-actin expression. For each real-time PCR assay, the threshold cycle Ct was determined for each reaction. Ct values for each gene of interest were normalized to the housekeeping gene, β-actin and PCR amplification efficiencies were taken into account by amplifying various amounts of target cDNA for each reaction. The fold differences in mRNA expression of samples were relative to the internal control sample, which was included in all runs.

Ion Concentration - The concentrations of Al, Fe, Cu, Zn, and Mg in liver, kidney, spleen, lung, heart, muscle, brain, and fat tissue were determined by inductively coupled plasma optical emission spectrometry using nitric acid–perchloric acid–based wet digestion. Approximately 200 μL or 0.5 g of each sample was digested with nitric acid (75%) and perchloric acid (25%) in a microwave digester. The same part of the organ was used from the control and treated animals.

Statistical Analysis - The data were expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test, which was performed with GraphPad Prismsoftware. Differences were considered to be statistically significant when $p< 0.05$.

Results

Body weight and body weight gain were statistically significantly decreased at 500 mg/kg bw/day and at 50 and 500 mg/kg bw/day, respectively. Absolute and relative organ weight was statistically significantly reduced for liver, spleen and kidney at 500 mg/kg bw/day.
Liver sections of rats exposed to glyphosate showed apoptosis of some hepatocytes, focal necrosis and mononuclear cell infiltration. At 5 mg/kg bw/day rats showed mild periportal expansion and apoptosis of some hepatocytes. At 50 and 500 mg/kg bw/day apoptosis of some hepatocytes and monocyte infiltration was observed in liver tissue. In the kidney, marked histological changes were observed, including proximal and distal tubular necrosis and glomerular toxicity. The histologic score of hepatic and renal damage was statistically significantly increased at all dose levels. Serum activity of GOT and GPT was statistically significantly increased at 500 mg/kg bw/day.

Assessment of oxidative stress - In serum, total SOD activity was statistically significantly decreased at

500 mg/kg bw/day. MDA content was significantly increased at 50 mg/kg bw/day but not at 500 mg/kg bw/day and CAT activity was significantly increased at 500 mg/kg bw/day. In liver, total SOD activity was statistically significantly decreased and H_2O_2 levels increased at 500 mg/kg bw/day. In kidney, total SOD and GSH-px activities were significantly decreased at 50 and 500 mg/kg bw/day. A statistically significant decrease in GSH levels was observed at 50 mg/kg bw/day but not at 500 mg/kg bw/day.

Serum IL-1β and IL-6 levels - In serum, the level of IL-1β was statistically significantly increased at 500 mg/kg bw/day. There was no statistically significant change for IL-6.

Expression of genes related to inflammation in the Liver - Hepatic IL-1α and IL-1β mRNA expression were statistically significantly increased at all dose levels. IL-6, MAPK3, SIRT1, TNF-α, GPX2, and *Caspase*-3 mRNA expression was significantly increased at 50 and 500 mg/kg bw/day. NF-κB mRNA expression showed only a significant increase 50 mg/kg bw/day and *Keap*1 mRNA expression was only increased at 5 mg/kg bw/day.

Expression of genes related to lipid metabolism in the liver - PPARα, *SREBP1c*, and *SCD1* mRNA expression were significantly increased at 50 and 500 mg/kg bw/day and *DGAT* mRNA expression was significantly increased at 500 mg/kg bw/day.

Concentration of Al, Fe, Cu, Zn and Mg in tissues – The Al concentration was statistically significantly increased in liver at 50 and 500 mg/kg bw/day but significantly decreased in lung at 50 mg/kg bw/day and in muscle at 500 mg/kg bw/day. Fe was significantly increased at 5 mg/kg bw/day only in liver, at 50 mg/kg bw/day only in kidney and spleen and at 500 mg/kg bw/day in lung. Cu was significantly increased in brain and fat tissue at 500 mg/kg bw/day and Zn was only significantly increased in liver at 50 and 500 mg/kg bw/day. Mg levels were only increased in brain tissue at 500 mg/kg bw/day.

Discussion and Conclusions

The results of this study showed that exposure to glyphosate for 35 days at doses up to 500 mg/kg bw/day led to a statistically significant reduction in body weight and body weight gain and in absolute and relative weight of liver, kidney and spleen. Histopathological examination of the tissues also revealed effects in liver and kidneys. Liver effects were corroborated by a significant increase in GOT and GPT. The results of this study showed that SOD activity was significantly decreased in serum, liver and kidney of rats treated with glyphosate when compared with the control group. MDA content was significantly increased in serum and kidney. CAT activity was also significantly elevated in serum and H_2O_2 levels were increased in liver tissue, suggesting oxidative stress. Taken together, the data demonstrated that glyphosate exposure could result in liver and kidney damage due to oxidative stress. The level of IL-1β was significantly increased at 500 mg/kg bw/day. Therefore, the relationship was investigated between oxidative stress and the transcription of genes related to inflammation. In this study, mRNA expression of *IL-1α, IL-1β, IL-6, MAPK3, NF-κB, SIRT1, TNF-α, Keap1, GPX2* and *Caspase-3* were all increased upon exposure to glyphosate. Genes related to lipid metabolism such as *PPARα, SREBP1c, DGAT*, and *SCD1* were significantly upregulated in rats exposed to glyphosate. The results from this study show that the liver toxicity induced by glyphosate is mediated by inflammation, oxidative stress and lipid related pathways. Tissue concentrations of Al, Fe and Zn were significantly increased in liver tissue of rats exposed to glyphosate. Concentrations of Fe were also increased in kidney, spleen, and lung tissue although not always in a dose-related manner. Al concentration was decreased in lung and muscle tissue whereas Cu concentrations were increased in brain and fat tissue and Mg in brain tissue of rats exposed to glyphosate. Combined, these results suggest that glyphosate exposure impaired the ion-balance of Al, Fe, Mg, Cu, and Zn.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to investigate the toxicity, oxidative stress and metal ion concentrations in tissues of rats after oral exposure to glyphosate for 35 days at doses up to 500 mg/kg bw/day. Oxidative stress was studied by the determination of markers of oxidative stress such as SOD, CAT, H_2O_2, MDA, GSH and GSH-px and the transcription of genes related to inflammation and lipid metabolism. Statistically significant effects were found on body weight, body weight gain, organ weight, serum indicators of liver toxicity and histopathology of the liver and the kidney. Significant changes were also reported on markers of oxidative stress and transcription of genes related to inflammation and lipid metabolism. Many of the effects reported were mild in nature and/or didn't show a clear dose-effect relationship. Also the effects on metal ion concentrations in organ tissues were not always consistent and often didn't show a dose-effect relationship.

This publication is considered relevant but reliable with restrictions because the test material was not sufficiently characterized, the number of animals used for this study duration is not sufficient, the results were not always accurately reported and are not corroborated by regulatory toxicology studies of similar test durations and dose ranges.

Reliability criteria for *in vivo* toxicology studies

Publication: Tang *et al*., 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Number of animals per dose level lower than minimum required for 4-week testing
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	?	Not described in sufficient detail
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Only source reported: Shanghai Ryon Biological Technology Co. Ltd., China.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	
Dose levels reported	Y	
Number of animals used per dose level reported	Y	
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	Sometimes inaccurate reporting of data in tables. Results not concordant with short term regulatory toxicology studies
Statistical methods described	Y	

Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant but reliable with restrictions because the test material was not sufficiently characterized, the number of animals used for this study duration is not sufficient, the results were not always accurately reported and are not corroborated by regulatory toxicology studies of similar test durations and dose ranges.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Thongprakaisang S. *et al.*
Report year	2013
Report title	Glyphosate induces human breast cancer cells growth via estrogen receptors
Document No	Food and Chemical Toxicology (2013) Vol. 59, 129–136
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10-12 to 10-6 M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity.

Materials and Methods

Chemicals - Glyphosate (purity >98%) was obtained from AccuStandard (New Haven, CT, USA).

Cell lines and culture conditions - A hormone-dependent human breast cancer cell line (T47D), a stably ERE-luc construct transfected hormone-dependent breast cancer cell line (T47D-KBluc) and a hormone-independent human breast cancer cell line (MDA-MB231) were obtained from the American Type Culture Collection (ATCC).

In vitro estrogen receptor activation-reporter assay - To study the estrogenicity and/or anti-estrogenicity of glyphosate, the T47D-KBluc cell line, stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct was used in this study. To minimize the effect of estrogen in the medium, 5 days prior to the start of the assay, cells were incubated in a non-phenol red RPMI modified medium with replacement of 10% FBS by 10% dextran-charcoal treated FBS (CSS). One day prior to the assay, cells were seeded at 10^4 cells/100 µL/well and were allowed to attach overnight. The dosing medium was further modified by reduction to 5% CSS and then replaced with 100 µL/well of dosing medium containing glyphosate at concentrations ranging from 10^{-12} to 10^{-6} M. Estradiol (E2) in the same range of concentrations was used as the positive control. Dosing medium without glyphosate was used as the negative control and wells without cells were used as the blank. After 24 hours of incubation, cells were washed with 100 µL PBS and harvested in 25 µL lysis buffer. The luciferase assay was performed by injecting 50 µL of reaction buffer and 50 µL of 1 mM D-luciferin and fluorescent intensity was measured by means of the microplate luminometer. Luciferase activity was quantified as relative light units (RLU).

Cell viability MTT assay - Cell growth and cell viability were tested using the 3-(4,5-dimetylthiazol, 2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent assay. Cells were seeded at 10^4 cells/100 µL/well

in 96-well microtiter plates. For the E2 withdrawal condition, cells were cultured in 10% CSS and a non-phenol red RPMI medium for 4 days before seeding. After 24 hours the cells were treated with E2 or glyphosate at concentrations ranging from 10^{-12} to 10^{-6} M. For E2 receptor antagonist conditions, E2- or glyphosate-treated cells were co-incubated with ICI 182780 at 1 and 10 nM. Cell sensitivity to a chemical was expressed as the % cell viability compared to control cells.

Cell number counting - T47D Cells were prepared in E2 withdrawal conditions 4 days before the start of the assay. Cells were placed in a 24 well culture plate with 10^4 cells/mL/well and incubated overnight. The medium was then replaced with 1 mL of treatment solution and incubated for 72 hours. Afterwards, the cells were washed with 1 mL PBS and then 100 µL of a trypsin–EDTA solution was added to detach. An aliquot of the cells was taken for counting using a counter analyzer.

Western blot analysis - Whole-cell extracts were prepared from cells treated for 6 and 24 hours with 10^{-12}, 10^{-9}, and 10^{-7} M glyphosate and non-treated control. The cells were lysed, incubated on ice and centrifuged. The supernatants were collected and either processed or stored at -80 ^{o}C until use. The protein concentration was measured using Bradford reagent and each lysate was aliquot for an equal amount of protein, 30 µg, before mixing with Laemmli loading buffer and then boiled at 95 °C for 5 minutes. The samples were resolved over 7.5% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell. The membrane was treated with blocking solution for one hour at room temperature and subsequently probed overnight with primary antibody (ERα, ERβ or β-actin) and then rinsed. HRP-conjugated secondary antibodies were added to the membrane for 2 hours and the membranes rinsed with TBS-T. Protein visualization was achieved by using enhanced chemiluminescence and the emitted light was captured on film. The signals on the films were quantified using densitometry.

Statistical analysis - Data are presented as the means ± SE. Statistical significance was determined using the Student's t-test. A two-tailed $p<0.05$ was evaluated as a statistically significant difference.

Results

T47D, hormone-dependent breast cancer cell growth - The hormone-dependent T47D and hormone-independent MDA-MB231 cell lines were studied both in completed medium and estrogen withdrawal medium to differentiate the effect of glyphosate from that of endogenous estrogen. Estrogen in a concentration range of 10^{-12} to 10^{-6} M was used as the positive control. Cell growth was assessed using the MTT cell viability assay. The results showed that T47D and MDA-MB231 cells exhibited different patterns of responses to glyphosate. In the absence of E2, glyphosate produced cell proliferation in T47D cells of approximately 15–30%. This effect was about half of the E2 response which is the most potent agonist in hormone dependent ER-positive breast cancer cells. No effect was observed on cell proliferation in MDA-MB231 cells both in the absence or presence of E2.

Cell proliferation via estrogen receptors - Due to the fact that the proliferative effect of glyphosate occurred only in T47D cells in the absence of E2, it was hypothesized that ER signaling may be involved in glyphosate-induced cell proliferation. Therefore, the effect of glyphosate on T47D cells was investigated in the presence of an ER antagonist, ICI 182780, to inhibit the estrogen receptor mediated response. The effective concentration of 1 nM of ICI 182780 was added to varying concentrations of glyphosate and E2 to observe its antagonistic activity. The results showed that ICI 182780 at 1 nM mitigated the proliferative effects of both glyphosate and E2. A higher concentration of ICI 182780 (10 nM) completely inhibited the growth promoting effects of glyphosate. These results suggest that glyphosate may produce the proliferative effect via the ER.

ERE-transcription activity via estrogen receptors - T47D-KBluc cells, stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct, were treated with the concentrations of glyphosate that produced cell proliferation. The results showed that glyphosate in a concentration range of 10^{-12} to 10^{-6} M induced ERE activation 5- to 13-fold of control and these effects were less than about half of that induced by E2. Glyphosate co-incubation with the ER antagonist, ICI

182780, exhibited a significant reduction in responses. ICI 182780 at 10 nM completely inhibited the ERE transcriptional activity of glyphosate. Since glyphosate was shown to induce cell proliferation and ERE activation via the ER, the potential effects of glyphosate on endogenous E2 signaling was investigated. Cells were co-incubated with glyphosate and E2 and the results revealed that glyphosate suppressed E2-induced ERE activation suggesting that glyphosate behaves as an ER antagonist in the presence of E2.

Expression of ERα and ERβ in human breast cancer cells - The expression of proteins involved in ERs including ERα and ERβ, was studied with the Western blot technique. After 6 hours of exposure, glyphosate increased the levels of both ERα and ERβ in a concentration-dependent manner while after 24 hours only ERα showed a significant induction at the highest glyphosate concentration tested (10^{-7} M). This result suggests that glyphosate alters the expression of both ERα and ERβ in human breast cancer cells.

Discussion

In this study, glyphosate was found to increase cell proliferation of a hormone dependent breast cancer T47D cell line at concentrations ranging from 10^{-12} to 10^{-6} M while this effect was not observed in a hormone independent breast cancer MDA-MB231 cell line. The results from the ERE luciferase assay confirmed ER activation because the responses seen could be blocked by the ER antagonist ICI 182780. Glyphosate induced rapid activation of ERβ while activation of ERα was slower but prolonged. It is suggested that glyphosate may behave like a weak xenoestrogen which can activate both subtypes of ER but with a different time course. Although the nature of the binding of glyphosate to the ER is still unknown, the ability of glyphosate to stimulate the ERE-gene transcription activity and upregulation of ERα protein expression suggests that glyphosate may exert the stimulatory effects via an ER-dependent mechanism.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to investigate the possible estrogenic effect of glyphosate and its mode of action. The endpoints explored were the cell proliferation of hormone-dependent and hormone-independent cell lines with and without an ER antagonist, ERE-transcription activity with and without an ER antagonist, and expression of ERs. Glyphosate was found to produce cell proliferation in a hormone-dependent cancer cell line but not in a hormone-independent cancer cell line in the absence of E2. In the presence of a potent ER antagonist the cell proliferation caused by glyphosate in a hormone-dependent cancer cell line was reduced. The interaction of glyphosate with the ER was confirmed by ERE activation with and without an ER antagonist. When cells were co-incubated with glyphosate and E2, glyphosate suppressed the E2-induced ERE activation suggesting that glyphosate behaves as an antagonist in the presence of an endogenous agonist. It was demonstrated that glyphosate alters the expression of both ERα and ERβ in human breast cancer cells.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test results are not corroborated by *in vivo* regulatory ED toxicology studies such as the uterotrophic assay and the female pubertal assay (U.S. EPA Endocrine Disruptor Screening Program).

Reliability criteria for *in vitro* toxicology studies

Publication: Thongprakaisang *et al*., 2013	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of >98%. Source: AccuStandard, New Haven, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	The slight estrogenic effect of glyphosate reported was not confirmed in *in vivo* studies such as the uterotrophic assay and the female pubertal assay.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	Results not consistent with other publications on ED.
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test results are not corroborated by *in vivo* regulatory ED toxicology studies such as the uterotrophic assay and the female pubertal assay.		

1. Information on the study

Data point:	CA 5.4
Report author	Townsend M. *et al.*
Report year	2017
Report title	Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity
Document No	Regulatory Toxicology and Pharmacology (2017) Vol. 85 79-85
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

The resulting DNA damage and cytotoxicity of various glyphosate concentrations on human cells was studies to evaluate DNA damaging potential. Utilizing human Raji cells, DNA damage was quantified using the comet assay, while cytotoxicity was further analyzed using MTT viability assays. Several glyphosate concentrations were assessed, ranging from 15 mM to 0.1 µM. It was found that glyphosate treatment is lethal to Raji cells at concentrations above 10 mM, yet has no cytotoxic effects at concentrations at or below 100 µM. Treatment concentrations of 1 mM and 5 mM induce statistically significant DNA damage to Raji cells following 30-60 min of treatment, however, cells show a slow recovery from initial damage and cell viability is unaffected after 2 h. At these same concentrations, cells treated with additional compound did not recover and maintained high levels of DNA damage. While the cytotoxicity of glyphosate appears to be minimal for physiologically relevant concentrations, the compound has a definitive cytotoxic nature in human cells at high concentrations.

