1. Information on the study

Data point:	CA 5.8.2
Report author	Forsythe S. D. *et al.*
Report year	2018
Report title	Environmental Toxin Screening Using Human-Derived 3D Bioengineered Liver and Cardiac Organoids
Document No	Frontiers in Public Health (2018) Vol. 6, 103
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
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 the toxicity of glyphosate for liver and cardiac organoids was investigated in the concentration range from 25 μM to 25 mM. The endpoints considered were cell viability, ATP activity and beating rate of the cardiomyocytes. Glyphosate was shown to reduce organoid integrity and viability at doses from 250 μM to 2.5 mM. The IC50 values based on ATP activity of liver and cardiac organoids were found to be 10.53 and 10.85 mM, respectively. When cardiac organoids were exposed to glyphosate at 0.25 mM a non-statistically significant effect was found on beating rate. Exposure to 2.5 mM for 2 days resulted in all organoids stopping beating.

Materials and methods

Chemicals – The purity and source of glyphosate were not reported.

Liver and cardiac cell sources, culture and organoid formation - For liver organoids, all cells used were commercially sourced, human primary cells. Hepatic stellate cells (HSCs) were expanded in culture for two passages before cryopreservation for use in organoid formation. During expansion, HSCs were cultured in 90% high glucose DMEM and 10% fetal bovine serum on a rat tail collagen I coating (10 g/cm^2) at 37°C with 5% CO_2. Primary human hepatocytes were thawed according to the manufacturer's instructions using hepatocyte thawing medium. Kupffer cells were also thawed following the manufacturer's instructions. After thawing, primary human hepatocytes were plated on collagen coated (10 g/cm^2) 6-well culture plates, using hepatocyte plating medium at a density of ~ 150,000 cells/cm^2. Cells were incubated at 37°C with 5% CO_2 for 4 hours before adding matrigel as an overlay. Following further incubation for 24 hours, fresh HCM medium was added. For cardiac organoids, induced pluripotent stem cell-derived cardiomyocytes (iPSC CMs) were commercially sourced from Axiogenesis (cat. #COR.4U Cardiomyocytes). Human primary cardiac fibroblasts were commercially sourced from ScienCell (cat. #6330). Prior to organoid formation, iPSC CMs were cultured on tissue culture plastic for 48 hours in COR.4U medium until cells began beating spontaneously. At this point, iPSC CMs were harvested using trypsin-EDTA. The liver cells were combined in a cell seeding mixture comprised of 90% HCM medium, 10% heat-inactivated fetal bovine serum, and rat tail collagen I (10 ng/μL). Liver organoids were produced with a mixture of 80% hepatocytes, 10% hepatic stellate cells, and 10% Kupffer cells. Approximately 1,500 cells per 40 μL medium were used to form aggregates in each well of non-adherent, round-bottom, 96-well plates to produce spherical organoids. Cardiac organoids were produced similarly. IPSC CMs were suspended in cardiomyocyte maintenance medium. Fibroblasts were added as 10% of the total cell number, and the volume was adjusted to reach a cell density of 10,000 cells/mL. 100 μL of cell suspension was pipetted into each well of non-adherent, round-bottom, 96-well plates to produce spherical organoids resulting in approximately 1,000 cells/organoid. Well plates were incubated and observed daily until organoid formation, and then immediately used in experiments.

Preparation of test item stock solutions – Glyphosate was dissolved in DI H_2O for environmental toxins to reach 50 mM. Serial dilutions were performed in media for each cell type until all concentrations were created at 2X final concentration. Glyphosate was assessed in the concentration range from 25 μM to 25 mM.

Live/dead staining - Organoids were isolated from 96-well low adhesion round bottom plates, suspended in Hystem hydrogel in a 20 μL construct, and placed into 12-well plates to immobilize organoids in a 3D extracellular matrix environment. Each concentration of glyphosate premixed at 1X concentration in media according to organoid type was added to individual wells and organoids were allowed to incubate under their respective conditions for 2 days at 37°C with 5% CO_2. Studies were performed using n = 5 or higher for all conditions. Medium was then removed and organoids were assessed by LIVE/DEAD® Viability/Cytotoxicity Kit assays. Specifically, 2.0 μM calcein AM and 4.0 μM ethidium homodimer in PBS was added to each well and was allowed to incubate for 1 hour. Imaging was then performed by macro-confocal microscopy and composite images were created with ethidium bromide red fluorescence representing dead nuclei and calcein AM green fluorescence representing live cells.

ATP activity assays – Glyphosate was added in a premix 2× concentration of 100 μL solution to each well of a 96-well plate containing an organoid with 100 μL of medium and allowed to incubate for 2 days at 37°C with 5% CO_2 with n = 6 or higher. Medium was then removed from each well leaving 100 μL of media remaining along with the organoid. Next, 100 μL of Cell-Titer Glo Luminescent Cell Viability Assay solution was added to each well along with 100 μL added to 100 μL DMEM in a Costar Black Polystrene 96-well assay plate and allowed to incubate for 10 minutes at room temperature shielded from light. The entire contents of the wells containing organoids were then added to the Costar Black Polystrene 96-well assay plate wells and the contents were read on a Vertias Microplate Luminometer using default settings. Values were then averaged among the different groups and graphed for analysis using Graph Pad Prism© software.

Heart beat assay - Fully formed cardiac organoids in the wells of a 96-well non-adhesive round bottom plate were placed under a Leica DMIL LED microscope to allow for the recording of natural beat rates in 20 s videos (n = 3). The plate was then returned to the 37°C, 5% CO_2 incubator for 5 minutes to ensure that organoids did not experience significant temperature decreases, which can detrimentally impact beating rates. The process was repeated until all experimental subject organoids under the test compound concentrations described above, but at varying time points, had been recorded. Glyphosate was added as 2 × 100 μL concentration to each well of a 96-well low adhesion round bottom plate containing 100 μL of normal medium with a cardiac spheroid, and allowed to incubate for 30 minutes at 37°C. The plate was then recorded, 3 organoids at a time, in the process listed above until all organoids were recorded and the plate was returned to the incubator. The process was then repeated 24 and 48 hours later. The 20 s videos were then analyzed by counting the beats for each video and multiplied by 3 to scale values to beats per minute. A beat was defined as the beginning of the contractile movement of the organoid. A beat did not need reach conclusion to be counted. If multiple beating regions were observed then the beating of the largest multi-cell structure was used to calculate the beating rate.

Statistical analysis - The data are generally presented as the means of number of replicates ± SD. All data were graphed and analyzed for significance using a Student's T-test. For ATP activity assays p-values were considered significant when < 0.01. For beating kinetic assays p-values were considered significant when < 0.05. Data samples were eliminated from the experimental groups if they fell outside of two SDs from the experimental group averages. Sample sizes (generally n = 5 or n = 6, depending on the experiment as described) were determined based on preliminary experiments. These sample sizes, with the typically observed SDs, allowed statistical significance at α = 0.05 with statistical power greater than 80%. The IC_{50} was calculated using the Graph Pad Prism© software.

Results

Organoid production and viability - Liver organoids were successfully formed after 4 days whereas it took 7 days for the cardiac organoids to allow for self-propagating beating to occur. Viability for both was confirmed to be greater than 95% using live/dead imaging.

Organoid viability - Live/dead staining of liver organoids was used to visualize indications of cytotoxicity due to exposure to glyphosate. Integrity and viability of liver organoids began to show a steady reduction when exposed to glyphosate concentrations from 250 µM to 2.5 mM. In contrast, cardiac organoids maintained integrity during all testing, but cell death occurred when exposed to glyphosate concentrations from 2.5 to 25 mM.