Materials and Methods

Chemicals and reagents - Glyphosate (95% w/w purity) was obtained from Sigma-Aldrich, Inc. (Milwaukee, WI).

Cell culture - Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Cells were cultured in RPMI 1640 and supplemented with 10% FBS and 2 mM L-glutamine. Media were replaced every 48 hours and the cells used for testing were placed in exponential growth with a minimum viability of 95%. Raji cells were selected for this study because the replication time is 18 hours allowing the assays to cover the entire cell cycle.

Cell viability assay - For use in the MTT viability assay, glyphosate was diluted in cell culture RPMI media to the final test concentrations and stored at 4 oC. Glyphosate stock solutions were diluted in RPMI media to their final test concentrations. Raji cells were incubated for 24 hours in a 96-well plate at 37 oC and 5% CO2. Afterwards, MTT reagent was added to each well. Following 3 hours of incubation, 100 µL of DMSO detergent was added to each well and incubated for 2 hours at 4 oC, and cytotoxicity evaluated at 570 nm absorbance.

Alkaline comet assay - Raji cells were incubated with either hydrogen peroxide as the positive control, PBS as the negative control, or glyphosate. The concentration and time points varied depending on the experimental run. The time intervals tested included 10, 20, 30, 40, 50, 60, 75, 90, 105, and 120 minutes. The concentrations of glyphosate used at each of the time points were 0.1, 1, 10, and 100 µM, and 1, 5, 10, and 15 mM. 200,000 cells per 100 µL were incubated with positive and negative controls and several concentrations of glyphosate at 37 ºC. Once treated, the cells were washed with PBS and suspended at 200,000 cells per 100 µL of PBS for the conduct of the comet assay. The test samples were mixed with

low melting point agarose and layered on double frosted microscope slides and placed in alkaline lysis buffer for 60 minutes, rinsed with purified water and then placed in alkaline electrophoresis buffer for 20 minutes and submitted to electrophoresis. After fixation and drying, the slides were stained with propidium iodide and imaged using a Zeiss Axioscope fluorescence microscope. All comets were scored using TriTek CometScore Freeware v1.5. Every experimental run tested a single concentration for multiple time points. Each time point contained a minimum of two slides as replicas. Approximately 50 comets were analyzed per slide, totaling 100 comets per time point and per treatment concentration. Each concentration was replicated multiple times to ensure consistency. Comet assay results are reported as tail moment which is defined as the product of the tail length and the percentage of DNA in the tail. A similar protocol was followed to test the effects of secondary glyphosate exposure at 1 mM and 5 mM by adding 200 µL glyphosate solution to the cells after 60 minutes of initial treatment, while 200 µL of PBS was added to the negative control.

Statistical analysis - Relationships between exposure time and tail moment were modeled statistically using a natural spline to account for nonlinearity. The number of knots was selected based on Akaike Information Criteria (AIC) and parameters were estimated using least squares. P-values of <0.05 were considered statistically significant.

Results

MTT analysis of Raji cells exposed to various glyphosate concentrations for 24 hours indicated a significant loss of cell viability at 10 and 15 mM. Glyphosate concentrations of 5 mM and lower did not have a significant effect on viability when compared to the negative control.

Comet assay analysis of Raji cells exposed to 10 and 15 mM glyphosate indicated severe DNA damage and cell death soon after exposure. Within 30 min of treatment, all cells had adopted an apoptotic profile which is characterized by a loss of a defined comet head and a large, fragmented DNA tail. Tail moments were significant after just 10 minutes of glyphosate exposure. Raji cells exposed to glyphosate concentrations of 1 and 5 mM produced statistically significant DNA damage after 40 minutes of treatment. Tail moments reached a maximum after 60 and 80 minutes of treatment at 5 mM and 1 mM, respectively. A steady decrease in tail moment was observed in later time points and the cells were able to recover to full viability after 120 minutes of treatment. Cells exposed to 10 and 100 µM of glyphosate did not show statistically significant DNA damage, and the cells retained full viability throughout 120 minutes of treatment. Raji cells exposed to physiologically relevant concentrations of glyphosate for 120 minutes did not experience any significant DNA damage. Following treatment with 10 µM glyphosate, Raji cells showed no signs of DNA damage and the ‘head’ of the cells stayed intact throughout the 2-hour exposure time. To further investigate the comet results at 1 and 5 mM, cells were treated again with glyphosate at these concentrations one hour after initial treatment. A significant difference could be observed between cells receiving only primary treatment and cells receiving the additional treatment. Raji cells exposed twice to glyphosate did not show the same pattern of recovery, with tail moments reaching levels above 20 for 1 mM and 25 for 5 mM. Cells with only primary exposure to glyphosate showed a decrease in DNA damage, with tail moments dropping from 15 to 5.8 for 1 mM and 23.67 to 6.74 for 5 mM.

Discussion

The results show that the DNA damaging and cytotoxic potential of glyphosate is related to exposure length and treatment concentration. Glyphosate only induced significant DNA damage at concentrations several orders of magnitude larger than those attainable *in vivo*. The data support the established evidence that glyphosate is not genotoxic in human cells at physiologically relevant concentrations. While these data ultimately support glyphosate's classification as a potential carcinogen, they suggest that its effects are negligible when exposure is minimal. Furthermore, study results obtained at 1 mM and 5 mM suggest that cells initially damaged by glyphosate may have the ability to repair and regain viability after single exposure. The results show also that the extent of DNA damage changed drastically across different incubation time points. For example, incubation at 1 mM for one hour produced severe DNA damage whereas no DNA damage was evident after 2 hours at the same concentration. If cells had

only been evaluated at this time point, results would suggest that there was no cytotoxic activity and the initial DNA damaging event would be missed. Cytotoxic activity might also be underestimated by standard viability assays in which the DNA damage is insufficient to induce cell death. Considering multiple time points made it possible to observe both DNA damage as well as the ensuing recovery.

Conclusion

Exposure of human cells to glyphosate produces minimal cytotoxicity and DNA damage at concentrations at or below 100 µM.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The purpose of this study was to investigate the concentration and time dependent DNA damaging potential of glyphosate in Burkitt's B Cell Lymphoma (Raji) cells using the comet assay and MTT viability assay. The cells were exposed to glyphosate concentrations ranging from 0.1 µM to 15 mM and resulting DNA damage and loss of cell viability were measured after various lengths of exposure. DNA damage could only be observed at 1mM and higher which are concentrations that cannot be attained in vivo. The DNA damage seen at 1 and 5 mM reached its maximum between 60 and 80 minutes of incubation which returned to control values thereafter. To reach 1 mM of systemic concentration *in vivo* experimental animals have to be treated orally with glyphosate at dose levels that are much higher than those used in long term carcinogenicity studies which showed no carcinogenic effect of glyphosate.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria for *in vitro* testing although no historical control data were reported. The significance for the risk assessment of glyphosate is limited because DNA damage has only been demonstrated at concentrations of glyphosate that cannot be attained in *in vivo* test systems.

Reliability criteria for *in vitro* toxicology studies

Publication: Townsend *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95%. Source: not reported.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y/N	0.1 µM to 15 mM for alkaline comet assay
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	

Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Human cell exposure to glyphosate has minimal cytotoxicity and DNA damage at concentrations at or below 100 μM. Only effects found beyond 1 mM.
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria for in vitro testing although no historical control data were reported.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Trasande L. *et al.*
Report year	2020
Report title	Glyphosate exposures and kidney injury biomarkers in infants and young children
Document No	Environ Pollution (2020), 256, 113334
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	Yes, all research was performed in accordance with relevant guidelines/regulations.
Acceptability/Reliability:	Yes/Reliable without restriction

2. Full summary of the study according to OECD format

The goal of this study was to assess biomarkers of exposure to glyphosate and assess potential associations with renal function in children. While previous studies have indicated that glyphosate may have nephrotoxic effects, few have examined potential effects on kidney function in children. In this study, three cohorts across different phases of child development and measured urinary levels of glyphosate. Associations of glyphosate with three biomarkers of kidney injury was evaluated: albuminuria (ACR), neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury marker 1 (KIM-1). Multivariable regression analyses examined associations of glyphosate with kidney injury biomarkers controlling for covariates. Glyphosate was identified in 11.1% of the total participants. The herbicide was detected more frequently in the neonate population (30%). Multivariable regression models failed to identify significant associations of log-transformed glyphosate with any of the kidney injury biomarkers, controlling for covariates age, sex, and maternal education. While detectability of glyphosate in children's urine at various ages and stages of life was confirmed, there was no evidence for renal injury in children exposed to low levels of glyphosate.

Materials and methods

Study populations; We briefly describe a longitudinal birth cohort of children (Starting Early), in whom urine samples were measured at 10-19 months of age, as well as two cross-sectional studies Preventing Environmental Exposures in Pregnancy (PEEPS), and Bright Start which represent older children (ages 3-8) and newborns (<30 days), respectively. The three cohorts are comprised of chronologically ordered categories of age in children as follows: Bright Start (newborns), Starting Early, and PEEPS. Inclusion in the present study was determined if sufficient urine was available for the experimental testing. All research was performed in accordance with relevant guidelines/regulations. In particular, informed consent was obtained from participants' parents or their legal guardians for use of collected samples for use in future studies such as this one of glyphosate exposure.

Urine collection and storage; Urine samples for each cohort were obtained at study visits using age-appropriate methods (urine bags and cotton balls in untrained babies and infants, freshly voided urine in trained children) and immediately transferred to sterile polyethylene cups. Within 2 h after collection from the infants, urine was transferred to cryovials for storage at -80 °C prior to laboratory analysis. We measured urinary markers of kidney injury NGAL, and KIM1 using Luminex xMAP technology (see below). Urine albumin and creatinine were measured using standard methods of quantitative spectrophotometry at the ARUP National Reference Laboratory (Salt Lake City, Utah) and the results were used to calculate ACR (Albumin-to-Creatinine Ratio [mg:mg]).

Analysis of urine samples for glyphosate; 200 μL of urine sample was transferred into a 15 mL polypropylene (PP) tube and spiked with the labeled internal standard mixture (2-^{13}C, 99%; ^{15}N, 98+% Glyphosate), allowed to stand at room temperature for 30 min and then diluted to 1.0 mL (5-fold dilution) with 1% formic acid in water. The diluted sample was vortexed for 1 min, centrifuged and

filtered through a nonsterile regenerated cellulose (RC) membrane filters (0.2 mm; Phenomenex, Inc., Torrance, CA, USA). The filtrate then transferred into an auto sampler vial for LC-MS/MS analysis. An Agilent 1260 Series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with an ABSCIEX 4500 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) running under negative mode electrospray ionization was used for the analysis. An anion-exchange column, Dionex IonPac AS 21 (2 mm x 250 mm, 7 μm) was employed for the separation of target chemicals under isocratic elution condition with a mobile phase consist of 1% formic acid in water and acetonitrile (95:5) mixture. Isotopic dilution mass spectrometry with MRM mode of analysis was used for selective quantification of the target chemicals. The QA/QC protocols included matrix spike (mean spike recoveries (n=5) for glyphosate was 109.5%) and procedural blanks (non-detected or < LOQ). Midpoint calibration standard and HPLC grade water were injected between every 20 samples analyzed to check the instrument detection linearity and carry-over effects, respectively. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as three and ten times of signal to noise ratio, respectively. LOD for glyphosate was 0.1 ng/mL, and LOQ was 0.33 ng/mL.

Analysis of kidney injury biomarkers; NGAL was measured in human urine using the Luminex performance human kidney Biomarker kit (FCSTM16-01, R&D Systems). Urine samples were thawed and diluted 1:10 in calibrator diluent and protocol followed manufacturer's instructions. Data were captured on a Luminex MAGPIX instrument with xPonent 4.2 software. Standard curve R^2 values were 0.98 and high- and low-quality controls were within expected range. KIM-1 was measured in human urine using the Milliplex MAP Human Kidney Injury Magnetic Bead Panel Kit (HKI1MAG-99K, EMD Millipore). Urine samples were thawed and diluted 1:2 in assay buffer and protocol followed manufacturer's instructions. Data were captured on a Luminex MAGPIX instrument with xPonent 4.2 software. Standard curve R2 values were 0.97 and high- and low-quality controls were within the expected range.

Statistical analysis; We first described the demographics of our study population (N = 108), including age, sex, maternal education, race/ethnicity, BMI category, and intervention arm in the case of the Starting Early group. Body mass index (BMI), calculated as weight in kilograms divided by height in meters squared, was used to measure adiposity in the Starting Early group. We used standardized BMI *z* scores given that BMI varies widely by age and sex, following the Centers for Disease Control and Prevention year 2000 norms. Overweight and obese were categorized as BMI *z*-score of 1.036 or greater (85th percentile for age and sex) and 1.64 or greater (95th percentile), respectively. We calculated median glyphosate, albuminuria, KIM-1, and NGAL levels, as well as interquartile ranges for all biomarkers and percent detected for glyphosate levels by study group. We also described mean and standard deviation in each one of the mentioned biomarkers of interest in the total population. Univariate regressions examined log-transformed glyphosate concentrations and their detectability (using logistic regression) as dependent variables with covariates examined singly. We created dummy variables for maternal education with one category for those children whose mothers did not complete a high school education and another for those whose mothers at least completed high school, and created a dummy variable for missing values (N = 34) that we used as reference. We also created an ordinal variable reflecting the age of the children in increasing order from youngest to oldest type as follows: Healthy Start, Starting Early, and PEEPS (N ¼ 108). We also performed univariate analyses by study arm in the Starting Early sample (N = 66). Multivariable analyses of continuous log-transformed glyphosate exposure examined age, sex, and maternal education as predictors (N ¼ 108). We assessed differences in detectability of glyphosate using multivariable logistic regressions with age, sex, and maternal education as predictors. All regressions examining predictors of log-transformed glyphosate were retransformed from the logarithmic scale. We then examined associations of glyphosate with kidney injury. First, we calculated Pearson correlation coefficients for all log-transformed kidney injury markers KIM-1, NGAL, and ACR, as well as log-transformed glyphosate levels. For glyphosate levels below the limit of detection (LOD), we imputed values equal to LOD/square root of 2. We also performed univariate and multivariable regressions of urinary log-transformed glyphosate as predictor of log-transformed kidney injury markers KIM-1, NGAL, and ACR, controlling for age, sex and maternal education. Sensitivity analyses were also included; we performed univariate and multivariable regressions of creatinine-adjusted urinary log-transformed glyphosate as predictor of log-transformed kidney injury markers KIM-1, NGAL, and ACR. Statistical analysis was performed with Stata/IC Version 14 (StataCorp: College Station, TX).

Results

The population was largely Hispanic, and comprised roughly equal numbers of males and females (Table 1). Only 4% of our participants had a mother with a college degree but the majority of parents in our study population graduated from high school (62.1%). The mean urinary glyphosate concentration was 0.278 ± 0.228 µg/mL, with a range of 0.105-2.125 µg/mL. We identified glyphosate in 11.1% of the participants. The herbicide was detected in 7.6-30% of the three cohorts: 30% of neonates had glyphosate exposure above the LOD, followed by PEEPS cohort (12.5%) and Starting Early (7.6%). We also observed a wide detection range in NGAL biomarkers. We also noted that 40% of children in the Starting Early cohort were overweight or obese. Younger children had higher urinary levels of glyphosate in both univariate and multivariable regressions. Children with mothers without a high school diploma had slightly lower levels of urinary glyphosate relative to those with mothers who attained a high school education in our multivariable regression. However, we found no other significant associations of demographic covariates with glyphosate in univariate or multivariable models. Similarly, we found no statistically significant associations of demographic factors in relationship to detectability of urinary glyphosate in children. No univariate regressions with urinary glyphosate identified a statistically significant association with increased renal biomarker injury (Fig. 1A-C respectively and Table 2). Multivariable regressions with urinary glyphosate failed to identify statistically significant associations of log-transformed glyphosate with KIM-1, NGAL, or ACR (Table 2). In sensitivity analyses of univariate and multivariable regressions with creatinine-adjusted log-transformed glyphosate, there was also no statistically significant evidence for increased levels of markers for renal injury (Table 2). In additional sensitivity analyses without the newborns similar results were reported with no statistically significant associations of log-transformed glyphosate with kidney injury biomarkers (Table 2).

Table 1: Study population (n=108)[a]

Demographics			N	%
Male (Sex)			56	51.9
Study	Bright Start (Neonates)		10	9.3
	Starting Early		66	61.1
		Intervention Arm	39	59.1
		Control Arm	27	40.9
	PEEPS		32	29.6
Maternal Education	None		7	9.5
	Elementary/Middle		21	28.4
	High School/General Equivalency Diploma		35	47.3
	Some college		7	9.5
	College grad		3	4.1
	Other		1	1.4
Race/Ethnicity	Hispanic		67	90.5
	Non-Hispanic White		5	6.8
	Non-Hispanic Black		1	1.4
	Non-Hispanic Asian		1	1.4
BMI Categories[b]	Underweight		2	3.0
	Normal		40	60.6
	Overweight		12	18.2
	Obese		12	18.2

Distribution of Glyphosate and Kidney Injury Biomarkers	Mean (SD)	Detection Range (Min. & Max. Concentrations)
Glyphosate (ng/mL)	0.278 (0.228)	0.105–2.125
KIM-1 (ng/mL)	80.17 (76.92)	12.38–560.6
NGAL (pg/mL)	12,486 (21,934)	530.7–131,196
ACR[c] (mg/g)	26.23 (25.43)	1.414–119.0

Distribution of Glyphosate and Kidney Injury Biomarkers by Study Groups (N = 108)			
Study	Healthy Start (Neonates)	Starting Early	PEEPS
Glyphosate (ng/mL), Median (IQR)	<LOD (<LOD-1.06)	<LOD (<LOD-LOD)	<LOD (<LOD-LOD)
Percent Detected	30.0%	7.58%	12.5%
KIM-1 (ng/mL), Median (IQR)	88.9 (71.0–114)	47.1 (33.8–71.7)	76.5 (40.3–140.2)
NGAL (pg/mL), Median (IQR)	3770 (2520–7200)	9080 (3880–18,700)	1920 (1240–5770)
ACR[c] (mg/g), Median (IQR)	9.45 (6.00–34.3)	28.0 (19.1–42.4)	5.66 (3.54–9.00)

[a] Maternal Education 34 missing, Race/Ethnicity 34 missing, BMI 42 missing, KIM-1 9 missing, NGAL 13 missing, and ACR 12 missing.
[b] Underweight (Below − 1.036 SD); Normal (Between −1.036 and + 1.036 SD); Overweight (Between + 1.036 and + 1.64 SD); Obese (Above + 1.64 SD).
[c] Albumin-to-creatinine ratio.

Table 2: Multivariable regressions of creatinine-adjusted urinary log-transformed glyphosate as predictor of log-transformed KIM-1, NGAL, ACR biomarkers[a]

All ages N = 108 [a]			
Increment per one log unit increase	KIM-1	NGAL	ACR
Glyphosate Univariate	0.974 (−17.92, 27.78)	−663.8 (−2462, 2641)	−0.287 (−6.773, 10.91)
Glyphosate Multivariable	−1.55 (−14.93, 17.62)	−887.8 (−1800, 662.2)	−5.05 (−14.14, 9.85)
Creatinine-adjusted models[b]			
Glyphosate Univariate	−1,648 (−15.22, 17.47)	−640.5 (−3242, 4079)	7.603 (−9.522, 32.88)
Glyphosate Multivariable	−3.70 (−13.47, 10.26)	−819.8 (−1688, 657.8)	0.858 (−13.80, 22.43)
Non-neonates N = 98[c]			
Increment per one log unit increase	KIM-1	NGAL	ACR
Glyphosate Univariate	−5.351 (−24.13, 27.39)	1489 (−4383, 17944)	3.002 (−10.66, 35.99)
Glyphosate Multivariable	−3.568 (−14.90, 15.96)	395.3 (−5128, 12782)	12.05 (−37.98, 106.6)
Creatinine-adjusted models[d]			
Glyphosate Univariate	−4.89 (−20.66, 22.43)	2083 (−5679, 23234)	8.24 (−16.60, 54.17)
Glyphosate Multivariable	−3.73 (−14.45, 14.88)	132.8 (−5165, 11668)	10.37 (−25.33, 72.33)

[a] KIM-1 N = 99; NGAL N = 95; ACR N = 96. Multivariable regression models controlling for demographic covariates age, sex, and maternal education, where maternal education missing observations were imputed as the majority category "Equal or Greater Than High School".
[b] Creatinine-adjusted regression models KIM-1 N = 95; NGAL N = 92; ACR N = 96.
[c] KIM-1 N = 89; NGAL N = 86; ACR N = 86. Multivariable regression models controlling for demographic covariates age, sex, and maternal education, where maternal education missing observations were imputed as the majority category "Equal or Greater Than High School".
[d] Creatinine-adjusted regression models KIM-1 N = 85; NGAL N = 83; ACR N = 86.

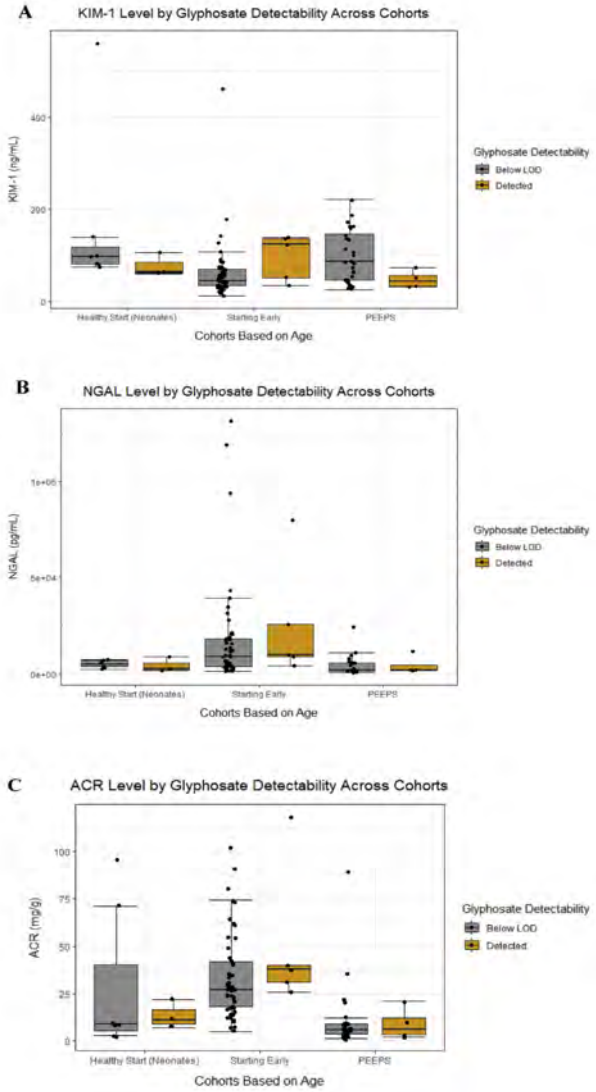

Figure 1: A. The dot plot illustrates the urinary KIM-1 level in participants enrolled in the different studies, 1B. The dot plot illustrates the urinary NGAL level in participants enrolled in the different studies, 1C. The dot plot illustrates the urinary albumin:creatine ratio (ACR) in participants enrolled in the different studies.

Discussion

The main findings of this study are the substantial and frequent prevalence of glyphosate in children across the first decade of life in three independently assembled samples. We identified glyphosate in 11.1% of the total participants, and the herbicide was detected more frequently in the neonate population (30%). However, urinary concentrations detected in the neonates, toddlers, and young school age children was significantly lower than levels documented in adults with occupational or environmental exposure. No association of urinary glyphosate was found with any of the three renal biomarkers (KIM-1, NGAL or ACR). Urinary excretion of albumin and serum creatinine concentration are conventional biomarkers for renal dysfunction. Novel biomarkers such as urinary excretion of KIM-1 and NGAL have been developed and it has been that they have diagnostic utility for earlier detection of acute kidney injury (AKI) prior to significant loss of function. KIM-1 also predicted renal damage from acute exposures in animal studies. This study has a number of limitations.

We used maternal education as a proxy for family educational attainment or socioeconomic status (SES) which are well-known factors for different levels of chemical exposures in children or renal injury outcome. We emphasize that low sample size also limited us to perform analyses in the three cohorts in conjunction, in lieu of stratified analysis. We acknowledge that the strength of any conclusion about the frequency of detecting glyphosate in newborns is limited by the sample size. The source of the glyphosate in the newborns, which was detected more frequently than in the other groups but not at high levels, could be via lactation or infant formula. It is possible that immature or different metabolic pathways for glyphosate in neonates, kidney injury during delivery, or lactation may have affected the findings in this age group. However, the addition of neonates in our study evaluating the association of glyphosate and kidney injury biomarkers may have biased findings towards the null. Our results also suggest that glyphosate levels declined with increasing age. The differences by age may represent differences in dietary behavior and access to foods free of contamination or improved elimination of the herbicide, though further research is needed. Previous larger scale case-control studies have shown in the past changes in renal function, kidney injury, or chronic kidney disease of unknown etiology (CKDu) upon glyphosate exposure, especially of adult occupational exposures. Several strengths in this study are notable. Our study is the first to document the prevalence of glyphosate exposures in young children through age 8 years. Our frequent detection of glyphosate suggests the need for inclusion and biomonitoring of glyphosate exposures in national surveys or screenings in populations of interest, which could provide more information in regards to these exposures and outcomes. Glyphosate biomonitoring could be added into studies such as the National Health and Nutrition Examination Survey (NHANES). Our study is novel in that we examine specific renal biomarkers in relationship to glyphosate in a young population. The lack of evident renal toxicity in association with glyphosate exposure in young children does not exclude a potential adverse impact of longer term exposure to the pesticide. Problems during labor and delivery can compromise renal perfusion leading to increased excretion of tubular injury biomarkers in the neonatal period. This could interfere with the ability to detect an independent effect of glyphosate in this age group. In addition, the regenerative capacity of the renal tubular epithelium in infants and young children may mask an adverse effect of the pesticide on tubule integrity. Urinary biomarkers such as NGAL and KIM-1 have been demonstrated to be useful biomarkers of tubular injury even without reduced kidney function, highlighting their utility in detecting subclinical insults to the kidney. Further research could explore more in detail the association between glyphosate and kidney injury biomarker types across different age groups in larger cohorts, and assess whether more prolonged exposure could have progressive deleterious effects.

Conclusion

In this study, we identify frequent prevalence of levels of glyphosate (11.1%), present across all three age ranges, and particularly present in the youngest population of neonates. There is no evidence for renal injury in young children exposed to low levels of glyphosate. Further studies of larger sample size are indicated to better understand the potential deleterious effects of the herbicide after different levels, routes, and duration of exposure.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This study evaluated three cohorts across different phases of child development and measured urinary levels of glyphosate. They evaluated associations of glyphosate with three biomarkers of kidney injury: ACR, NGAL, and KIM-1. Sample collection and analysis as well as statistical evaluation of data have been conducted using well described methodologies. Multivariable regression models failed to identify significant associations of log-transformed glyphosate with any of the kidney injury biomarkers, controlling for covariates age, sex, and maternal education. The authors confirm detectability of glyphosate in children's urine at various ages and stages of life, there is no evidence in this study for renal injury in children exposed to low levels of glyphosate.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good monitoring study.

Reliability criteria of exposure studies

Publication: Trasande et al., 2020.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	Y	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.	Y	
Exposure to formulations with glyphosate combined with other a.i.	Y	
Exposure to various formulations of pesticides	Y	Environmental exposure.
Study		
Study design clearly described	Y	Longitudinal birth cohort and 2 cross sectional studies. Study of the association between renal biomarkers and glyphosate in urine.
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	Environmental exposure.
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Statistical analysis	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with the quality criteria of a good monitoring study.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Vanlaeys A. *et al.*
Report year	2018
Report title	Formulants of glyphosate-based herbicides have more deleterious impact than glyphosate on TM4 Sertoli cells
Document No	Toxicology in Vitro (2018) Vol. 52, 14–22
Guidelines followed in study	None
Deviations from current test guideline	NA
Previous evaluation	No
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Roundup and Glyphogan are glyphosate-based herbicides containing the same concentration of glyphosate and confidential formulants. Formulants are declared as inert diluents but some are more toxic than glyphosate, such as the family of polyethoxylated alkylamines (POEA). In this study glyphosate alone, glyphosate-based herbicide formulations and POEA on the immature mouse Sertoli cell line (TM4) was tested, at concentrations ranging from environmental to agricultural-use levels.

Materials and methods

Chemicals - Glyphosate was purchased from Sigma-Aldrich, St Louis, USA. The purity was not reported.

Culture of TM4 Sertoli cells and treatments - The murine TM4 Sertoli cell line was obtained from the American Type Culture Collection (ATCC Manassas, USA). Cells were maintained in DMEM/HamF12 medium containing 0.2% glutamine, 1.2 g/L $NaHCO_3$, 15mM Hepes, 5% horse serum and 2.5% fetal calf serum, 100 U/mL of antibiotics and fungizone (complemented DMEM/Ham F12 medium) at 37 °C (5% CO2, 95% air) during 24 hours to 80% confluence in 24-well plates or in 6-well plates (for measurement of GST activity). Cells were then exposed to various concentrations of glyphosate.

Crystal violet cell viability assay - After incubation with glyphosate at different concentrations, culture supernatants were discarded and cells incubated in medium containing crystal violet solution (0.1% w/v in PBS 0.01 M, pH 7.4) for 30 minutes at 20 °C with gentle rocking. Excess dye and non-adherent dead cells were removed through 3 washing steps with PBS. Diluted acetic acid solution (10%) was then added to release the crystal violet taken up by cells, and the optical density reflecting living adherent cells was determined by absorption at 600 nm using a plate reader.

MTT cytotoxicity assay - This enzymatic test is based on the cleavage of MTT (tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a blue-colored product (formazan) by the mitochondrial enzyme succinate dehydrogenase. Activity of mitochondrial dehydrogenase enzymes indirectly measures activity of mitochondrial respiration and antioxidant defense systems. Culture medium was removed, and cells were washed once with PBS and then incubated with 500 µL MTT solution per well after each treatment. The plates were incubated for 3 hours at 37 °C. The reaction was stopped by placing the plates for 10 minutes at 4 °C followed by adding to each well 500 µL 0.04 N hydrochloric acid-containing isopropanol. The plates were then vigorously shaken for 40 minutes to solubilize the blue formazan crystals formed. Absorbance was measured by spectrophotometry at 570 nm.

Glutathione-S-transferase (GST) activity - For preparation of S9 fractions enriched in GST, the medium was removed, and cells were detached by treatment with trypsin-EDTA and washed twice with PBS at room temperature. Cells were then resuspended in 500 µL of 50 mM phosphate buffer at pH 7.2 containing 0.25 M sucrose and 1mM DTT. Then, they were homogenized and centrifuged at 9000g at

4 °C for 30 minutes. Supernatants corresponding to the S9 fraction were collected and stored at -80 °C. The protein concentration of each S9 fraction was determined using the Bradford assay. For the determination of GST activity 250 µg of S9 cell fraction was mixed with 10 µL of 100 mM reduced L-glutathione and 990 µL phosphate buffer (pH 6.5). The reaction was initiated by the addition of 10 µL of 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution in 95% ethanol. After incubation for 90 seconds at 37 °C absorbance was measured at 340 nm every 60 seconds for 3 minutes.

Lipid Sudan Black B staining - Sudan Black B stains lipids, including phospholipids and sterols. Culture medium was removed and cells were washed once with PBS. Cells were then incubated for 5 minutes at room temperature with 500 µL of Sudan Black solution with gentle shaking. This solution was removed, and cells were washed 3 times or more with 70% ethanol to remove excess stain and then at least 3 times with PBS. Then, intracellular Sudan Black B was extracted by incubation with DMSO for 30 minutes with gentle rocking and absorbance measured at 600 nm using a microplate reader.

Statistical analysis - The experiments were repeated at least in triplicate in different weeks on three independent cultures on each occasion (n=9). All data are presented as the mean ± standard error (SEM). Statistical differences from controls were determined by an ANOVA test. Results were statistically significantly different from controls when $p < 0.05$.

Results

Viability of TM4 cells - Glyphosate was found to have no impact on cell viability of TM4 cells after 24 hours of exposure at concentrations ranging from 10 to 10,000 ppm.

Mitochondrial succinate dehydrogenase activity - The measurement of succinate dehydrogenase (SD) activity was used to assess the effect of glyphosate on mitochondrial function and viability after 24 hours of exposure. Reduced SD activity was seen for glyphosate over the entire concentration range from 0.001% (approx. 85%) to 1% (approx. 75%) as derived from the graph.

Inhibition of glutathione-S-transferase (GST) activity – The effect of glyphosate on GST activity involved in the anti-xenobiotic defense system was evaluated. At an LC_{50} concentration glyphosate was found to have no impact on GST activity.

Lipid droplet accumulation - After staining with Sudan Black B exposure of TM4 cells to glyphosate for 24 hours at 2,500 or 5,000 ppm induces an increase in cytoplasmic lipid droplets as assessed microscopically.

Discussion and conclusions

Measurements of cell viability, respiratory chain activity, detoxification system and lipid accumulation were undertaken in immature murine TM4 Sertoli cell line following 24 hours of exposure to glyphosate at concentrations ranging from environmental levels to agricultural use concentrations (1%, 10,000 ppm). This study demonstrated that at sub-agricultural use levels (10-10,000 ppm) TM4 cell viability is not affected by glyphosate. Exposure of TM4 cells to glyphosate reduces mitochondrial succinate dehydrogenase (SD) activity at the lowest concentrations tested in TM4 cells, as compared with other cell types. The formulants present in commercial herbicides are known to increase glyphosate penetration into cells by membrane disruption and thus probably potentiate perturbation of mitochondrial permeability induced by glyphosate. This mechanism may explain the higher toxicity of formulations on mitochondrial activity. In this study, we also demonstrated that 24 hours exposure of TM4 cells to glyphosate induced lipid droplet accumulation.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study the effect of glyphosate on murine TM4 Sertoli cells was investigated *in vitro*. The endpoints were cytotoxicity, glutathione transferase activity and lipid accumulation. In contrast to the glyphosate-based formulations and co-formulants tested glyphosate was found to have no impact on cell viability after 24 hours of exposure at concentrations ranging from 10 ppm to 10,000 ppm.

Glyphosate reduced succinate dehydrogenase to some extent over the entire concentration range from 10 (approx. 85% of control) to 10,000 ppm (approx.75% of control) with no dose-effect relationship and was found to have no impact on glutathione transferase activity. Exposure of TM4 cells to glyphosate for 24 hours at 2,500 or 5,000 ppm induces an increase in cytoplasmic lipid droplets. These concentrations are far beyond what is physiologically feasible *in vivo*.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterized and no positive controls were used in any of the assays conducted.

Reliability criteria for *in vitro* toxicology studies

Publication: Vanlaeys *et al*., 2018	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also co-formulants and formulations were tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y (partly)	
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive controls used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterized and no positive controls were used in any of the assays conducted.		