Organoid ATP activity - After testing original 3 doses ($n \geq 6$) centered on doses described in the literature, 2 further trials narrowed the range containing the IC_{50}. The IC_{50} values were found to be similar for cardiac and liver organoids for glyphosate, i.e. 10.85 and 10.53 mM, respectively. For liver organoids, 10 mM of glyphosate resulted in a statistically significant decrease in ATP activity ($p < 0.001$) and for cardiac organoids, 5 mM of glyphosate resulted in statistically significant decrease in ATP activity ($p < 0.001$).

Cardiac organoid beating rates – The time points to test physiological reactions to glyphosate exposure were chosen based on previous studies testing calcium channel impairment in 2D cardiomyocyte populations and defined as immediate (30 minutes), short term (1 day), and long term (2 days). Although not statistically significant, glyphosate demonstrated toxicity at 250 µM and higher, with beat rates at 250 µM slowing on day one among all organoids and 2 of the 3 organoids ceasing beating on day 2. At 2.5 mM, exposure for one day caused two spheroids to cease beating entirely, with one-third showing a 50% reduction in beat rate. By day 2 all organoids ceased beating.

Conclusion

Glyphosate live/dead imaging varied greatly, with significant cell death in liver organoids and little cell death in cardiac organoids at 2.5 mM. ATP values recorded at 10 mM showed a large difference between liver and cardiac organoids (0.568 and 0.804, respectively), displaying a more gradual decrease in cell viability for the liver organoids as compared to the cardiac organoids. The IC_{50} values of liver and cardiac organoids were found to be similar i.e. 10.53 and 10.85 mM, respectively. When cardiac organoids were exposed to glyphosate at concentrations from 0.25 mM on an effect on beating rate was observed with a complete stop of beating at 2.5 mM after 2 days of exposure.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study the toxicity of glyphosate for liver and cardiac organoids was investigated in the concentration range from 25 µM to 25 mM. The endpoints considered were cell viability, ATP activity and beating rate of the cardiomyocytes. Glyphosate was shown to reduce organoid integrity and viability at doses from 250 µM to 2.5 mM. The IC_{50} values based on ATP activity of liver and cardiac organoids were found to be 10.53 and 10.85 mM, respectively. When cardiac organoids were exposed to glyphosate at 0.25 mM a non-statistically significant effect was found on beating rate. Exposure to 2.5 mM for 2 days resulted in all organoids stopping beating.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not characterized, no positive controls were used to validate the organoid test systems and the concentrations at which most of the effects have been observed are physiologically not feasible in *in vivo* experimental models.

Reliability criteria for *in vitro* toxicology studies

Publication: Forsythe *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 and source were not reported.
Only glyphosate acid or one of its salts is the tested substance	N	Also lead, mercury and thallium were assessed.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y?	Liver and cardiac organoids. Origin of hepatic cells not sufficiently documented.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Partly	Test concentrations at which effects were seen were > mM.
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 was not characterized, no positive controls were used to validate the organoid test systems and the concentrations at which most of the effects have been observed are physiologically not feasible in *in vivo* experimental models.		

1. Information on the study

Data point:	CA 5.3
Report author	Gao H. *et al.*
Report year	2019
Report title	Activation of the N-methyl-D-aspartate receptor is involved in glyphosate-induced renal proximal tubule cell apoptosis
Document No	Journal of Applied Toxicology (2019) Vol. 39(8), 1096-1107
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate-based herbicides have been used worldwide for decades and have been suggested to induce nephrotoxicity, but the underlying mechanism is not yet clear. In this study, a human renal proximal tubule cell line (HK-2) was treated with glyphosate for 24 hours at concentrations of 0, 20, 40 and 60 μM. A cell culture model and an animal exposure model were used to investigate the influences of glyphosate on renal proximal tubule cells and the potential role of NMDAR in response to glyphosate exposure.

Materials and methods

Chemicals – Glyphosate monoisopropylamine salt (purity 96%), as a 40% w/w solution in water, purchased from Millipore Sigma, St. Louis, USA.

Cell culture and treatment - The human renal proximal tubular epithelial cell line HK-2 was obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C. Glyphosate monoisopropylamine salt solution (40% w/w in water) was used to prepare a glyphosate stock solution (200 mM), which was then was diluted in complete medium to the final concentrations. HK-2 cells were exposed to glyphosate at 0, 20, 40, 50, 60, 70, 80, 90 or 100 μM for 24 hours. For intervention experiments, HK-2 cells were pretreated with MK-801 at 100 μM for 12 hours, BAPTA-AM at 2 μM for 12 hours or NAC at 2 mM for 12 hours followed by treatment with glyphosate at 40 μM.

Cytotoxicity assays - Cell viability and death were evaluated by Cell Counting Kit-8 (CCK-8) and lactate dehydrogenase (LDH) cytotoxicity assay kit following the manufacturer's instructions. To determine cell viability, HK-2 cells were seeded into a 96-well plate (1×10^4 cells/mL) and then exposed to various concentrations of glyphosate for 24 hours. Cell-free medium and cells treated with water served as the blank and solvent controls, respectively. A mixture of 10 μL of CCK-8 solution and 90 μL of culture medium was added to each well and then incubated at 37°C for 2 hours. Optical density at 450 nm was measured with a Synergy™ HT Microplate Reader. During the detection of glyphosate cytotoxicity, cell supernatants from each well were collected and then incubated with the LDH assay solutions for 30 minutes at 25°C. Cell-free medium and cells treated with water served as the blank and solvent controls, respectively. Cells treated with lysis buffer and lysis buffer alone served as the positive and positive blank controls, respectively. Optical density at 490 nm was measured using a Synergy™ HT Microplate Reader.

Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis assay - Cell apoptosis was measured using an Annexin V-fluorescein isothiocyanate apoptosis detection kit. Glyphosate- or vehicle-treated HK-2 cells were harvested and suspended in binding buffer. Annexin V-fluorescein isothiocyanate (5

μL) was added to the cells, followed by the addition of propidium iodide (PI, 5 μL). Water served as the solvent control, and apoptosis inducer A (Apopida) in the Apoptosis Inducer Kit served as the positive control. Cells without Annexin V and PI were used as negative controls. Subsequently, the cells were labeled for 15 minutes at 37°C. The fluorescence intensity of Annexin V and PI was recorded using a flow cytometer. Data from 10,000 events per sample were analyzed using FlowJo™ software.

Cellular reactive oxygen species measurement - Intracellular ROS production was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) using flow cytometry. HK-2 cells were incubated with 100 nM DCFH-DA for 30 minutes at 37°C. Cells were then harvested and resuspended in basal medium. Water and hydrogen peroxide were used as the solvent and positive controls, respectively. Cells without the DCFH-DA probe were used as a negative control. The fluorescence intensity of 3 × 104 cells per sample was acquired using a flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Data were analyzed using FlowJo™ software.

Detection of intracellular Ca^{2+} levels -
Intracellular Ca^{2+} concentration was analyzed using a Fluo-4/AM fluorescent probe. The cells were incubated with 2 μM Fluo-4/AM in Hanks' balanced salt solution at 37°C for 30 minutes and then suspended in Hanks' balanced salt solution and incubated at 37°C for an additional 20 minutes. H_2O and ionomycin (5 μM) were used as the solvent and positive controls, respectively. Cells without Fluo-4/AM probe were used as a negative control. Cell analysis was performed on a flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The $[Ca^{2+}]_i$ value is represented by the mean fluorescent intensity.