1. Information on the study

Data point:	CA 5.5
Report author	Wang L. *et al.*
Report year	2019
Report title	Glyphosate induces benign monoclonal gammopathy and promotes multiple myeloma progression in mice
Document No	Hematol Oncol 12, 70 (2019)
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate is the most widely used herbicide in the USA and worldwide. There has been considerable debate about its carcinogenicity. Epidemiological studies suggest that multiple myeloma (MM) and non-odgkin lymphoma (NHL) have a positive and statistically significant association with glyphosate exposure. As a B cell genome mutator, activation-induced cytidine deaminase (AID) is a key pathogenic player in both MM and B cell NHL. Vk*MYC is a mouse line with sporadic MYC activation in germinal center B cells and considered as the best available MM animal model. Vk*MYC mice and wild-type mice were treated with drinking water containing 1000 mg/L of glyphosate and examined animals after 72 weeks. Vk*MYC mice under glyphosate exposure developed progressive hematological abnormalities and plasma cell neoplasms such as splenomegaly, anemia, and high serum IgG. Moreover, glyphosate caused multiple organ dysfunction, including lytic bone lesions and renal damage in Vk*MYC mice. Glyphosate-treated wild-type mice developed benign monoclonal gammopathy with increased serum IgG, anemia, and plasma cell presence in the spleen and bone marrow. Finally, glyphosate upregulated AID in the spleen and bone marrow of both wild-type and Vk*MYC mice. Conclusions: These data support glyphosate as an environmental risk factor for MM and potentially NHL and implicate a mechanism underlying the B cell-specificity of glyphosate-induced carcinogenesis observed epidemiologically.

Materials and methods

Mouse model and treatments; All chronic and acute animal experiments were performed in accordance with NIH guidelines and under protocols approved by the Cleveland Clinic Institutional Animal Care and Use Committee. Wild-type (WT) C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Vk*MYC mice in the C57Bl/6 genetic background were obtained from Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ). Vk*MYC and WT mice were intercrossed to obtain WT and Vk*MYC littermates. Sex-matched WT and Vk*MYC mice (8 weeks old) were assigned to treatment or control groups based on body weight. For chronic study of glyphosate effects, treatment groups were provided 1.0 g/L glyphosate (Sigma-Aldrich, St. Louis, MO) in their drinking water for 72 weeks. Regular drinking water was provided for the control groups (Fig. 1a). Every 6 weeks, blood was collected from the tail vein of mice, and the serum IgG level was measured. At week 72, the remaining 3 surviving Vk*MYC mice reached humane endpoints. These 3 treated Vk*MYC mice were used for M-spike detection and pathologic analyses, along with mice from other groups. Other Vk*MYC mice that were sacrificed before week 72 were analysed for total serum IgG levels, complete blood cell count, and total serum creatinine. For comparison, mice from other groups were euthanized at week 72 and their tissues and blood analyzed. For acute treatment, 8-week old mice (n = 5 per group) were given 0, 1.0, 5.0, 10.0, or 30.0 g/L of glyphosate for 7 days before sacrifice. The same variables were analyzed in the acute study.

Blood and post-mortem assays; Whole-blood complete blood count (CBC), IgG enzymelinked immunosorbent assay (ELISA), serum protein electrophoresis, flow cytometry, and histological examinations of relevant tissues were performed as described previously. Serum creatinine was measured by ELISA using a creatinine assay kit (#ab65340, Abcam, Cambridge, MA) according to the manufacturer's protocol.

Western blotting analyses; Mouse tissues were processed for Western blotting. The antibodies were from Cell Signaling Technology (Danvers, MA, USA): AID (L7E7) (#4975) and β-actin (#3700). Blotting was run with 3 technical replicates. Horseradish peroxidase- conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody.

Statistics; Statistical analysis was carried out using GraphPad InStat 3 software (GraphPad Software, Inc., San Diego, CA, USA). The statistical significance between the groups was determined by one-way or two-way analysis of variance (ANOVA) with the appropriate post hoc testing using Tukey's test. Statistical significance was accepted at $P \leq 0.05$. All data are shown as mean ± SEM unless otherwise indicated.

Results

*Chronic glyphosate exposure reduces survival and induces splenomegaly in Vk*MYC mice*; Eight-week-old Vk*MYC mice and their WT littermates were provided 1.0 g/L glyphosate in drinking water for 72 weeks, and animals were monitored at regular intervals before sacrifice (Fig. 1a). Glyphosate significantly affected the health of Vk*MYC mice, all of which had to be euthanized by week 72 (Fig. 1b). Surviving mice in other groups were sacrificed at week 72 (at age 80 weeks) for necropsy. Inspection of organs revealed a marked increase in spleen weight and size in Vk*MYC mice treated with glyphosate compared to the other 3 groups (Fig. 1c, e). Glyphosate significantly augmented the splenocyte number in Vk*MYC mice (Fig. 1d). Findings indicate that glyphosate induces splenomegaly in both WT and Vk*MYC mice.

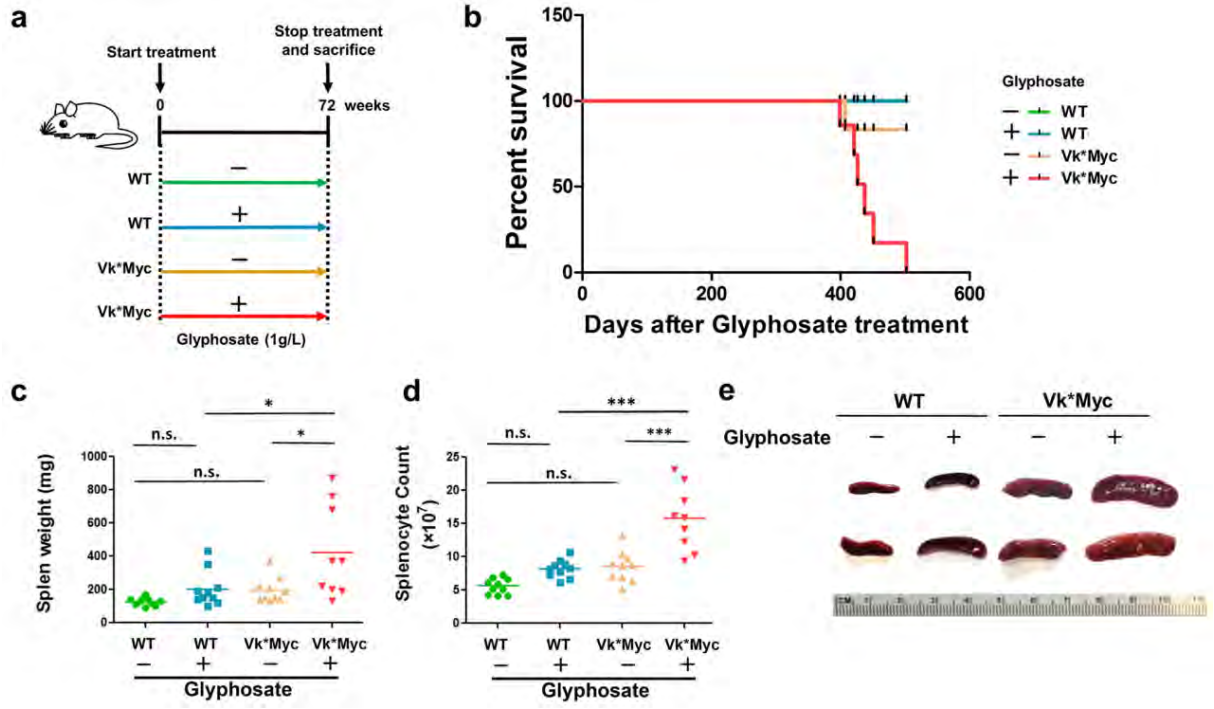

Fig. 1: Glyphosate reduced survival and induced splenomegaly in Vk*MYC mice. a Schematic diagram of the chronic glyphosate exposure regimen in 4 groups of mice. b The percentage of mice surviving under glyphosate exposure. The line (blue) to indicate untreated WT mice aligned directly with that for WT treated mice and so was not visible. c Mouse spleen weight at sacrifice. d The total number of splenocytes per spleen from mice at sacrifice. e Representative images of spleens from 4 groups (2 per group).

*Hematological abnormalities occur in Vk*MYC mice with chronic glyphosate exposure*; As illustrated in Fig. 2a, untreated Vk*MYC mice exhibited higher IgG levels than untreated WT mice. Upon glyphosate exposure, WT mice showed moderate yet steady increasing in IgG levels, suggesting that

glyphosate induces benign monoclonal gammopathy, a mouse equivalent to human MGUS. Vk*MYC mice receiving glyphosate had greater IgG elevation, and by week 30, IgG levels jumped to 11.78 g/L, more than 5-fold the 2.07 g/L observed in untreated Vk*MYC mice. From week 36 to week 72, the mean IgG level was significantly higher in treated WT and Vk*MYC mice compared to the untreated control groups, and Vk*MYC mice, treated or untreated, had higher IgG levels than their WT counterparts. Overt MM diagnosis was determined by serum protein electrophoresis (SPEP) analysis to detect the M-spike, which is a significant IgG monoclonal peak. SPEP results showed that Vk*MYC mice treated with glyphosate had a clear M-spike, whereas weaker M-spike was observed in glyphosate-treated WT mice. No clear M-spike was present in the untreated WT mice or Vk*MYC mice (Fig. 2b). This is the direct in vivo evidence that glyphosate exposure leads to M-spike, a cardinal hematological abnormality consistent with MM. Hematological abnormalities were present in glyphosate treated mice as compared to untreated control mice (Fig. 2c–i). Data support the notion that glyphosate induces multiple hematological abnormalities characteristic of MM in mice.

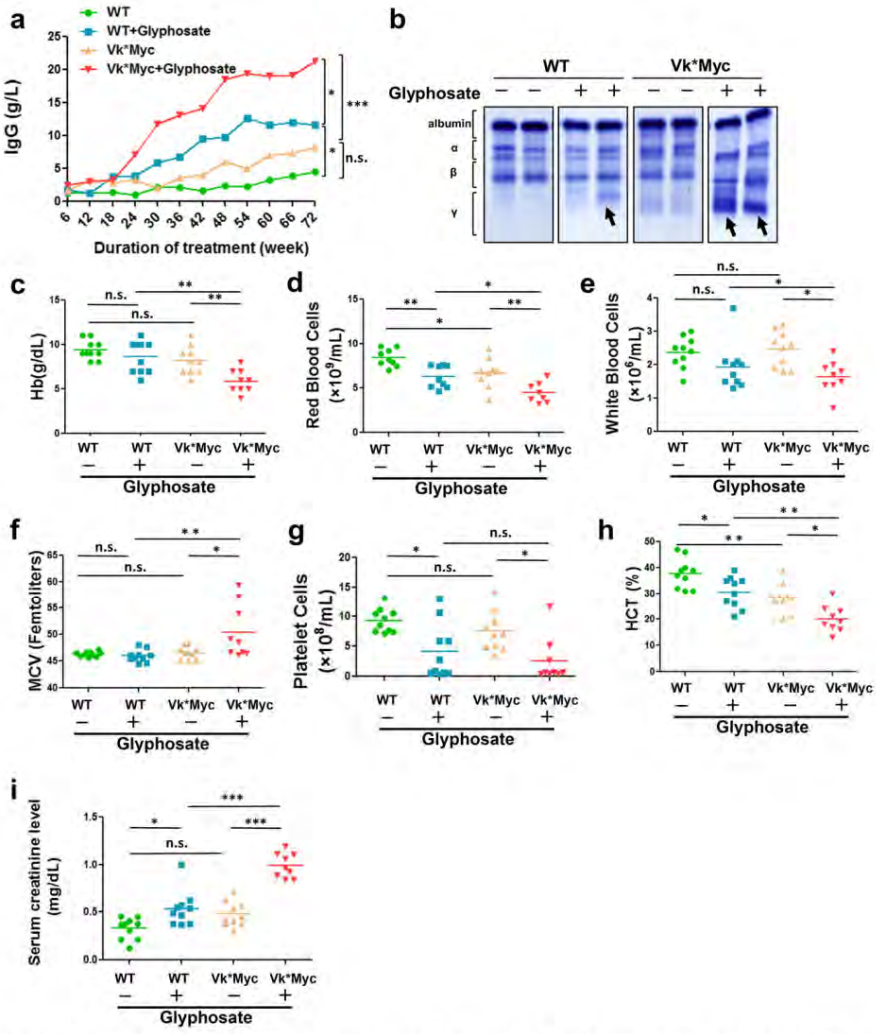

Fig. 2. Hematological abnormalities found in Vk*MYC mice treated with glyphosate. **a** Total serum IgG in mice during 72 weeks of glyphosate treatment. Mouse blood samples were collected and assayed for IgG every 6 weeks. **b** Immunoglobins from mice as determined by SPEP at week 72. Arrows indicate IgG clonal peaks (M-spike; γ-globulin peak). SPEP was performed for all mice in each group, and representative results of 2 mice per group are shown. **c**–**h** Complete blood cell counts in mice. Hemoglobin concentration (Hb, **c**), red blood cell count (**d**), white blood cell count (**e**), mean red cell volume (MCV, **f**), platelet cell count (**g**), and hematocrit (HCT, **h**) are shown. **i** Total serum creatinine in mice at week 72. The horizontal lines indicated the mean value. Data were analyzed by two-way ANOVA (**b**) or one-way ANOVA (**a**, **d**, **e**). $n = 10$ mice per group

*Vk*MYC mice chronically exposed to glyphosate develop progressive plasma cell neoplasms;* Plasma cells exhibit CD138hi B220–(high CD138 expression without B220 expression). Flow cytometric analyses of cells harvested from the spleens and bone marrow showed expansion of plasma cells in mice under glyphosate exposure. A marked increase in the numbers of CD138hi B220– cells was detected in both WT and Vk*MYC mice treated with glyphosate (Fig. 3a). These data demonstrate that glyphosate treatment expands the plasma cell population in the spleen and bone marrow in both WT and Vk*MYC mice.

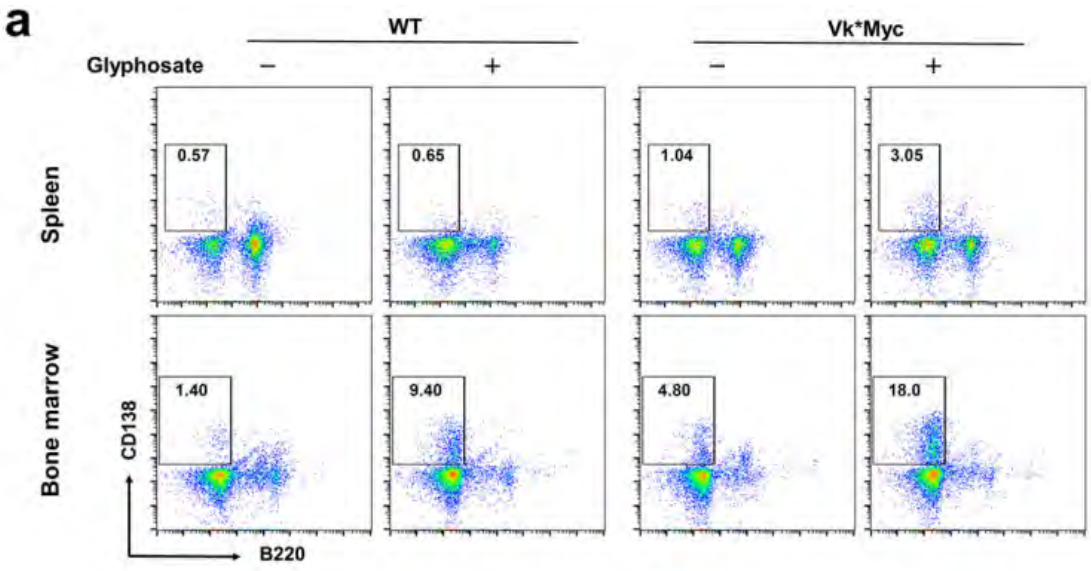

Fig. 3 Glyphosate-treated Vk*MYC mice developed progressive plasma cell neoplasms. **a** Representative flow cytometry plots detecting cell surface markers CD138 (*Y*-axis) and B220 (*X*-axis) in splenocytes (upper panel) and bone marrow cells (lower panel). The numbers on the axes denoted the $\log_{10}$ values of fluorescence. The numbers in the inserts show the percentage of $CD138^{high}B220^{-}$ cells in the entire cell population.

Chronic glyphosate exposure triggers multiple organ dysfunction; To determine whether target organ damage occurred in glyphosate-treated mice, the femoral shaft, spleen, liver, lung, and kidney were sectioned and stained with hematoxylin and eosin (H&E). Severe destructive osteolytic bone lesions in the femoral shaft were readily detectable in glyphosate-treated Vk*MYC mice. Treated WT mice showed smaller bone lesions. No lesions were observed in the control groups (Fig. 4a). Plasma cells with a perinuclear clear zone and eccentric round nucleus were observed in glyphosate-treated WT and Vk*MYC mice (Fig. 4b, c). Next, the histopathologic changes in the liver, lung, and kidney were analyzed. In glyphosate-treated mice, hepatic fibrosis and collagen deposition were observed in Vk*MYC mice, whereas WT mice showed less severe liver damage; the 2 control groups had normal hepatic tissue architectures (Fig. 4d). The lungs in treated Vk*MYC mice were severely damaged, with large distal air spaces filled by lymphocytes, neutrophils, cell debris, and hyperplastic pneumocytes; those from untreated WT mice had normal alveolar spaces and alveolar septa lined with normal pneumocytes. The lungs from treated WT mice and untreated Vk*MYC mice showed an intermediate phenotype (Fig. 4e). Renal tubular obstruction by large casts, indicative of necrotic tubular cells, were detected in glyphosate-treated WT and Vk*MYC mice, but not in the untreated groups; there were more and larger casts in treated Vk*MYC kidneys than in WT kidneys (Fig. 4f). Taken together, these data indicate that glyphosate treatment damages multiple organs in both WT and Vk*MYC mice with more severe damage occurring in Vk*MYC mice.

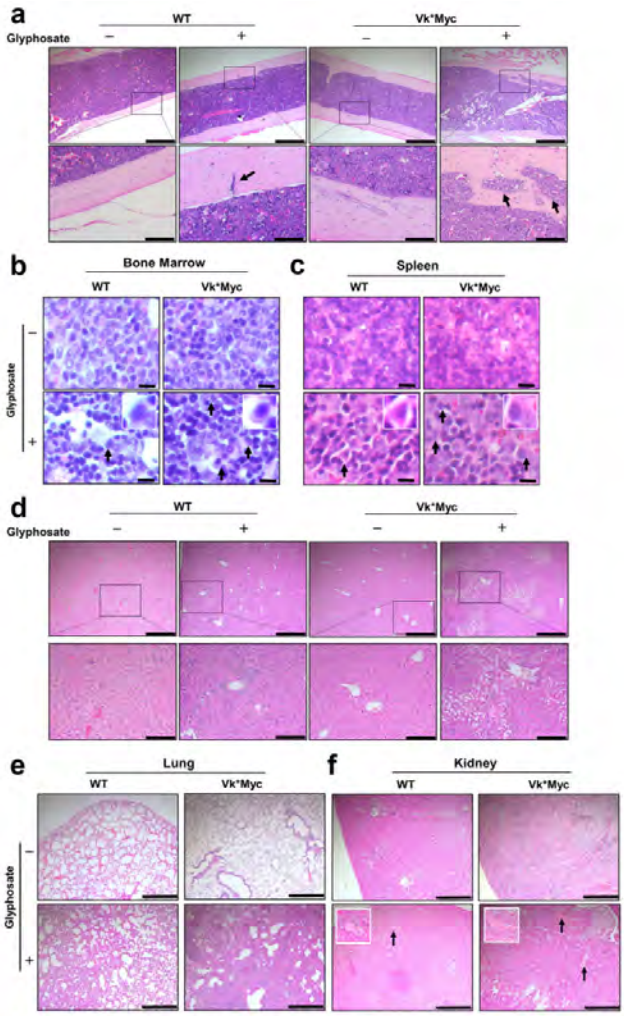

Fig. 4 Glyphosate led to multiple organ dysfunction. **a** Histological evaluation of bone morphology from 4 groups of mice. Bone lytic lesions (indicated by arrows) were detected in the femoral shaft of Vk*MYC mice treated with glyphosate. Scale bar = 500 μm (top) or 100 μm (bottom). **b** Infiltrating plasma cells in the bone marrow of glyphosate-treated mice. Scale bar = 20 μm. Arrows pointed to plasma cells. **c** Infiltrating plasma cells in the spleen of glyphosate-treated mice. Scale bar = 20 μm. Arrows point to plasma cells. **d** Collagen deposition in the liver was observed in glyphosate-treated Vk*MYC mice. *n* = 10 mice per group. Scale bar = 500 μm (top) or 200 μm (bottom). **e** Destruction of lung morphology was observed in glyphosate-treated Vk*MYC mice. *n* = 10 mice per group. Scale bar = 500 μm. **f** Protein deposition (indicated by arrows) in the kidney was observed in glyphosate-treated Vk*MYC mice. *n* = 10 mice per group. Scale bar = 500 μm. All panels show 1 representative image each from 4 groups of mice unless otherwise indicated

Chronic glyphosate exposure induces AID upregulation; To investigate the underlying mechanisms of glyphosate-mediated MGUS induction and MM progression, expression of activation-induced cytidine deaminase (AICDA, also known as AID) in mice treated with 1.0 g/L glyphosate for 72 weeks was examined. It was found that AID was upregulated in both the bone marrow and the spleen of WT and Vk*MYC mice (Fig. 5a).

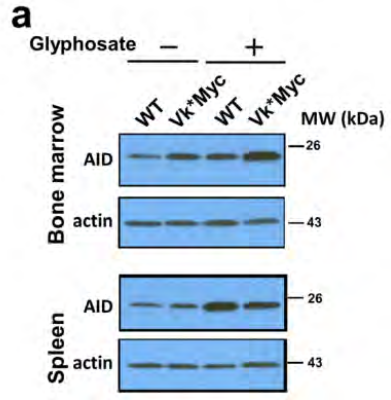

Fig. 5 Glyphosate-induced AID upregulation. **a** Western blotting analysis of mice treated with 1.0 g/L of glyphosate for 72 weeks.

Acute glyphosate exposure induces AID upregulation; To determine the acute effect of glyphosate, 8-week-old WT and Vk*MYC mice were treated with increasing doses of glyphosate (1, 5, 10, and 30 g/L) in drinking water for 7 days. This acute treatment neither increased spleen weight nor affected body weight significantly. Next, expression of AID in the spleen, bone marrow, and lymph nodes were analysed. It was found that AID was upregulated in a glyphosate dose-dependent manner in the spleen and bone marrow of WT and Vk*MYC mice treated with 10 and 30 g/L of glyphosate (Fig. 5c). Given the role of AID in MM pathogenesis in the context of its capacity to induce mutations and chromosome translocations, these results from mice with chronic and acute glyphosate treatment support an AID mediated mutational mechanism in the etiology of MGUS and MM under glyphosate exposure.

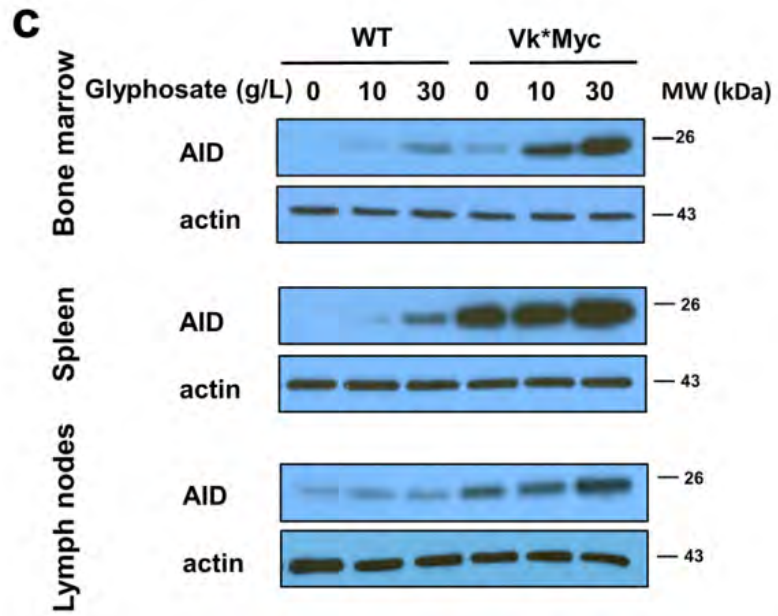

Fig. 5 c Western blotting analysis of mice treated with glyphosate for 7 days. One representative mouse

Discussion

In this study, it was demonstrated that glyphosate induces benign monoclonal gammopathy (mouse equivalent to MGUS in human) in WT mice and promotes MM progression in Vk*MYC mice. In Vk*MYC mice, glyphosate causes hematological abnormalities like anemia and multiple organ dysfunction like lytic bone lesions and renal damage, which are hallmarks of human MM. Beyond epidemiology and animal models, the mechanism of action is the third pillar required to define a compound as a carcinogen. Numerous studies have revealed that glyphosate may induce DNA damage, oxidative stress, inflammation, and immunosuppression, as well as modulate cell proliferation and death and disrupt sex hormone pathways. However, these mechanistic studies have failed to explain why glyphosate exposure is only positively associated with MM and NHL. Our results demonstrate that glyphosate treatment, either at a chronic low dose or acute high doses, upregulates the expression of AID in the bone marrow and spleen of both WT and Vk*MYC mice. The data disclose, for the first time, that glyphosate elicits a B cell-specific mutational mechanism of action in promoting carcinogenesis, as well as offering experimental evidence to support the epidemiologic finding regarding its tissue specificity in carcinogenesis (i.e., only increasing the risk for MM and NHL). The "acceptable daily intake (ADI)" of glyphosate currently allowed in the USA, defined as the chronic reference dose as determined by EPA, is 1.75 mg/kg body weight/day; an average adult male or female in the USA who weighs 88.8 or 76.4 kg and drinks 2 L (8 glasses) water daily containing 77.7 (for male) or 66.9 (for female) mg/L glyphosate would reach the ADI. A dose of 1,000 mg/L glyphosate in drinking water (~ 15-fold the ADI) was chosen in this study, which caused significant adverse effects and accelerated MM progression in Vk*MYC mice, i.e., animals predisposed to MM.

Conclusion

The data provide in vivo evidence to support that glyphosate induces MGUS and promotes disease progression to MM. A B cell-specific mutational mechanism for glyphosate exposure that increases MM and NHL risk was uncovered, providing a molecular basis for human epidemiological findings.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to investigate the pathogenic role of glyphosate in multiple myeloma using Vk*MYC mice. The study did demonstrate the ability of glyphosate to impact measured parameters in the tested models. However, this study is not appropriate for human health risk assessment. The number or animals per group was below the recommended number for guideline toxicity studies and to perform sufficient statistical analysis. Only one dose level was used in the chronic study. It was not possible to correlate effects with a glyphosate dose-response as the water consumption of animals was not provided and is therefore impossible to calculate a dose on a mg/kg bw basis for risk assessment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not characterized, only one dose was considered for the chronic study and the number of animals used per group was either too low (acute study) or not reported (chronic study).

Reliability criteria for *in vivo* toxicology studies

Publication: Wang *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance		
AMPA is the tested substance		
Study		
Test species clearly and completely described	Y	Wild-type (WT) C57Bl/6 mice and Vk*MYC mice.
Test conditions clearly and completely described		
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	For the chronic study: 1 g/L in drinking water for 72 weeks.

		For the acute study: 1, 5, 10, 30 g/L for 7 days.
Number of animals used per dose level reported	Y	5/group for the acute study, no/group not reported for the chronic study.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	Not possible. Only one dose level used.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not characterized, only one dose was considered for the chronic study and the number of animals used per group was either too low (acute study) or not reported (chronic study).		

1. Information on the study

Data point:	CA 5.5
Report author	Wozniak E. *et al.*
Report year	2020
Report title	Glyphosate affects methylation in the promoter regions of selected tumor supressors as well as expression of major cell cycle and apoptosis drivers in PBMCs (in vitro study)
Document No	Toxicology in Vitro 63 (2020)
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

The effect of glyphosate on selected epigenetic parameters and major cell cycle drivers in human peripheral blood mononuclear cells (PBMCs) was determined. The cells were incubated with glyphosate at 0.5, 10 and 100 μM. The analysis included: global DNA methylation, methylation in the promoter regions of tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1) by the Real-Time PCR and the expression profile of the indicated genes by Real-Time PCR. The obtained results have revealed significant reduction of global DNA methylation level in PBMCs exposed to glyphosate. Tested compound changed methylation pattern of the P21 and TP53 suppressor gene promoters, but in case of other analyzed genes: P16, BCL2 and CCND1 we did not identify any statistically significant changes. Gene profiling showed that glyphosate changed the expression of genes involved in the regulation of cell cycle and apoptosis. Glyphosate decreased expression of P16 and TP53 as well as an increase in the expression of BCl2, CCND1 and P21. Summing up, our results have shown a potential disturbance in methylation processes and gene expression in human PBMCs exposed to glyphosate, but the observed changes do not prejudge about the final metabolic effects, which are depended on many other factors.

Materials and methods

Chemicals; N-(phosphonomethyl)glycine (glyphosate) (purity 95%), fetal bovine serum (FBS), penicillin - streptomycin, TRIzol™ and using primers were bought from Sigma-Aldrich, (USA). RPMI 1640 medium with L-glutamine and lymphocyte separation medium (LSM) (1.077 g/cm^3) were purchased in Cytogen (Germany). Invisorb Spin Tissue Mini Kit was bought in Stratec (Germany). Methylated DNA Quantification Kit was bought in Abcam (United Kingdom). Transcriptor First Strand cDNA Synthesis Kit and FastStart Essential DNA Green Master was purchased from Roche (Basel, Switzerland). EZ DNA Methylation™ Kit was bought from Zymo Research (USA). Methyl Primer Express®, v.1.0 was obtained from Life Technologies. TRIzol™ Reagent was purchased from Thermo Fischer Scientific, Waltham, MA, USA. Other chemicals were from Roth (Germany) and POCh (Poland) and were of analytical grade.

Cells isolation; PBMCs were isolated from leucocyte-buffy coat obtained from blood purchased in Blood Bank in Lodz, Poland. Blood was obtained from four healthy volunteers (aged 18–55), who showed no signs of infection disease symptoms at the time the blood samples were collected. The investigation was approved by the Bioethics Committee of the University of Lodz No. 1/KBBN-UŁ/II/2017. Cells isolation was determined. The final PBMCs density used in the experiments (after addition of glyphosate) was 1×10^6 cells/mL.

Cells treatment; Glyphosate was dissolved in PBS, pH 7.4. The concentrations of glyphosate were from 0.5 to 100 μM (0.085–17 mg/L). In a previous study, no changes in cell viability after treatment of PBMCs with glyphosate were observed at the above mentioned concentrations. The cells were incubated

with investigated xenobiotic for 24 h in four independent experiments (four blood donors). During incubation, the cells were resuspended in RPMI supplemented with 10% FBS and penicillin/streptomycin solution (50 U/mL and 50 μg/mL, respectively) at 37°C, 5% CO_2. After incubation, the cells were centrifuged, glyphosate was discarded, and the cells were resuspended in RPMI medium.

Methylation levels

Global DNA methylation; Genomic DNA from human PBMCs was isolated using Invisorb Spin Tissue Mini Kit (Stratec Molecular GmbH, Berlin, Germany). Global DNA methylation was determined by colorimetric measurement of 5-methylcytosine in DNA using Methylated DNA Quantification Kit (Abcam). For global DNA methylation analysis, 100 ng of genomic DNA was used, following the protocol provided by the manufacturer. Methylation levels were calculated relatively to the methylated control DNA (included in the kit) and expressed as a percentage of total methylated DNA using the following formula:

$$5-\mathrm{mC}\% = \frac{(\text{Sample OD} - \text{Negative Control OD}) \div S)}{\text{Positive Control OD} - \text{Negative Control OD}) \times 2 \div P} \times 100\%$$

where:
S - the amount of input sample DNA in ng; P - the amount of input positive control in ng; 2 - a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC.

Methylation at promoter regions of tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1); Chemical modification of 500 ng of genomic DNA was analysed using EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, US), according to manufacturer's instruction. For methylation analysis, methylation-specific real-time PCR assay (MSP-PCR) was conducted with FastStart SYBR Green Master (Roche, Basel Switzerland). Bioinformatic analysis of the potential methylation sites within the promoter regions of the proto-oncogens BCL2 and CCND1 as well as methylated and unmethylated primers were designed by utilizing of Methyl Primer Express®, v.1.0 (Life Technologies, Carlsbad, CA, US). The DNA sequences around the transcription start sites (from -1000 to 300 bp) of both genes, were obtained from the DBTSS. The primer list is presented in Supplementary Table 1. All samples were amplified in duplicate and in the presence of positive control (CpG methylated Jurkat genomic DNA, fully methylated), negative control (5-Azadc-treated Jurkat genomic DNA) (NEB, Ipswich, MA, US) and blank control (water). The methylation status of a particular gene is expressed as methylation index (MI):

$$\Delta Ct = CtU - CtM$$

$$MI = \frac{1}{1 + 2^{-\Delta Ct}} \times 100\%$$

where: MI - methylation index [%]; Ct values of the methylated gene (M) were compared with the Ct values of the unmethylated gene (U).

Gene expression: RNA was extracted with TRIzol™ Reagent (Thermo Fischer Scientific, Waltham, MA, USA). RNA samples with a 260/280 nm ratio in the range of 1.8–2.0 were used for further analysis. cDNA synthesis was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel Switzerland). The cDNA was quantified by real-time PCR using FastStart SYBR Green Master (Roche, Basel Switzerland). Gene expression was normalized to the mean expression of all three housekeeping genes (GAPDH, RPL13, RPLP0) and was presented as a relative gene expression. The 2ΔCt (Ctgene–Ct mean from GAPDH, RPL13, RPLP0) method was used to calculate the expression levels of studied genes. The $2^{-\Delta Ct} \times 100$ values were re-calculated into relative copy number values.

Statistical analysis; The statistical analysis was performed with STATISTICA 13.1 data analysis software (2000 Stat-Soft, Inc., Tulsa, USA). Statistical analysis was conducted using the one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparisons procedure. The

difference was considered to be significant for $P < .05$. The individual analysis was performed on blood from four donors, while each experiment (conducted for blood from one donor) was repeated twice or three times depending on the method.

Results

Analysis of global DNA methylation level; Statistically significant changes in 5-mC level were observed in PBMCs treated with glyphosate (Fig. 1). As compared to control cells, the level of global DNA methylation was significantly decreased after glyphosate treatment at all concentrations tested: 0.5 μM, 10 μM and 100 μM.