Western blotting - Untreated and treated cells were washed three times with ice-cold PBS and then lysed by RIPA lysis buffer containing 1% protease inhibitors. The cell lysate was centrifuged and the supernatant was collected. The total cellular protein concentration was determined using a BCA assay kit. Equal amounts of total proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis through a 12% gel and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in PBS + Tween 20 (pH 7.5) at room temperature for 2 hours. Subsequently, the membranes were washed with PBS + Tween 20 and incubated with primary antibodies against human NMDAR1, Bcl-2, Bax, Bcl-xl, Bad, cleaved caspase-3 or β-tubulin overnight at 4°C. The membranes were then incubated with antirabbit horseradish peroxidase-conjugated IgG antibodies for 2 hours at room temperature. The protein bands were visualized using an ECL detection kit. β- Tubulin served as an internal loading control.

Animals and treatment - Adult male ICR mice (aged 8 weeks) were obtained from the Shanghai Jiesijie Laboratory Animal Company (Shanghai, China) with license number SCXK (Hu)2013-0006. The mice were provided food and water ad libitum and maintained in a controlled environment at a temperature of 24 ± 1°C, humidity of 45% ± 5% and a 12-hour light/dark cycle. The animals were randomly assigned to the control group or the glyphosate exposure group (6 mice per group). The mice in the glyphosate exposure group received 400 mg/kg bw/day glyphosate via oral gavage once per day for a period of 28 days. The mice in the control group received distilled water. Body weight and food intake were measured daily. Urine was collected once a week using metabolic cages. At the end of the exposure period, blood was collected from the orbital venous plexus under anesthesia to prepare serum. Then, the animals were sacrificed and kidneys excised and washed with saline. The kidney samples were fixed by immersion in a 4% paraformaldehyde solution for 24 hours at 4°C for histology, immunohistochemistry and TUNEL examinations. The remaining samples were snap-frozen in liquid nitrogen and maintained at −80°C for subsequent laboratory analysis.

Urine biomarker measurement - Urine and serum were frozen and stored at −80°C immediately after collection and centrifugation. Urine creatinine, uric acid, urea and serum creatinine levels were determined using the respective assay kits. Urine β2-microglobulin and albumin levels were measured using enzyme-linked immunosorbent assay kits.

Superoxide dismutase, catalase, glutathione peroxidase and malondialdehyde measurements - At the end of glyphosate treatment, the culture supernatants of treated and untreated HK-2 cells for the *in vitro*

experiment or renal tissue for the *in vivo* experiment were freshly collected on ice for measurements of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) activities using the corresponding assay kits. SOD activity was determined according to the xanthine oxidase method, CAT activity was determined using the colorimetric method, GSH-Px activity was determined according to the ultraviolet spectrophotometric method, and MDA levels were determined using the thiobarbituric acid method. All experiments were performed following the manufacturer's instructions.

Kidney histopathology – Kidney tissue fixed for histopathological examination was dehydrated and embedded in paraffin. Tissue sections of 5 μm were mounted on poly-L-lysine-coated slides, deparaffinized with xylene, and stained with hematoxylin and eosin. A trained pathologist blinded to the treatments evaluated the tissue slides using an optical microscope.

Immunohistochemistry - Because the chemical structure of glyphosate and that of its metabolite AMPA are similar to glycine and glutamate, which are agonists of the N-methyl-D-aspartate receptor (NMDAR), the potential role of the NMDAR pathway in mediating the proapoptotic effect of glyphosate on proximal tubule cells was investigated. The kidney samples fixed for immunohistochemical examination were dehydrated, embedded in paraffin and cut in 5-μm thick slices. The slides were then deparaffinized and incubated with rabbit monoclonal anti-NMDAR1 primary antibodies at 4°C overnight, washed in PBS and incubated with goat antirabbit IgG-horseradish peroxidase secondary antibodies for 50 minutes at room temperature. Next, 50 mL of 3,3'- diaminobenzidine was added to each kidney section, which was stained for 5 minutes. After the slides were washed, they were counterstained with hematoxylin for 3 minutes. The slides were then mounted and examined under a microscope. NR1 expression was quantified using Image-Pro Plus 6.0 software. Two negative controls were used: PBS treatment in place of primary antibody and an isotype-matched nonspecific antibody (normal rabbit IgG). Brain tissue from mice was used as a positive control.

TUNEL assay - Apoptotic cells were detected with a TUNEL assay kit according to the manufacturer's instructions. 4'-6-diamidino-2-phenylindole was used for counterstaining. Kidney tissue samples fixed for TUNEL examination were dehydrated, embedded in paraffin and cut in tissue sections of 5 μm. The sections were then deparaffinized, rehydrated and treated with Protease K, after which they were incubated with the TUNEL reaction mixture in a humidified chamber at 37°C for 2 hours. The sections were washed with PBS (pH 7.4) to terminate the reaction and then treated with 4'-6-diamidino-2-phenylindole. Sections treated with DNase before TUNEL examination served as a positive control, and sections without terminal deoxynucleotidyl transferase were used as a negative control. From each section, 5 randomly selected fields (200 × magnification) were photographed with a fluorescence microscope. The number of TUNEL-positive cells in each field was counted using Image-Pro Plus 6.0 software and divided by the field area. The average apoptotic cell density of the 5 fields was then obtained for each group.

Results

Cytotoxicity and apoptosis in HK-2 cells – Glyphosate reduced statistically significantly the number of viable cells at ≥ 40 μM and increased LDH release from 40 μM on. In addition, the cytotoxic effect of glyphosate on HK-2 cells was found to be dose- and time-dependent upon prolongation of the exposure times. Cell viability of both the 40 and 60 μM exposure groups was reduced but still greater than 50% after 24 hours of exposure. Therefore, subsequent experiments were conducted with 0, 20, 40 and 60 μM with 24-hour exposure periods. In comparison with the control group, the glyphosate exposure groups showed increased percentages of apoptotic cells (Annexin V+, PI+/−), with significant differences occurring at concentrations greater than 40 μM. Furthermore, the expression of apoptosis-related proteins was investigated using Western blotting where in comparison to the control group glyphosate exhibited upregulated proapoptotic proteins (Bax and Bad) at 20 and 40 μM while downregulated antiapoptotic proteins (Bcl-2 and Bcl-xl) were evident at 40 and 60 μM. Cleaved caspase-3 levels were significantly upregulated at 40 and 60 μM.

Oxidative stress, NMDAR1 expression and calcium influx in HK-2 cells - In comparison with the control group, glyphosate increased statistically significantly cellular ROS levels at 40 and 60 μM. SOD, CAT and GSH-Px levels were statistically significantly reduced whereas there was a statistically significant increase in the MDA level. Glyphosate exposure increased NMDAR1 expression in a dose-dependent manner. Because NMDAR is involved in calcium influx and calcium homeostasis, $[Ca^{2+}]_i$ levels were determined after glyphosate exposure using flow cytometry-based measurement with the Ca^{2+}- sensitive probe Fluo-4/AM. It was found that glyphosate exposure increased $[Ca^{2+}]_i$ levels.