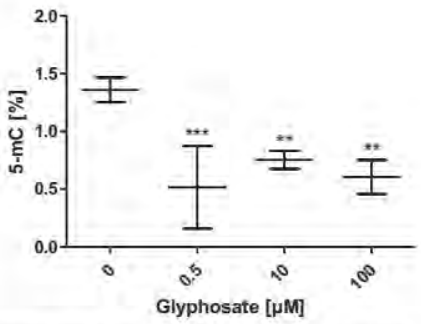

Fig. 1. 5-methylcytosine (%) in human PBMCs incubated with glyphosate (0.5–100 μM) for 24 h. Mean ± SD was calculated for four individual experiments. Statistically different from control at $^{**}p < .01$; $^{***}p < .001$. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Analysis of methylation at promoter regions of the selected tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1); Statistically significant ($p < .05$, one-way ANOVA) decrease of methylation within P21 gene promoter was found in PMBCs treated with glyphosate from its lowest concentration of 0.5 μM (Fig. 2A). The opposite response to the investigated xenobiotic (0.5 μM) such as hypermethylation of gene promoter, was observed on TP53 tumor suppressor gene (Fig. 2A). In the case of other analyzed genes: P16, BCL2 and CCND1 we did not determine statistically significant changes in gene promoter methylation level (Fig. 2A, Fig. 3A).

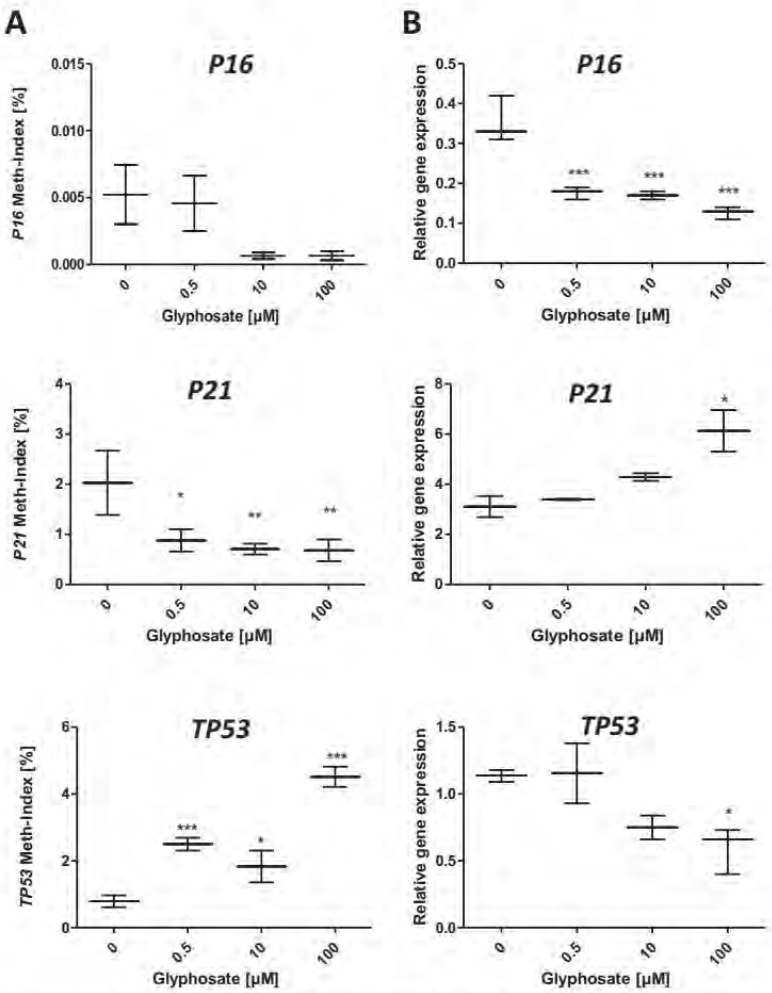

Fig. 2. Methylation (A) and expression (B) of suppressor genes (*P16*, *P21* and *TP53*) in human PBMCs incubated with and glyphosate (0.5–100 µM) for 24 h. Mean ± SD was calculated for four individual experiments. Statistically different from control at *p < .05; **p < .01; ***p < .001. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Analysis of gene expression of the selected tumor suppressor genes (P16, P21, TP53) and proto-oncogenes (BCL2, CCND1); The analysis of methylation of the selected gene promoters using Real-Time PCR let us assess their expression at the transcript level. A statistically significant decrease of P16 expression in PBMCs treated with all concentrations of glyphosate were identified (Fig. 2B). Glyphosate induced significant decrease in TP53 expression only at the highest concentration of 100 µM ($p < .05$, one-way ANOVA). Expression of other cell cycle drivers like P21 demonstrated a significant increase only at the highest concentration of glyphosate ($p < .05$, one-way ANOVA; Fig. 2B), which was also effective in increasing of BCL2 expression (Fig. 3B). Expression of the cyclin was significantly increased by two tested concentrations (10 µM and 100 µM) of glyphosate ($p < .05$, oneway ANOVA) (Fig. 3B).

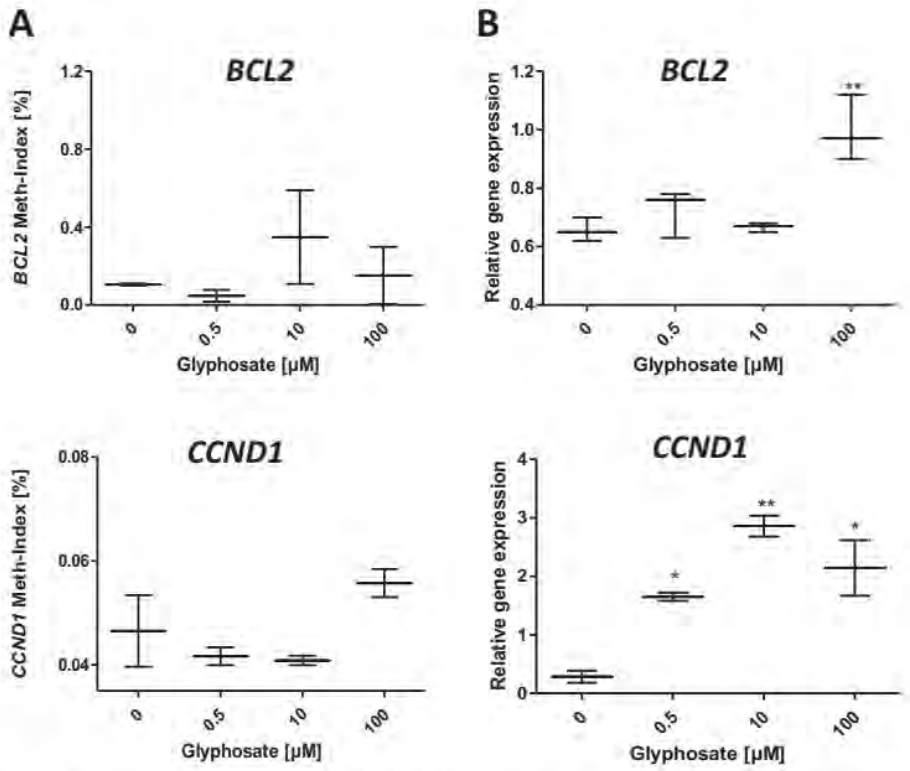

Fig. 3. Methylation (A) and expression (B) of proto-oncogenes (*BCL2* and *CCND1*) in human PBMCs incubated with and glyphosate (0.5–100 µM) for 24 h. Mean ± SD was calculated for four individual experiments. Statistically different from control at *p < .05; **p < .01. Statistical analysis was conducted using one-way ANOVA and a posteriori Tukey test.

Discussion

The knowledge that pollutants may influence the epigenome raises grave concerns concerning their long-term effects on chronic diseases development. A study showed that glyphosate can predispose breast cells to tumorigenesis via epigenetic reprogramming, which occur through TET3-mediated global and local DNA hypomethylation. Just recently, changes in the DNA methylation machinery due to glyphosate treatment were also identified in the fish model of Japanese medaka (Oryzias latipes), where upregulation of DNMT1, DNMT3a, Tet1 and Tet3 gene expression was shown. The current results have shown that treatment of PBMCs with glyphosate changed global DNA methylation profile. Decreased 5-mC level in PBMCs treated with low concentration of glyphosate that is comparable to that determined in human body after environmental exposure (0.5 µM) were found. Moreover, the obtained results are in agreement with previous findings, showing that glyphosate at high concentrations (determined in blood during glyphosate acute poisoning at 250–500 µM), reduces total DNA methylation in PBMCs. Recently, a study observed that glyphosate causes global DNA hypomethylation in non-neoplastic mammary epithelial MCF10A cells and triggers tumorigenesis in a "two-hit oncogenic model". Also a specific DNA hypomethylation signature of genes (i.e., local DNA hypomethylation), which was linked to the TET3 pathway that potentially can be used as an epimark to glyphosate exposure, was identified. Similar findings i.e. global hypomethylation of DNA were found in blood cells of tobacco farmers (n = 40) using pesticides and other xenobiotics during plant cultivation. However, literature data regarding potential epigenetic effects exerted by herbicides became contradictory. Recent epidemiological studies point to an increase of DNA methylation level under the combined effects of various pesticides, including glyphosate. It was found that long-term exposure of farmers (n = 137), employed at growing soybeans cultivation, to complex mixtures of pesticides, resulted in DNA hypermethylation and micronuclei formation in their blood cells probably due to impairment of DNA repair mechanisms. Nevertheless, the farmers were exposed to the mixture of different pesticides and heavy metals that are known to induce DNA hypermethylation and to disturb the cell cycle progression. These findings were confirmed in a study on children living in an industrial areas. In blood samples of young subjects exposed to increased heavy metals and polycyclic aromatic hydrocarbons concentrations, DNA hypermethylation was observed. Detailed analysis of selected genes promoters involved in cellular

metabolism regulation including cell cycle, i.e. TP53, P21, P16, CCND1 or BCL2, revealed significant changes in their methylation profiles. Beside global DNA hypomethylation we have found an increased methylation within the CpG islands of the TP53 gene promoter in PBMCs treated with glyphosate in all range of concentrations. The lowest concentration (0.5 μM) of glyphosate induced 5-mC methylation of the TP53 gene promoter. This result is in agreement with previous studies, where observed statistically significant increase of CpG islands methylation within the TP53 gene promoter after treatment of PBMCs with 250 μM and 500 μM glyphosate were identified. TP53 is known to be genome guard and tumor suppressor gene. It has been shown that hypermethylation of CpG islands within its promoter results in the silencing of gene transcription, but it is not clear-cut relationship in this case. Glyphosate reduced TP53 expression only from the concentration of 100 μM. Changes in TP53 expression and identified functions of p53 protein in the regulation of cell cycle directed us towards the findings of an author, who have shown that only Roundup but not glyphosate changed cell cycle of sea urchin embryonic cells due to delayed activation of the CDK1/cyclin B complex, acting downstream from p53. The effect of glyphosate on cell cycle was also excluded in the experiments on human lymphocytes; however the tested concentrations range of this compound was between 0.0125 and 0.5 μg/mL (0.07–2.9 μM), while the current study showed that glyphosate at 100 μM caused downregulation of TP53 expression in PBMCs. Beside hypermethylation of TP53 gene promoter and down regulation of its transcript level in PMBCs treated with glyphosate (100 μM), hypomethylation within P21 gene promoter was detected for all glyphosate concentrations. Changes in methylation of the P21 gene promoter were correlated with increased expression of the cell cycle regulator at 100 μM of glyphosate, that would suggest inhibition of PBMCs cycling in G0/G1 phase. Further analysis of molecular drivers of the cell cycle revealed a decreased expression of the P16 gene encoding the cell cycle inhibitor in glyphosate-treated cells, staring from its lowest concentration comparable to that occurring during environmental exposure. However, the analysis carried out did not show any changes in the methylation level within the P16 gene promoter region in PBMCs due to glyphosate treatment. These results correlate with previous findings, in which higher concentrations of glyphosate (250 μM and 500 μM) also did not cause any changes in methylation of P16 gene promoter. The protein product of P16 gene, p16, is an inhibitor of cyclin D. Literature data suggests that low expression of P16 may result in overexpression of cyclin D1 (CCND1), activation of its complexes with CDK4 and CDK6 complexes (cyclin D1/CDK4, cyclinD1/CDK6) and overcoming the G0/G1 checkpoint of the cell cycle. In the tested experimental conditions, an increased expression of the CCND1 due to glyphosate treatment at its whole tested range of the concentrations (0.5–100 μM) was identified, but no statistically significant changes in the methylation level of the gene promoter has been shown. Beside of changes in the expression level of the major cell cycle regulators and/or methylation pattern within their gene promoters, significant upregulation of the BCL2 expression at the mRNA level at the highest tested concentration of glyphosate (100 μM) was also identified. Therefore, this data may suggest a disturbance of apoptosis induction, due to sustained antiapoptotic abilities of BCL2. In contrast, in vitro studies on the effect of glyphosate on apoptosis in mature rat testicular cells and PBMCs showed that glyphosate only at very high concentrations (29.57 mM/0.5 mM, in rat testicular cells and PBMCs; respectively) induced apoptosis. Thus, the obtained data may not result in specific metabolic effects until the exposure to high doses or cumulative long-term exposure of humans to glyphosate will be preceded.

Conclusion

This study has attempted to assess epigenetic mechanisms of action of glyphosate in human PBMCs, which has been poorly studied in cellular models including blood cells. The conducted analysis have shown that glyphosate significantly affected global DNA methylation of PBMCs as well as methylation in the promoter regions of selected tumor suppressors (P21 and TP53) as well as expression of major cell cycle and apoptosis drivers (P16, TP53, BCl2, CCND1 and P21). Changes in the DNA methylation profile were minimally correlated with gene expression level, however, regulation of transcription process is performed at many different levels and further and more global analysis (genome-wide based on) are necessary to give clear answer about epigenetic-transcriptomic changes induced by glyphosate. It should be noted that glyphosate induced changes in concentrations corresponding to environmental or occupational exposure. Conducting of further in vitro studies on various epigenetic modifications caused by glyphosate with different target cell cultures is still warranted.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective was to assess epigenetic mechanisms of action of glyphosate in human PBMCs, which has been poorly studied in cellular models including blood cells. The study was conducted using an *in vitro* test system. The ability of glyphosate to impact the measured parameters was demonstrated (global DNA methylation of PBMCs, methylation in the promoter regions of selected tumor suppressors (P21 and TP53), and expression of major cell cycle and apoptosis drivers (P16, TP53, BCl2, CCND1 and P21). However, a positive control was not used, and a clear dose-response was not established for all of the measured parameters. Additionally, the measured effects in vitro are not clearly linked to an adverse outcome in vivo. While it is stated that the concentrations used are comparable to environmental exposure, external exposure was not linked to a corresponding internal concentration. Therefore, it is not possible to calculate a dose for risk assessment purposes. The study is useful for supplemental information on *in vitro* effects resulting from glyphosate exposure, but, is not appropriate for derivation of an endpoint in human health risk assessment.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because the assays conducted comply in general with the quality criteria for *in vitro* testing.

Reliability criteria for *in vitro* toxicology studies

Publication: Wozniak *et al*., 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95%. Source: Sigma-Aldrich, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	PBMC.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.5 to 100 µM
Cytotoxicity tests reported	Y	Ref. to earlier paper.
Positive and negative controls	Y	Methylated control DNA as positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because the assays conducted comply in general with the quality criteria for in vitro testing.

1. Information on the study

Data point:	CA 5.6
Report author	Zhang, J-W. *et al.*
Report year	2019
Report title	The toxic effects and possible mechanisms of glyphosate on mouse oocytes
Document No	Chemosphere 237 (2019) 124435
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities.
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The effects of glyphosate on oocyte maturation, as well as its possible mechanisms remain unclear. The present study revealed that mouse oocytes had reduced rates of germinal vesicle breakdown (GVBD) and first polar body extrusion (PBE) after treatment with 500 mM glyphosate. Reactive oxygen species (ROS) were found in mouse oocytes exposed to glyphosate, as shown by changes in the mRNA expression of related antioxidant enzyme genes (*cat, sod2, gpx*). After 14 h of exposure to glyphosate, metaphase II (MII) mouse oocytes displayed an abnormal spindle morphology and DNA double-strand breaks (DNA-DSBs). Simultaneously, mitochondria showed an aggregated distribution and decreased membrane potential in mouse oocytes exposed to glyphosate. The protein expression levels of apoptosis factors (Bax, Bcl-2) and the mRNA expression levels of apoptosis-related genes (*bax, bcl-2, caspase3*) were measured by Western blot and qRT-PCR, respectively. Meanwhile, the expression levels of autophagy-related genes (*lc3, atg14, mtor*) and proteins (LC3, Atg12) were significantly decreased in the glyphosate treatment group compared with the control group.

Materials and methods

Antibodies and chemicals: Rabbit polyclonal anti-LC3A/B (light chain 3, LC3), anti-p-MAPK, anti-g-H2AX and anti-b-actin antibodies were purchased from Cell Signaling Technology. Mouse monoclonal anti-Bax, anti-Bcl-2, antiAtg12, anti-Annexin V and anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology. The Reactive Oxygen Species Assay Kit was purchased from Beyotime Biotechnology (S0033). The Mitochondrial Membrane Potential Assay Kit with JC-1 was obtained from Beyotime (C2005). Mito Tracker Red CMXRos was purchased from Cell Signaling Technology (#9082).

Mice: Female Kunming mice (25-30 g) were purchased from the Institute of Zoology, Chinese Academy of Sciences. They were housed in a temperature-controlled room with 12D:12L (dark vs. light) and had unrestricted access to food and water under conditions of constant temperature (23 ± 2° C).

Oocyte collection and treatment; To collect fully grown GV oocytes, the mice were superovulated by injecting them intraperitoneally with 10 IU Pregnant Mares Serum Gonadotropin (PMSG) 48 h earlier. The mice were sacrificed by cervical dislocation and the ovaries were placed in M2 medium. Oocytes were released from the ovaries by puncturing the follicles with a fine needle, and denuded oocytes were collected by gentle pipetting. GV oocytes were cultured in an incubator in 50 μL droplets of culture medium under liquid paraffin oil at 37° C in 5% CO_2. Glyphosate was dissolved in M16 medium and diluted to a final concentration of 50, 100, 200 or 500 μM. After culturing for 2 h or 14 h, GVBD and MII oocytes were used for the subsequent experiments.

Immunofluorescent staining: To determine the levels of intracellular ROS production, denuded MII oocytes from 6 mice were incubated with M16 medium that contained 10 μM dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37° C in the dark. After washing three times with 1% Bovine Serum Albumin/Phosphate-buffered saline (BSA/PBS), the oocytes were placed in 50 μL of M16 medium droplets and the fluorescence was observed with a confocal laser-scanning microscope (Zeiss LSM 710

META, Germany) with the same scanning parameters. The fluorescence intensity of each oocyte was analysed using ImageJ software. For mitochondrial staining, MII oocytes from 6 mice were fixed in 4% paraformaldehyde (PFA) for 1 h and then placed in membrane permeabilization solution containing 0.5% Triton X-100 for 20 min at room temperature. After washing three times with 1% BSA/PBS, the oocytes were incubated in 200 nM Mito Tracker Red CMXRos in M16 medium for 30 min in the dark. After several washes, the oocytes were costained with DAPI (4', 6-diamidino-2-phenylindole) for 5 min. Images were captured by the confocal laser-scanning microscope. To measure mitochondrial membrane potential (MMP), MII oocytes from 6 mice were incubated at 37° C for 20 min with the 1 x JC-1 probe, then washed twice with JC-1 buffer (5 min each) to remove surface fluorescence. Images of fluorescence were captured using confocal microscopy as above. MMP was quantified as the ratio of red to green fluorescence using ImageJ software. To measure the spindle, apoptosis, autophagy and DNA damage, MII oocytes from 24 mice were stained with anti-α-tubulin, antiAnnexin-V, anti-LC3and anti-ɣ-H2AX antibodies, respectively. The denuded MII oocytes were fixed with 4% PFA for 30 min, permeabilized in 0.5% Triton-X 100 for 20 min at room temperature, and blocked with 1% BSA/PBS for 1 h at 4 C. The oocytes were incubated with anti-α-tubulin (1:200), anti-LC3 A/B (1:200), antiAnnexin-V (1:200) and anti-ɣ-H2AX (1:200) at room temperature for 2 h followed by three washes with 1% BSA/PBS. The oocytes were then incubated with an appropriate secondary antibody for 2 h at room temperature and then costained with DAPI for 5 min in the dark. After three washes, oocytes were mounted on a glass slide and representative images were captured by the confocal laser-scanning microscope. ImageJ software was used to quantify the fluorescence intensity in the images.

Quantitative real-time PCR: Total RNA was extracted from 100 MII oocytes from 12 mice using the TRNpure Total RNA Kit (Nobelab, Beijing, China) according to the manufacturer's instructions. The first strand cDNA was generated with the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, China). All gene expression was determined using the FS Universal SYBR green PCR master mix (Roche, Canada) under the following conditions: 50°C for 2 min, 95° C for 10 min, 40 cycles of 95° C for 15 s and 60° C for 1 min. Each sample was tested in triplicate and *gapdh* was used as an internal control gene. Relative expression levels were analysed by the $2^{-\Delta\Delta Ct}$ method.

Western blot: A total of 200 MII oocytes from 24 mice were lysed in 1 x SDS sample buffer containing 1 x protease inhibitor Cocktail (cwbiotech) on ice for 20 min. They were boiled for 5 min at 95° C, then subjected to 12% SDS-PAGE and the separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked for 2 h with 5% nonfat milk in TBST and then probed with primary antibodies for 2 h at room temperature (anti-p-MAPK antibody, 1:500; anti-Bcl-2 antibody, 1:1000; anti-Bax antibody, 1:1000; anti-LC3 antibody, 1:1000; anti-Atg12 antibody, 1:500; anti-α-tubulin antibody, 1:1000; and anti-β-actin antibody, 1:1000). After washing three times with TBST (10 min each), membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibodies (1:1000). Three washes later, the protein bands were visualized using BeyoECL Plus (Thermo Fisher Scientific), and the signals were acquired by Tanon 5500. The images were quantified using ImageJ software.

Statistical analysis; At least 50 oocytes were analysed for each experiment. For each treatment, at least three biological replicates were performed and data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using the GraphPad Prism software followed by Student's unpaired two-tailed t-test and two-way ANOVA. Statistical significance was set at P value: *<0.05, **<0.001, ***<0.0005, ****<0.0001.

Results

Glyphosate composition analysis: The chemical composition of glyphosate was analysed before the start of the experiment by Ultra Performance Liquid Chromatography/Quadrupole-Time-of-Flight-Mass Spectrometry (UPLC/Q-TOF-MS). Specific results from this analysis were not reported.

Glyphosate treatment reduced the GVBD and PBE in mouse oocytes: To test the effect of glyphosate on the mouse oocytes maturation, the oocytes were cultured in culture medium supplemented with increasing concentrations of glyphosate (50, 100, 200 or 500 μM). Typical images of exposure to glyphosate on GVBD and PBE are shown in Fig. 2. After treatment with glyphosate, GVBD (Fig. 2A

and B) and PBE (Fig. 2C and D) were significantly decreased in the 200 mM and 500 mM groups, while there was no significant variation in the 50 μM and 100 μM treatment groups. Hence, the 500 μM glyphosate treatment was adopted for subsequent experiments. These observations were considered to show that glyphosate exposure decreased mouse oocyte developmental competence.

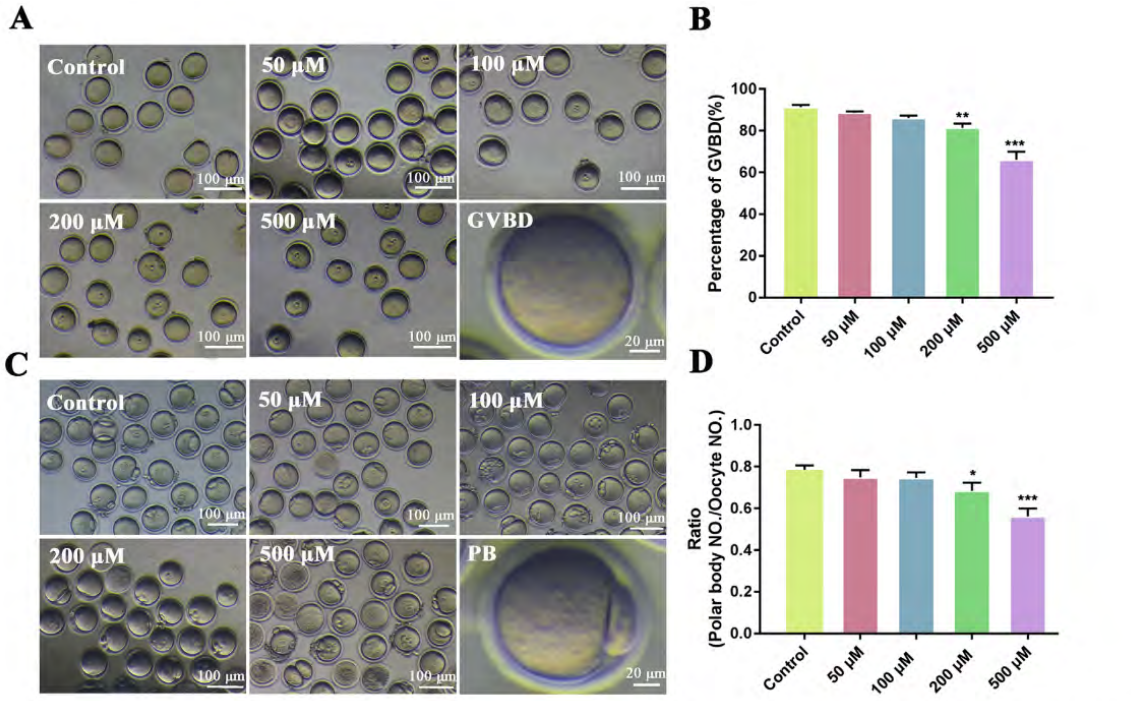

Fig. 2. Effects of glyphosate on GVBD and PBE in mouse oocytes. (A) Representative images of mouse oocytes after exposure to different concentrations of glyphosate for 2 h. (B) GVBD rates of different groups after 2 h of treatment. The data are expressed as the mean ± SEM. (C) Representative images of mouse oocytes after exposure to different concentrations of glyphosate for 14 h. (D) Quantification of the presence of PBE in control and treatment oocytes. The data are expressed as the mean ± SEM.

Glyphosate treatment increased ROS generation and DNA damage in mouse oocytes: It was tested whether defects observed in glyphosate-exposed mouse oocytes were mediated by oxidative stress. As shown in Fig. 3A and B, the fluorescence intensity of DCFH-DA was significantly higher in the 500 μM glyphosate treatment group than in the control oocytes. The levels of *sod2* and *gpx* mRNA expression were significantly increased in glyphosate-exposed oocytes when compared with the control group (Control: sod2: 1.005 ± 0.066, gpx: 1.003 ± 0.052; 500 μM: sod2: 1.702 ± 0.148, gpx: 1.545 ± 0.181); the expression of *cat* mRNA was also increased (Control: 1.001 ± 0.036; 500 μM: 1.241 ± 0.111). These results suggested that glyphosate administration enhanced ROS production.As described above, glyphosate is cytotoxic to oocytes. We further glyphosate treatment would cause DNA damage in mouse oocytes using g-H2AX staining, which indicates the presence of DNA-DSBs. As shown in Fig. 3D, there were no significant DSB foci in the control oocytes; however, there was a dense mass of γ-H2AX foci associated with chromatin in glyphosate-exposed oocytes. As shown in Fig. 3E, the fluorescence intensity of γ-H2AX also significantly increased after glyphosate treatment compared to that in the control group. These results were considered to indicate that glyphosate treatment results in DNA damage in mouse oocytes.

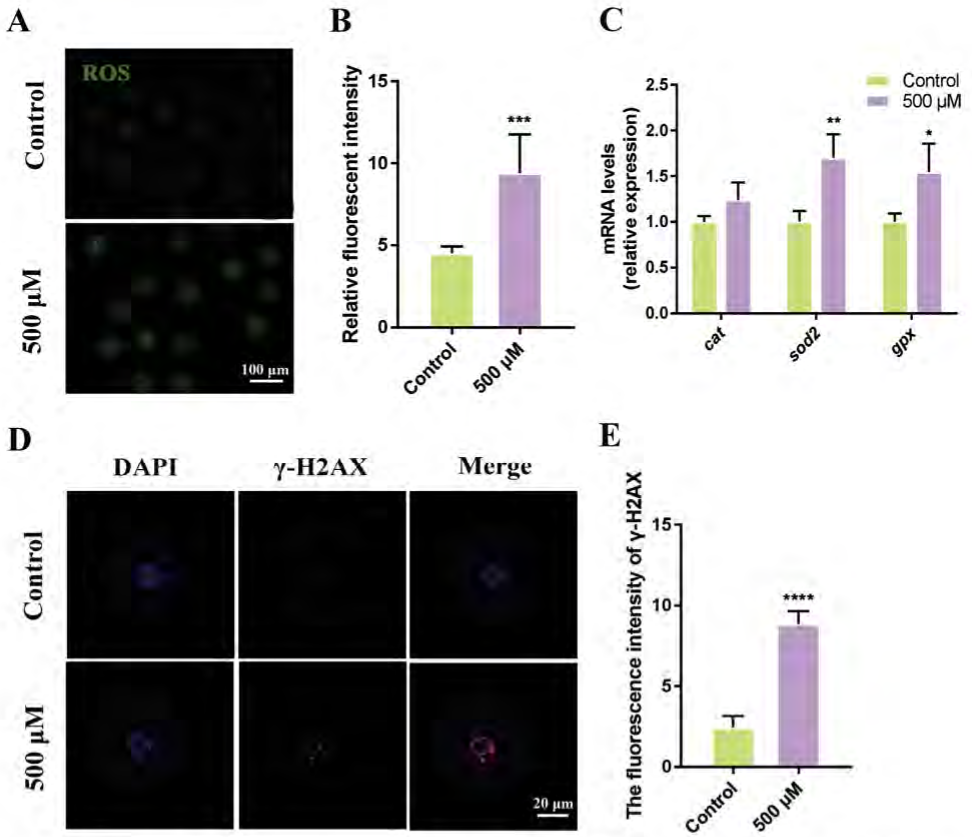

Fig. 3. Effects of glyphosate on ROS generation and DNA damage in mouse oocytes. (A) Representative images of DCFH-DA fluorescence in control and glyphosate-exposed mouse oocytes. (B) Quantitative analysis of ROS fluorescence intensity in control and glyphosate-exposed groups. The data are expressed as the mean ± SEM. (C) Relative expression levels of oxidative stress-related genes were examined by qRT-PCR. The data are expressed as the mean ± SEM. (D) Representative images of control and glyphosate-treated oocytes exhibiting γ-H2AX immunostaining. (E) The γ-H2AX fluorescence intensity obviously increased after glyphosate treatment. The data are presented as the mean ± SEM.

Glyphosate treatment disturbs spindle morphology in mouse oocytes: To investigate whether the oocytes with glyphosate treatment showed defects in spindle positioning and chromosome scattering, MII oocytes were assessed by immunocytochemical staining with anti-α-tubulin antibody and DAPI. In contrast to oocytes from the control group, which presented a normal spindle appearance and well-aligned chromosomes at the equatorial plate, the glyphosate-exposed group showed misaligned chromosomes and abnormal spindle morphology(Fig. 4A). The rate of abnormal spindles in the glyphosate treatment group was also increased (Control: 9.333 ± 0.491%; 500 μM: 15.767 ± 1.369%). as shown in Fig. 4BThe effect of glyphosate on the expression levels of spindle assembly regulatory protein was also assessed. In Fig. 4C and D, Western blots revealed that the level of p-MAPK protein was significantly reduced after 500 μM glyphosate treatment (Control: 0.856 ± 0.053; 500 μM: 0.517 ± 0.070). These results were considered to suggest that glyphosate treatment disturbs spindle morphology in mouse oocytes.

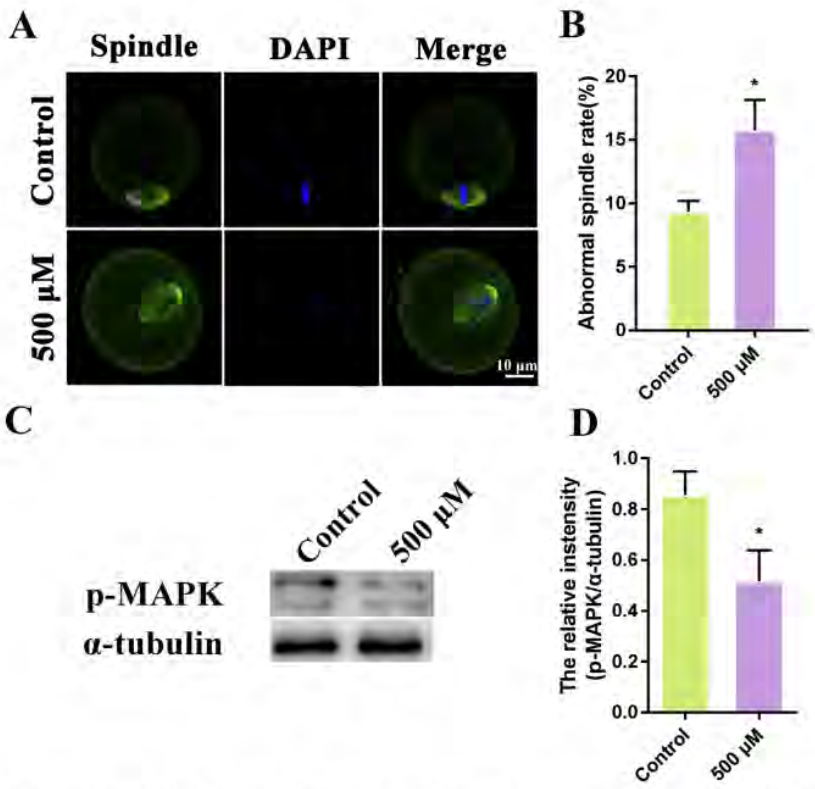

Fig. 4. Effects of glyphosate on spindle morphology and chromosome alignment in mouse oocytes. (A) Representative confocal images of spindle morphology in control and glyphosate exposed groups. (B) Percentage of cells exhibiting abnormal spindle/chromosome morphology. The data are expressed as the mean ± SEM. (C) The expression level of p-MAPK protein in oocytes from different groups was analysed by Western blot analysis. (D) The relative intensity of p-MAPK protein expression (p-MAPK/α-tubulin) was significantly reduced after treatment with 500 μM glyphosate. The data are expressed as the mean ± SEM.

Glyphosate treatment resulted in mitochondrial injury in mouse oocytes: A homogeneous distribution of mitochondria was present in the control group,but an aggregated distribution was seen in the glyphosate-exposed group by staining with Mito Tracker Red(Fig. 5A). Alterations in MMP of mouse oocytes from different groups were evaluated by staining with JC-1 (Fig. 5B). As shown in Fig. 5C, the result illustrated that the MMP was significantly lower in the glyphosate-exposed group compared with the control group (Control: 1.871 ± 0.082; 500 μM: 4.517 ± 0.155). Based on these data, glyphosate treatment was considered to interfere with mitochondrial function in mouse oocytes.