Effects after pretreatment with MK-801 - To examine the role of NMDAR in glyphosate-induced calcium influx and apoptosis, HK-2 cells were pretreated with the NMDAR inhibitor MK-801 at 100 μM for 12 hours followed by treatment with 40 μM glyphosate for 24 hours. MK-801 attenuated the upregulation of $[Ca^{2+}]_i$ in the HK-2 cells after 24 hours of glyphosate treatment. Inhibition of NMDAR was found to attenuate ROS increase in these cells and significantly decrease glyphosate-induced apoptosis.

Effects after pretreatment with BAPTA-AM and N-acetylcysteine - To examine the relationship between glyphosate-induced calcium influx and apoptosis, HK-2 cells were pretreated with 2 μM of an intracellular calcium chelator (BAPTA-AM) for 12 hours to decrease $[Ca^{2+}]_i$. Cells pretreated with BAPTA-AM and exposed to 40 μM glyphosate had lower $[Ca^{2+}]_i$ levels than cells exposed to glyphosate alone. BAPTA-AM pretreatment also significantly decreased glyphosate-induced apoptosis and ROS. To determine the role of ROS in glyphosate-induced apoptosis of HK-2 cells, cells were pretreated with 2 mM of a ROS scavenger (N-acetyl cysteine, NAC) for 12 hours before glyphosate treatment. NAC pretreatment was found to reduce glyphosate-induced ROS levels and apoptosis.

NMDAR1 expression and kidney damage - Glyphosate was administered orally to ICR mice for 28 days at a daily dose of 400 mg/kg bw to investigate its effects on the kidney *in vivo*. No statistically significant difference was found in body weight gain and relative liver and kidney weight between the control and the glyphosate exposure groups. Histopathological examination of the kidney identified exfoliation of renal tubular cells. The TUNEL assay confirmed the increase in renal tubular cell apoptosis in mice exposed to glyphosate. No statistically significant changes were observed in urine creatinine, uric acid, urea nitrogen, serum creatinine and blood urea nitrogen levels. A transient increase in urine albumin was observed after 7 and 14 days of treatment and urinary β2- microglobulin levels were statistically significantly increased after 7, 21 and 28 days of treatment. In addition, statistically significant reductions in the levels of SOD, CAT and GSH-Px, and a statistically significant increase in MDA was observed in the kidneys of the glyphosate treated group. Besides, the average optical density for NMDAR1 was found to be increased in kidneys from glyphosate treated mice.

Discussion and conclusion

Glyphosate exposure was found to increase the production of ROS. In *in vitro* and *in vivo* it was shown that MDA levels increased and that the activities of the major endogenous antioxidant enzymes SOD, CAT and GSH-Px decreased as a result of glyphosate exposure which is indicative of the disturbance of the pro-oxidant/antioxidant balance. To understand how this balance is disturbed the role of the N-methyl-D-aspartate receptor (NMDAR) was investigated because the structure of glyphosate is similar to that of glycine and glutamate which are both agonists of this receptor. NMDAR has been reported to mediate some renal diseases, such as hyperhomocysteinemia-induced glomerulosclerosis, gentamicin nephrotoxicity and lipopolysaccharide-induced renal insufficiency. In this study, using the human renal tubular epithelial cell line HK-2, the increased expression of NMDAR1 protein was demonstrated upon glyphosate exposure. In the animal tests, a clear upregulation of NMDAR1 in renal tissue was observed in tubular epithelial cells using immunohistochemical staining. Accompanied by the increase in NMDAR1 upon glyphosate exposure, [Ca2+]i, oxidative stress markers and apoptosis were all increased. Blocking NMDAR not only ameliorated glyphosate-induced increases in [Ca2+]i and ROS levels but also attenuated apoptosis. The increase in β2-microglobulin in the urine as observed in the *in vivo* study is indicative of an impairment of tubular reabsorption. From the results of this study it can be concluded that glyphosate could affect renal tubule epithelial cells via the NMDAR1/$[Ca^{2+}]_i$/ROS pathway both *in vitro* and *in vivo*. These findings provide a theoretical basis and reference data to assess the risk of

glyphosate and to explore the ethiology of chronic kidney disease.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study the effect of glyphosate on human proximal tubular epithelial cells was studied *in vitro* and on kidney *in vivo*. Tubular epithelial cells (HK-2) *in vitro* were exposed to glyphosate at concentrations ranging from 20 to 100 µM whereas mice were orally treated with glyphosate at 400 mg/kg bw/day for 28 days. The endpoints investigated for the *in vitro* study were cell viability, apoptosis, oxidative stress, intracellular Ca^{2+}, expression of the N-methyl-D-aspartate (NMDA) receptor and expression of proteins involved in apoptosis. The endpoints explored in the *in vivo* study in mice were kidney pathology biomarkers, oxidative stress in kidney tissue, kidney histopathology, NMDA receptor immunochemistry and apoptosis in kidney tissue. The results were that glyphosate was found to reduce cell viability, increase the incidence of apoptotic cells with an increase in the expression of apoptosis-related proteins, increase of oxidative stress in a concentration-related manner, increase of the expression of the NMDA receptor and increase Ca^{2+} influx. Kidney histopathology in mice treated with glyphosate at 400 mg/kg bw/day for 28 days revealed the exfoliation of renal tubular cells. It is postulated by the authors that glyphosate could affect renal tubule epithelial cells via the NMDAR1/$[Ca^{2+}]_i$/ROS pathway.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because some of the biochemical methods are not completely described, only one dose was used in the *in vivo* study and the pathology results from the *in vivo* study are not corroborated by regulatory 90-day repeated dose toxicity studies where no renal effects were seen in rats dosed up to more than 4,000 mg/kg bw/day and mice dosed up to more than 7,000 mg/kg bw/day.

Reliability criteria for *in vitro* toxicology studies

Publication: Gao *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)	Y	Purity of 96% as glyphosate monoisopropylamine salt. Source: Millipore Sigma, St. 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	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Concentration range *in vitro* from 20 to 100 µM. Only one dose (400 mg/kg bw/day) was used in the oral

		toxicity study in mice.
Cytotoxicity tests reported	Y	
Biochemical methods described	Y?	Some could be better documented.
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	*In vitro* but not *in vivo* (only one dose used).
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 some of the biochemical methods are not completely described, only one dose was used in the *in vivo* study and the pathology results from the *in vivo* study are not corroborated by regulatory 90-day repeated dose toxicity studies where no renal effects were seen in rats dosed up to more than 4,000 mg/kg bw/day and mice dosed up to more than 7,000 mg/kg bw/day.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Gigante P. *et al.*
Report year	2018
Report title	Glyphosate affects swine ovarian and adipose stromal cell functions
Document No	Animal Reproduction Science (2018) Vol. 195, 185–196
Guidelines followed in study	None
Deviations from current test guideline	NA
Previous evaluation	No
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The effect of glyphosate (GLY) at different doses (0.2, 4 and 16 μg/mL) was evaluated on granulosa cells growth (BrDU incorporation and ATP production), steroidogenesis (17-β estradiol and progesterone secretion) and redox status (superoxide and nitric oxide production and non-enzymatic scavenging activity). GLY has been shown to inhibit cell growth, 17-β estradiol and non-enzymatic scavenging activity and to increase progesterone and nitric oxide secretion ($P < 0.05$). In addition, GLY significantly decreased the viability of ASCs ($P < 0.001$), and inhibited their adipogenic differentiation. These data indicate that GLY alters the main features of granulosa cells and ASCS thus suggesting that GLY could affect both reproductive function and adipose tissues homeostasis.

Materials and methods

Chemicals – Glyphosate was purchased from Sigma Chemical Co, St Louis, USA. The purity was not reported.

Isolation and culture of granulosa cells - Swine ovaries were collected at a local slaughterhouse, placed in PBS at 4°C supplemented with penicillin (100 Ul/mL), streptomycin (100 Ul/mL) and amphotericin B (2.5 μg/mL) and transported to the laboratory within 1 hour. The samples were immersed for 1 minute in ethanol 70% before processing. Cystic or hemorrhagic follicles were discarded and the granulosa cells were harvested by aspiration of the follicles in the later state of maturation. The granulosa cells were then centrifuged at 450xg for 10 minutes and the cell pellet obtained treated with ammonium chloride to remove red blood cells. The cell number was estimated after vital staining with trypan blue whereafter the cells were plated and cultured in a validated serum free system composed of DMEM/Ham's F12 medium supplemented with penicillin (100 μg/mL), amphotericin B (2,5 μg/mL), streptomycin (100 μg/mL), sodium selenite (5 ng/mL) and transferrin (5 μg/mL) (CM). CM medium maintains the characteristics of granulosa cells and avoids luteinization.

Cell proliferation - Cell proliferation is measured using the BrdU cell proliferation ELISA assay. The granulosa cells were plated into 96-well plates (10^4 cells/100 μL CM) and incubated overnight with glyphosate at 0, 0.2, 4 or16 μg/mL. At the end, plates were centrifuged for 10 minutes at 400×g, the supernatants discarded and the cells dried and fixed. The DNA was denatured before the addition of the anti-BrdU antibody, conjugated with horseradish peroxidase (POD). The POD substrate used was tetramethyl-benzidine (TMB). Absorbance at 450 nm of the blue color formed is proportional to the amount of newly synthesized DNA. To quantify the viable cell number, the absorbance of each sample was related to a standard curve prepared by culturing, in quintuplicate, granulosa cells at different plating densities for 48 hours. The curve was repeated in four different experiments. The cell number/well was estimated from the resulting linear regression equation and was used to correct experimental data. The assay detection limit was 10^3 cell/well and the variation coefficient was less than 5%.

Cell viability - Granulosa cell viability was assessed using the ATP-lite bioluminescent assay based on the reaction of ATP luciferase and luciferin. The light emitted is proportional to the ATP concentration in the cells. The test was validated by plating different viable cell numbers (from 2.5×10^3 to 4×10^6/100 µL) three times. The relationship between cell number and luminescence was linear (r = 0.95). 2×10^5 cells/100 µL CM were seeded in 96-well plates and treated with glyphosate at 0, 0.2, 4 or 16 µg/mL for 48 hours. Kit reagents were added according to the instructions and luminescence was measured.

Granulosa cell steroid production - Granulosa cells at 10^4/200 µL in CM supplemented with androstenedione at 28 ng/mL were seeded in 96-well plates and treated with glyphosate at 0.2, 4 or16 µg/mL. After 48 hours incubation, culture media were collected, frozen and stored at −20 °C until determination of progesterone (P4) and 17-β estradiol (E2) by a validated RIA. P4 assay sensitivity and ED_{50} were 0.24 and 1 nmol/L, respectively. E2 assay sensitivity and ED_{50} were 0.05 and 0.2 nmol/L. The intra- and inter-assay coefficients of variation were less than 12% for both assays.

Granulosa cell superoxide (O_2^-) *production* - O_2^--production was measured using the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate) test which is based on the cleavage of the water soluble tetrazolium salt, WST-1, to the water soluble formazan. A total of 10^4 cells/200 µL CM were seeded in 96-well plates and incubated with glyphosate at 0, 0.2, 4 or 16 µg/mL for 48 hours. During the last 4 hours of incubation, 20 µL of WST-1 was added to the cells, and the absorbance determined at 450 nm against 620 nm. The coefficients of variation were less than 3%.

Granulosa cell nitric oxide (NO) production - 10^5 viable cells/200 µL CM were seeded in 96-well plates and treated with glyphosate at 0.2, 4 or 16 µg/mL for 48 hours. At the end, plates were centrifuged for 10 minutes at 400×g, the supernatants were collected and NO levels assessed by measuring nitrite after incubation with Greiss reagent (1% sulfanilamide, 5% phosphoric acid and 0.1% N-[naphtyl] ethylenediaminedihydrochloride). The absorbance was measured at 540 nm against 620 nm. A calibration curve ranging from 25 to 0.39 µM was prepared by diluting sodium nitrite in CM.

Non-enzymatic scavenging activity - The Ferric Reducing Activity of Plasma (FRAP) assay is a colorimetric method based on the ability of the antioxidant molecules present in a biological matrix to reduce ferric-tripiridyltriazine (Fe^{3+} TPTZ) to a ferrous form (Fe^{2+} TPTZ). 2×10^5 cells/200 µL CM were seeded in 96-well plates and treated with glyphosate at 0, 0.2, 4 or 16 µg/mL. At the end, the plates were centrifuged for 10 minutes at 400×g, the supernatants discarded and the cells lysed by adding, Triton 0.5% + PMSF in PBS at 200 µL/well in an ice bath for 30 minutes. The test was performed on 40 µL of cell lysates added to Fe^{3+} TPTZ reagent. After 30 minutes incubation at 37°C, absorbance of Fe^{2+} TPTZ was determined at 595 nm. The ferric reducing ability of cell lysates was calculated by plotting a standard curve of absorbance against $FeSO_4 .7H_2O$ standard solution.

Isolation and culture of adipose stromal cells (ASCs) from swine adipose tissue - Samples of about 5 g of subcutaneous abdominal adipose tissue were collected from pigs at a local slaughterhouse and placed in 20 mL of PBS supplemented with penicillin (100 Ul/mL), streptomycin (100 Ul / mL) and amphotericin B (2.5 µg/mL), transferred to Petri dishes in sterile conditions and shredded in fragments of about 3 mm^3. Thereafter, the adipose tissue fragments were distributed in 6-well plates, at a density of 10 fragments/well and then carefully covered with a minimal quantity (1 mL) of culture medium (CM_{ASC}) to avoid floating. CM_{ASC} was composed of low glucose DMEM + GlutaMax, supplemented with 10% FBS, penicillin (100 UI/mL), streptomycin (100 UI/mL) and amphotericin B (2.5 µg/mL). The plates were maintained at 37° in a humidified atmosphere at 5% CO_2 and 19% O_2. Every 48 hours CM_{ASC} was replaced. After 5 to 7 days, the adipose tissue fragments were removed from the culture plates and the remaining adherent cells, growing in monolayer, were cultured in fresh CM_{ASC} for 5 to 7 days, until 80% confluence was reached. The ASCs were the trypsinized and cultured in 25 cm^2 flasks with 5 mL CM_{ASC}. The ASCs obtained were subsequently either used to evaluate the expression of adipogenenesis-related marker genes or plated to study the effects of glyphosate at 4 µg/mL on cell viability and adipogenic differentiation.

Adipose stromal cells (ASCs) viability - Cell viability was evaluated using the MTT assay. ASCs were seeded in 96-well plates at 10^4 cells/well in 200 µL CM_{ASC} and incubated at 37°C in humidified atmosphere at 5% CO_2 and 19% O_2 for 48 and 72 hours with glyphosate at 0 or 4 µg/mL. At the end of each incubation period, 20 µL of 5 mg/mL of MTT were added and incubated for 4 hours. Subsequently, media were discarded and 100 µL of lysis solution (SDS10% in HCl 0.01 N) were added to the wells

and left overnight at 37°C. Absorbance was measured at 540 nm.