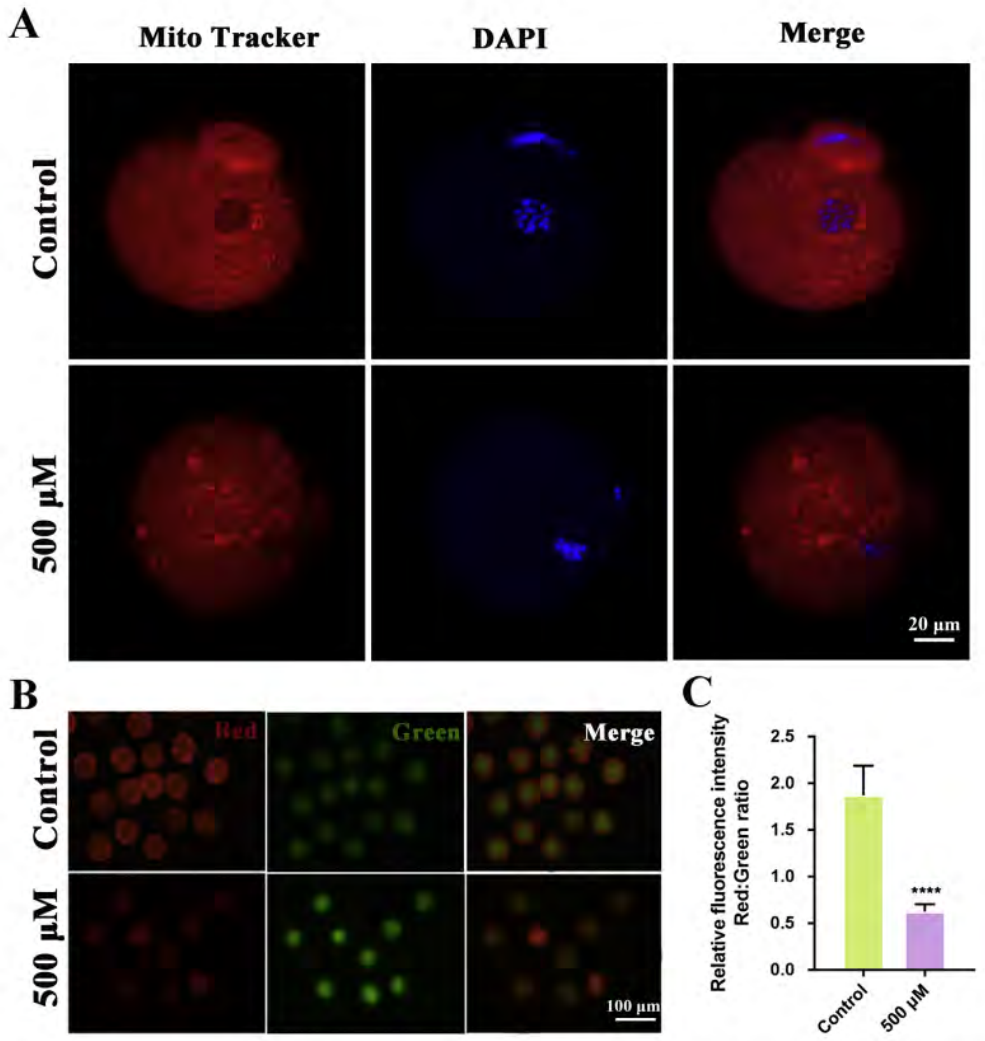

Fig. 5. Glyphosate treatment results in mitochondrial injury in mouse oocytes. (A) Representative images of mitochondrial distribution in control and glyphosate-treated oocytes by staining with MitoTracker Red. (B) Representative images of MMP in mouse oocytes from different groups stained with JC-1. (C) MMP levels (red/green fluorescence intensity) in glyphosate-exposed oocytes was significantly lower than that in the control group. The data are presented as the mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Glyphosate treatment induced early apoptosis and autophagy in mouse oocytes: Annexin-V staining was conducted to identify whether early apoptosis occurred in glyphosate-treated oocytes. In the control group, Annexin-V signals were detected only in the zona pellucida, whereas the treatment group had a clear green signal in the membrane and zona pellucida Fig. 6A). In Fig. 6B, the apoptotic fluorescence intensity of oocytes was notably higher in the treatment group than in the controls. Apoptosis-related protein expression levels were also assayed via As shown in Fig. 6G and H, Western blot analysis, which showed that the expression of Bcl-2 protein decreased and the expression of Bax protein increased after exposure to 500 μM glyphosate (Control: Bcl-2: 0.645 ± 0.009, Bax: 0.427 ± 0.020; 500 μM: Bcl-2: 0.549 ± 0.011, Bax: 0.556 ± 0.018). To confirm this result, the mRNA expression levels of apoptosis-related genes were tested by qRT-PCR. The qRT-PCR results were consistent with the Western blot results. Together, these results were considered to indicate that glyphosate induced early apoptosis in mouse oocytes. Whether autophagy had occurred in glyphosate-treated oocytes was assessed by LC3 immunofluorescent staining; As shown in Fig. 6D and E, the fluorescence intensity was significantly higher in the 500 μM glyphosate treatment group than in the oocytes from the control group. The mRNA expression levels of autophagy-related genes were also assessed by qRT-PCR and showed an increasing trend (Control: *lc3*: 0.860 ± 0.013, *atg14*: 1.024 ± 0.149, *mtor*: 0.847 ± 0.171; 500 μM: *lc3*: 1.591 ± 0.109, *atg14*: 1.800 ± 0.139, *mtor*: 1.268 ± 0.242 (Fig. 6F). Western blot analysis showed that the expression of LC3 and Atg12 protein increased after exposure to 500 μM glyphosate (Control: LC3: 0.280 ± 0.021, Atg12: 0.435 ± 0.007; 500 μM: LC3:0.504 ± 0.030, Atg12: 0.580 ± 0.016). Together, these results were considered to indicate that glyphosate induced autophagy in mouse oocytes.

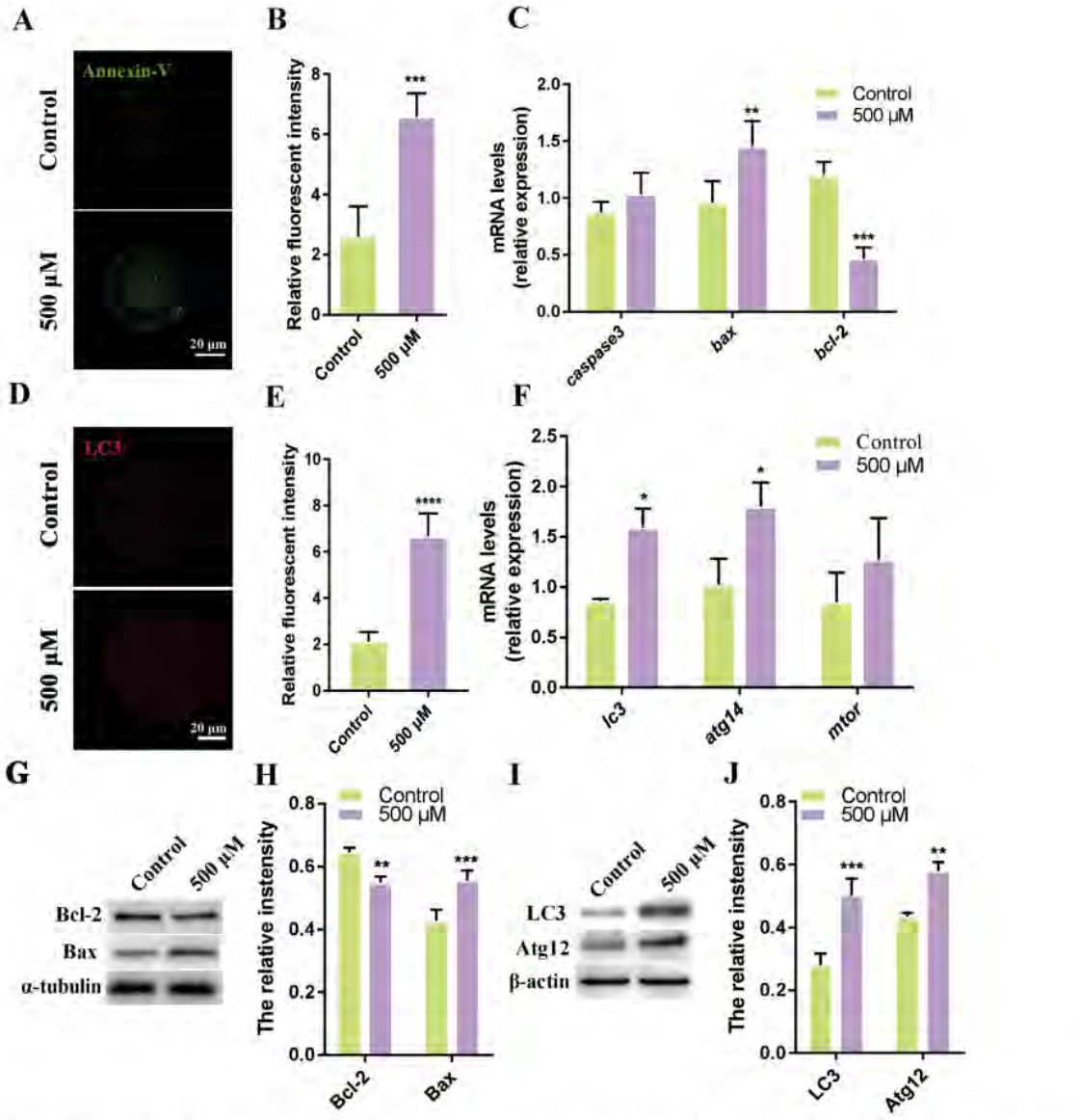

Fig. 6. Glyphosate treatment induces early apoptosis and autophagy in mouse oocytes. (A) Representative images of apoptosis (Annexin-V staining) in mouse oocytes. (B) Quantification of Annexin-V signal was recorded in control and glyphosate-exposed oocytes. (C) Relative mRNA levels of *caspase3*, *bax* and *bcl-2* in different groups of oocytes. (D) Representative images of autophagy (LC3 staining) in mouse oocytes. (E) Quantification of the fluorescence signal was recorded for the control and glyphosate-exposed oocytes. (F) The relative mRNA level of *lc3*, *atg14* and *mtor* in different groups oocytes. (G) The expression levels of apoptosis-related protein in oocytes from different groups were analysed by Western blot analysis. (H) The relative intensities of Bcl-2 and Bax protein expression were calculated. (I) The expression levels of autophagy-related proteins in oocytes from different groups were analysed by Western blot analysis. (J) The relative intensities of LC3 and Atg12 protein expression were calculated.

Discussion

In recent years, numerous studies have demonstrated the damaging effects caused by glyphosate in various organisms using different test systems. However, few studies evaluated the reproductive effects of glyphosate on female mammals. Therefore, we investigated the toxic effects of glyphosate on mouse oocyte maturation in vitro. Based on the experimental results, we believed that glyphosate contributed to the destruction of oocyte development . Germinal vesicle breakdown and the first polar body extrusion are markers of oocyte nuclear maturation. Our experimental results indicated that mouse oocytes exposed to glyphosate in vitro experienced a decrease in the proportion of GVBD and PBE. The decrease in the ratio of GVBD and PBE indicates that the growth and developmental ability of the oocyte is destroyed. Reactive oxygen species are excessively generated when cells are subjected to various noxious stimuli, and the oxidative system and the antioxidant system are out of balance, resulting in cell damage. Therefore, we examined the level of ROS in mouse oocytes after glyphosate exposure, and our results showed that ROS levels were increased. To survive, the expression of the antioxidant enzyme genes is increases increased. The mRNA expression levels of sod2, cat and gpx were significantly increased in mouse oocytes exposed to glyphosate. Mouse oocytes exposed to glyphosate produced

excessive ROS, which in turn affected their growth and development. Oocytes are rich in mitochondria. These mitochondria play a leading role in cell metabolism. The changes in their morphology, quantity, distribution and function are closely related to cell metabolism, proliferation and differentiation. Mitochondria are mostly uniformly distributed in the cytoplasm in oocytes with higher developmental potential, while they are aggregated in oocytes with lower developmental potential. A lack of mitochondrial rearrangement in the cytoplasm is a marker of immature cytoplasm. Our results showed that mouse oocytes exposed to glyphosate had aggregated mitochondria and a decrease in membrane potential. These findings indicated that glyphosate destroyed the function of the mitochondria in oocytes. The cytoskeleton plays an important supporting role in the oocyte maturation process. The morphology and kinetic changes of the spindle may reflect the quality of oocytes, and its integrity determines the correctness of chromosome segregation. Abnormalities in spindle morphology will lead to meiosis and fertilization failure. MAPK is a regulated protein involved in microtubule assembly and mitotic spindle assembly. The experimental results showed that after glyphosate exposure, the expression level of p-MAPK protein in mouse oocytes was decreased. So, we proposed that glyphosate affected formation of the spindle by altering the level of p-MAPK, which in turn resulted in a reduction in the rate of mouse oocyte maturation. Excessive accumulation of ROS products has an adverse effect on lipids, proteins, and DNA. In addition, ROS damages the sugarphosphate back-bone of DNA, resulting in single- and double-strand breaks. DNA damages in cells primarily originate from oxidative stress. g-H2AX produced by phosphorylation of H2AX can be used as a biomarker to clearly reflect the extent of DNA damage and repair. Oocytes exposed to glyphosate showed an increase in g-H2AX foci, showing that glyphosate could destroy DNA integrity and produce cytotoxicity in oocytes. Oxidative stress is often associated with apoptosis and autophagy. Therefore, we first detected apoptosis in oocytes by Annexin-V staining. Our experimental results showed that the apoptotic fluorescence intensity of oocytes was increased after glyphosate exposure. The Bcl-2 gene is an inhibitor of apoptosis, and Bax is a pro-apoptotic gene that plays an important role in regulating apoptosis. In the mitochondrial mediated apoptosis pathway, abnormal intracellular signals lead to the activation of Bax, inhibit the expression of Bcl-2, induce mitochondria release of cytochrome c, form apoptotic bodies or initiate caspase cascade activation, and then lead to the occurrence of apoptosis. Western blot analysis and qRT-PCR showed that the expression of Bcl-2 was decreased and the expression of Bax was increased in glyphosate-treated oocytes. Although there was no significant difference in caspase3 mRNA expression, there was an increasing trend after exposure to glyphosate. Autophagy mainly includes phagophore, autophagy formation, autophagosome formation and degradation. The combination of PI3K complex and Atg14 is involved in the formation of phagophore. Atg gene regulates the formation of autophagosomes by forming Atg12-Atg5 and LC3-II complexes. Microtubule associated light chain protein (LC3) is present in autophagosomes; therefore, the LC3 protein is widely used as a marker for autophagosomes. Mammalian target of rapamycin, mTOR, also plays a role in the autophagy pathway. Our results showed that the fluorescence intensity of LC3 was significantly increased, and the expression levels of autophagy-related proteins and genes were increased in the glyphosate treatment group. We considered that oocytes exposed to glyphosate-induced ROS, which could further activate autophagy and early apoptosis, experience a decrease in oocyte quality.

Conclusion

In summary, glyphosate exposure was considered to have caused a block in mouse oocyte development, spindle assembly disruption and chromosome scattered distribution, mitochondrial aggregation and membrane potential reduction, DNA damage, and increased oxidative stress levels, which then led to cellular apoptosis and autophagy. These Our results were considered to provided evidence for the toxic effects of glyphosate on reproductive systems.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In vitro intracellular changes in Kunming mice oocytes were evaluated after being cultured in medium supplemented with 500 μM glyphosate. Findings included: decreased germinal vesicle breakdown, decreased first polar body extrusion, increased mRNA expression of anti-oxidant enzyme related

genes, abnormal spindle morphology, increased DNA double strand breaks, aggregated mitochondria, decreased mitochondrial membrane potential, increased protein expression of apoptosis factors, increased mRNA expression of apoptosis related genes and decreased autophagy-related genes.

No dose-response could be determined seeing as only one concentration was tested, far in excess of that considered biologically relevant. Whilst some evaluations were conducted on oocytes harvested from a wider data set of 24 mice (protein expression levels of apoptosis factors by Western blot analysis), a number of the assessments were conducted on oocytes from just 12 mice (mRNA expression of oxidative stress-related, apoptosis-related and autophagy-related genes) or 6 mice (mitochondrial staining, measurement of mitochondrial membrane potential). This narrow source of oocytes limits the robustness of certain conclusions. Furthermore, there are insufficient details reported in the methods to establish whether mice were of the same age before oocyte harvesting or the purity of the glyphosate tested.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the poor characterization of the glyphosate tested, no cytotoxicity testing, the lack of a positive control and insufficient dose-response characterization at biologically relevant doses.

Reliability criteria for *in vitro* toxicology studies

Publication: Zhang JW *et al*., 2019.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity and source not reported. Chemical analysis was performed but the results were not clear.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	50, 100, 200, 500 µM. Most of the assays were carried out at 500 µM which is a concentration that cannot be reached systemically in the rat at 2000 mg/kg bw after oral intake.
Cytotoxicity tests reported	N	

Positive and negative controls	N	No positive control used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the poor characterisation of the glyphosate tested, no cytotoxicity testing and the lack of a positive control.		