Adipose stromal cells (ASCs) adipogenic differentiation - ASCs were seeded in CM_{ASC} and incubated for 48 hours at 37°C in a humidified atmosphere at 5% CO_2 and 19% O_2 to permit cell adhesion. Adipogenic differentiation was performed by exposing cells to 3 cycles of 4 days each, of the following treatments:

- 3 days with induction medium: CM_{ASC} supplemented with dexamethasone (DEX) at 1 μM, insulin (INS) at 1.7 μM, 3-iso-butyl-1-methylxanthine (IBMX) at 0.5 mM and indomethacin at 250 μM.
- 1 day with differentiation maintaining medium: CM_{ASC} supplemented with insulin (INS) at 1.7 μM.

Negative controls were performed using ASCs cultured in CM_{ASC}. Cell differentiation was evaluated by Oil Red O Staining to detect cytoplasmic lipid vacuoles as markers of adipogenesis.

Expression of adipogenic marker genes - In order to evaluate the expression of adipogenic marker genes crucial for the function of adipose tissue, PPARγ and Leptin total RNA was extracted from ASCs pellets (7×10^5 cells), subjected or not to adipogenic differentiation, using NucleoSpin RNA II. cDNA was then obtained by reverse transcription of 2 μg of the extracted RNA using the High-capacity cDNA reverse transcription kit. The expression of the genes was evaluated by PCR (Polymerase Chain Reaction) using the primers for PPARγ, leptin and GADPH. The occurrence of the amplification was verified by 2.5% agarose gel electrophoresis in TAE buffer (Tris base, glacial acetic acid, EDTA 0,5 M, pH 8), where the correct length of the amplicons was verified by comparison with Gene Ruler™ 100 bp DNA LaddeR markers. DEPC treated water was used instead of cDNA as a negative control for all reactions. The DNA patterns were displayed under UV light and the images were captured with a PowerShot A610 Canon camera.

Solubilisation and quantification of Oil Red O production - To evaluate intracellular lipid accumulation, 20×10^4 ASCs were plated in 24 wells plates and subjected to a differentiation process with glyphosate at 0 or 4 μg/mL. After 12 days the cells were washed 3 times with PBS, fixed with 4% formaldehyde for 1 hour and washed with 60% isopropanol. The cells were then dried and stained with Oil Red O staining solution for 30 minutes. After multiple washings, Oil Red O was solubilized in each well with 60% isopropanol for 2 hours and the absorbance measured at 540 nm. Absorbance was considered to be proportional to the amount of differentiated cells.

Adipose cell counting - 5×10^4 MSCs were seeded on a cover slip in 6-well plates and subjected to the adipose differentiation process with glyphosate at 0 or 4 μg/mL. After 12 days, cover slips were fixed with formalin (4%) and stained with Oil Red O. Then, cells were stained in Mayer haematoxylin for 3 minutes and images made by light microscopy (total magnification 10x) and quantified by counting the stained cells in 10 different fields.

Statistical analysis - The experiments were performed five times on granulosa cells and three times on ASCs and six replicate wells were used for the assessment of the effect of glyphosate. Every time the adipose tissue was collected from 3 pigs. Data are presented as the mean ± SEM. Statistical differences were calculated by ANOVA using Statgraphics software. In the presence of a significant difference ($p < 0.05$), the means were subjected to Scheffè' F test for multiple comparisons.

Results

The effect of glyphosate on swine granulosa cells - Glyphosate statistically significantly decreased cell proliferation ($p < 0.001$) as evaluated by BrdU incorporation and cell viability ($p < 0.05$) as measured by ATP production without a concentration-effect relationship. Granulosa cell steroidogenesis was affected by glyphosate. E2 secretion was statistically significantly inhibited ($p < 0.05$) whereas P4 secretion was statistically significantly increased ($p < 0.05$) both at all tested concentrations but without a concentration-effect relationship. While O_2^--production was not statistically significantly modified by glyphosate at any of the concentrations tested, NO production was significantly stimulated ($p < 0.001$) at all the tested concentrations although with no statistically significant differences amongst them. Scavenging activity, represented by non-enzymatic antioxidant power, was significantly inhibited ($p < 0.05$) by glyphosate at all the concentrations tested but also without significant differences among them.

The effect of glyphosate on swine adipose stromal cells (ASCs) - The histological analysis of the isolated fragments obtained from pig fat showed typical characteristics of adipose tissue. In particular, adipocytes containing a single large lipid droplet with thin cytoplasm and flattened peripheral nucleus are surrounded by a microvasculature dispersed in the stromal tissue. After 3 to 4 days of *in vitro* culture of the fragmented explants, there was a migration of cells characterized by typical mesenchymal fibroblast-like morphology, which proliferated in adhesion to the bottom of the well. After 5 to 7 days from fragment removal, ASCs reached 80% confluence in monolayer, maintaining their morphological characteristics. The proliferation of ASCs under basic culture conditions was statistically significantly increased ($p < 0.001$) (454 ± 12 vs 476 ± 7), while glyphosate treatment at 4 μg/mL significantly decreased ($p < 0.001$) the viability of proliferating ASCs both after 48 (432 ± 10) and 72 hours (451 ± 7). The expression of PPARγ and LEP adipogenic markers was not observed in undifferentiated ASCs, but was detected following adipogenic differentiation. Adipogenic differentiation of ASCs was achieved, as shown by the appearance of red lipid droplets in the differentiated cell cytoplasm. Differentiated cell counts showed a significant inhibition ($p < 0.05$) of the adipogenic process by glyphosate at 4 μg/mL. These data were confirmed by spectrophotometric evaluation of solubilized Oil RedO.

Discussion

This study shows that glyphosate is able to significantly stimulate P4 production. This is a critical effect for granulosa cells because P4 is a hallmark of luteinization. Ovarian steroidogenesis is a key function for the survival of GCs, and is essential for follicular development and growth. To verify the potential effects of glyphosate on follicular physiology, the present study also analyzed the possible effect of glyphosate on granulosa cell proliferation and metabolic activity. The data show an inhibition of DNA replication activity and ATP production at all concentrations analyzed suggesting a disruption of follicular development in vivo. Since endothelial cell characteristics have been recently attributed to granulosa cells, it is interesting to evaluate the increased NO production in granulosa cells following glyphosate exposure. It can be hypothesized that glyphosate exerts direct pro-angiogenic effects on pigs GCs at all tested concentrations as a result of a significant increase in NO, which is a key molecule of this process. The stimulation of NO production can also be associated with an increase in oxidative stress which seems to be supported by the glyphosate-induced reduction of the non-enzymatic antioxidant power in GCs at all concentrations tested. Adipose stromal cells (ASCs) were isolated from abdominal subcutaneous fat tissue of pig and characterized. Their ability to differentiate into adipocytes under appropriate stimuli has been evaluated on the basis of the expression of PPARγ and leptin genes that was not detected in undifferentiated ASCs. These results were essential to define an adequate experimental model to investigate potential effects of glyphosate on the expression of these adipogenic markers. The inhibitory effect of glyphosate during induction of ASCs adipogenic differentiation suggests that this substance, once accumulated as a consequence of environmental exposure, can interfere with adipose tissue biology *in vivo*. Moreover, glyphosate was found to significantly decrease the viability of ASCs after 48 and 72 hours of exposure. Such evidence appears to be critical since it suggests a direct interference of glyphosate with adipose tissue function.

Conclusion

In this *in vitro* study it is shown that glyphosate inhibits cell growth, 17-β estradiol production and non-enzymatic scavenging activity and increased progesterone production and nitric oxide secretion in granulosa cells. In addition, glyphosate significantly decreased the viability of adipose stromal cells (ASCs) and inhibited their adipogenic differentiation. These data are indicative of the interference of glyphosate with the main functional parameters of granulosa cells and ASCs and could affect both reproductive function and adipogenic processes *in vivo*.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The effects of glyphosate on functional parameters of granulosa cells and adipose stromal cells from swine were investigated *in vitro*. In granulosa cells the effect of glyphosate was studied on cell proliferation, cell viability, steroid production, superoxide anion production, NO production and ferric reducing activity. In adipose stromal cells the effect of glyphosate was studied on cell viability, adipogenic differentiation, adipogenic marker genes (PPARγ and leptin), intracellular lipid accumulation and adipose cell count. Glyphosate was found to significantly decrease cell proliferation, cell viability, estrogen production and ferric reducing capacity and increase progesterone and NO production in granulosa cells when tested at concentrations ranging from 0.2 to 16 µg/mL. However, in none of the assays with granulosa cells a concentration-effect relationship was established. Glyphosate treatment at 4 µg/mL significantly decreased ($p < 0.001$) the viability of proliferating adipose stromal cells after 48 and 72 hours. Differentiated cell counts showed a significant inhibition ($p < 0.05$) of the adipogenic process by glyphosate at 4 µg/mL. Since only one concentration of glyphosate was tested it was not possible to establish a concentration-effect relationship. In this publication it is suggested that glyphosate interferes with the main functional parameters of the granulosa cell which could affect reproductive function. No effects on female reproductive function were reported in the rat in regulatory reproductive toxicology tests at doses beyond 2,000 mg/kg bw/day producing systemic glyphosate concentrations that are higher than those tested in this study.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized, no positive controls were included in the assays and only one dose level was used for the testing of adipose stromal cells.

Reliability criteria for *in vitro* toxicology studies

Publication: Gigante *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 is not reported. Source: Sigma Chemical Co, St 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	Granulosa cells from swine ovaries, adipose stromal cells from swine adipose tissue.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.2, 4 or 16 µg/mL.
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No 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		
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, no positive controls were included in the assays and only one dose level was used for the testing of adipose stromal cells.		

1. Information on the study

Data point:	CA 5.6
Report author	Gorga A. *et al.*
Report year	2020
Report title	In vitro effects of glyphosate and Roundup on Sertoli cell physiology
Document No	Toxicology in Vitro 62 (2020) 104682
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 aim of the present study was to analyze whether glyphosate (G) and Roundup (R) are able to affect Sertoli cell functions, such as energy metabolism and blood-testis barrier (BTB) integrity. Sertoli cell cultures from 20-day-old rats were exposed to 10 and 100 ppm of G or R, doses which do not decrease cell viability. Neither G nor R caused impairment in lactate production or fatty acid oxidation. G and R decreased Transepithelial Electrical Resistance, which indicates the establishment of a Sertoli cell junction barrier. However, neither G nor R modified the expression of claudin11, ZO1 and occludin, proteins that constitute the BTB. Analysis of cellular distribution of claudin11 by immunofluorescence showed that G and R induced a delocalization of the signal from membrane to the cytoplasm.

Materials and methods

Materials: [2,6-3H]-2-deoxy-D-glucose (2-DOG) and [9,10(n)-3H] palmitic acid were purchased from NEN (Boston, MA, USA). Culture media, glyphosate and all other drugs and reagents were purchased from SigmaAldrich (St Louis, MO, USA). The glyphosate formulation used in this work was the formulation available on the market called Roundup Full II (Monsanto Argentina S.A.I.C.), which contains 54% w/v acid glyphosate.

Sertoli cell (SC) isolation and culture: Twenty-day-old Sprague-Dawley rats (Rattus norvegicus) were obtained from the Animal Care Laboratory, Facultad de Ciencias Veterinarias, Buenos Aires, Argentina. Animals were killed by CO2 asphyxiation according to protocols for animal laboratory use following the principles and procedures outlined in the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Comité Institucional de Cuidado y Uso de Animales de Laboratorio (CICUAL) from the Hospital de Niños “Dr. Ricardo Gutiérrez”. SC were isolated as previously described. Decapsulated testes were digested with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in Hanks' balanced salt solution (HBSS) for 5 min at room temperature by manual agitation. The enzymatic action was stopped by dilution with four volumes of HBSS. Seminiferous tubules were collected by sedimentation and washed twice with HBSS. Then seminiferous tubules were cut with a razor and submitted to 1M glycine-2mM EDTA (pH 7.4) treatment for 10 min to remove peritubular cells. At the end of the incubation period, nine volumes of HBSS were added and a 30 min sedimentation was performed. The washed tubular pellet was then digested again with 0.1% w/v collagenase and 0.006% w/v soybean trypsin inhibitor in HBSS for 10 min at room temperature by continuous pipetting. The enzymatic action was stopped by dilution with four volumes of HBSS. The cell suspension was collected by centrifugation at 200 x g for 3 min. The cell suspension was diluted with HBSS and submitted to a 10 min sedimentation to remove germ cells. The pellet containing SC was filtered through a nylon mesh and SC were recovered by centrifugation at 200 x g for 3 min. SC were resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 10mM HEPES, 100 IU/ml penicillin, 2.5 μg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 μg/ml transferrin, 5 μg/ml insulin, 5 μg/ml

vitamin E and 4 ng/ml hydrocortisone. SC were cultured on 6-, 24- or 96-multiwell plates (5 μg DNA/cm2), on Matrigel-coated cell culture inserts (15 μg DNA/cm2) placed on 24- multiwell plates or on glass coverslips coated with laminin at 34 °C in a mixture of 5% CO2:95% v/v air. No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to SC cultures using a specific antiserum to smooth muscle α actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5% after 48 h in culture as examined by phase contrast microscopy.

Culture conditions: SC were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. Treatment with glyphosate (G) and Roundup (R) was performed with variable doses and for variable periods of time. Cells incubated for 48 h with 10, 100 and 1000 ppm of G and R harvested on day five were used to evaluate cell viability and LDH leakage. The cells treated for 48 h with 100 ppm of G and R were harvested on day five and used to evaluate GLUT1, FAT/CD36 and CPT1 mRNA levels, glucose uptake and fatty acid (FA) oxidation and the 48 h-conditioned media were utilized to evaluate lactate production. For Western blot studies, cells cultured for 4 days under basal conditions and pretreated for 30 min with 10 and 100 ppm of G or R were used. To quantify Transepithelial Electrical Resistance (TER), SC were cultured at high cell density (15 μg DNA/cm2, corresponding to 1.2 × 106 cells/cm2) on Matrigel-coated (1,6 dilution with F12/DMEM v/v) cell culture inserts (Millicell HA inserts) (Millipore, Billerica, MA, USA) placed on 24-multiwell plates. On day 3 in culture, testosterone was added and TER across the SC monolayer was recorded every 24 h in culture. On day 5, when the tight junction barrier had been formed, different doses of G or R were added and TER was recorded until day 8. To study the distribution and localization of claudin11, the cells were cultured on glass coverslips coated with laminin and treated with 100 ppm G or R in the presence or absence of testosterone for 48 h and harvested on day 5.

Evaluation of Sertoli cell energetic metabolism: Energetic metabolism in SC has been considered to have features of its own. Lactate, produced by SC, provides the energetic substrate to germ cells in the adluminal compartment. Consequently, it has been postulated that SC utilizes FA as their energy source. In this context, lactate production, glucose uptake, FA oxidation and the expression of genes involved in these processes were evaluated.

a) Lactate determination: Conditioned media obtained from cells cultured in 24-multiwell plates were used to determine lactate production. Lactate was measured by a standard method involving conversion of NAD+ to NADH.

b) Measurement of 2-deoxyglucose (2-DOG) uptake: Glucose transport was studied using the uptake of the labelled non-metabolizable glucose analogue 2-DOG on cells cultured in 24-multiwell plates as previously described.

c) Fatty acid oxidation assay: FA oxidation was performed measuring the release of 3H2O to the incubation medium from [3H]-palmitate on SC cultured in 24-multiwell as previously described

Evaluation of BTB function: The main component of the BTB is the presence of tight junctions between neighboring SC. In order to evaluate BTB function, Transepithelial Electrical Resistance (TER), claudin11 cellular distribution and the expression of proteins that participate in tight junction assembly were evaluated.

a) Transepithelial Electrical Resistance (TER) measurement: The establishment of the SC junction barrier was assessed daily from day 3 to day 8 by measurement of TER across the SC monolayer by a Millicell electrical resistance system (Millipore), as described previously. Briefly, a short (~2 s) 20-μA pulse of current was passed through the epithelial monolayer between 2 silver-silver chloride electrodes and electrical resistance was measured. Electrical resistance was then multiplied by the surface area of the insert to yield the area of resistance in ohms.cm2. The net value of electrical resistance was then computed by subtracting the background, which was determined by Matrigel-coated cell-free inserts.

Each time point had quadruplicate bicameral units. This experiment was run four times on different batches of cells.

b) Immunofluorescent (IF) detection of claudin11 protein: Monolayers were fixed with methanol for 10 min at -20 °C. After washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature. After 3 washes with PBS for 1 min each, the cells were blocked with 5% bovine serum albumin (BSA). Then, the coverslips were incubated with a 1:50 dilution of polyclonal antibody against claudin11 in PBS overnight at 4 °C. After 3 washes with PBS for 1 min each, coverslips were incubated with an anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated (1,25; Vector Laboratories, Burlingame, CA, USA). For negative controls, primary antibodies were replaced by PBS. Finally, the coverslips were washed 3 times with PBS for 1 min each, mounted in buffered glycerine and observed using an Axiophot fluorescent microscope with epi-illumination (Carl Zeiss Inc., Oberkochen, Germany).

RT-Real-time PCR (RT-qPCR): The expression of genes that participate in energetic metabolism (GLUT1, FAT/CD36 and CPT1) and in BTB organization (occludin, claudin11 and ZO-1) was evaluated by RT-qPCR. Total RNA was isolated from SC cultured in 6-multiwell plates with TRI Reagent (Sigma-Aldrich). The amount of RNA was estimated by spectrophotometry at 260 nm. Amplification was carried out as recommended by the manufacturer: 25 µl reaction mixture containing 12.5 µl of SYBR Green PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µl of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalized to HPRT1. The amplification program included the initial denaturation step at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative ΔΔCt method was used to calculate relative gene expression.

Western blot analysis: Cells cultured in 6-multiwell plates were washed once with PBS at room temperature. Then, 200 µl of PBS containing 2 µl of protease inhibitor cocktail (P-8340; Sigma-Aldrich), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50 nM okadaic acid and 2 mM PMSF was added to each well. Cells were then placed on ice and disrupted by ultrasonic irradiation. Western blot analysis was performed as previously described. Membranes were probed with antibodies that allow specific recognition of total Akt and mTOR, phosphorylated p38-MAPK and ERK1/2 (Cell Signaling Technology, Inc., Danvers, MA, USA), claudin11 (Zymed Lab. Inc.), androgen receptor and GAPDH (Santa Cruz Biotechnology, Inc., USA). A 1:1000 dilution of primary antibodies, as indicated by the manufacturer, was used. For chemiluminescent detection of the blots, a commercial kit from Cell Signaling Technology was used. The intensities of the autoradiographic bands were estimated by densitometry scanning using NIH Image Software (Scion Corporation). Levels of the corresponding total Akt, mTOR and GAPDH served as loading controls.

Cytotoxicity: A cell viability test (MTT assay) was performed in cells cultured in 96-multiwell using a commercial kit (CellTiter 96® AQueous NonRadioactive Cell Proliferation Assay; Promega Corporation). Cell cytotoxicity was determined by measuring the activity of LDH enzyme leaked from the cytosol of damaged cells into the medium as previously described. Results were expressed as the percentage of activity detected in the media over the sum of the activities in the media and in cells.

Statistical analysis: All experiments were run in triplicates and repeated 3–4 times. One way ANOVA and post hoc analysis using Tukey-Krämer's multiple comparisons test were performed using InfoStat versión 2016 (Grupo InfoStat, FCA, UNC, Argentina). P values < 0.05 were considered statistically significant.

Results

Effects of glyphosate and Roundup on SC cell viability: SC cultures were exposed for 48 h to glyphosate (G) and Roundup (R) at concentrations ranging from 10 to 1000 ppm, corresponding to 0.01 to 1 g/L respectively. Cell viability was analyzed by MTT assay and by measuring LDH leaked from the cytosol of damaged cells into the medium. The highest dose tested for R (1000 ppm) caused a cell death (Fig.

1). Therefore, in the present investigation destined to analyze G and R effects on SC functions only doses of 10 and 100 ppm were utilized.

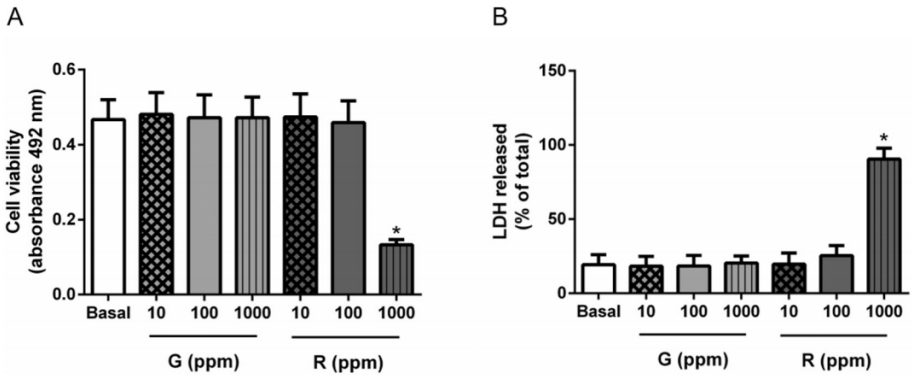


Fig. 1. Effect of G and R on SC cytotoxicity.
SC were maintained under Basal conditions or incubated with 10, 100 or 1000 ppm of G or R for 48 h. (A) Cell viability was determined by MTT assay. (B) LDH activity was determined in SC monolayer and in the culture medium. Values represent mean ± S.D. of one representative experiment out of three. *$p < 0.05$ versus Basal.

Effects of glyphosate and Roundup on SC energetic metabolism: SC cultures were exposed for 48 h to 100 ppm of G and R. Fig. 2 shows the results obtained for lactate production, glucose uptake and GLUT1 mRNA levels. The exposure to G or R did not modify lactate production neither glucose uptake nor GLUT1 expression.

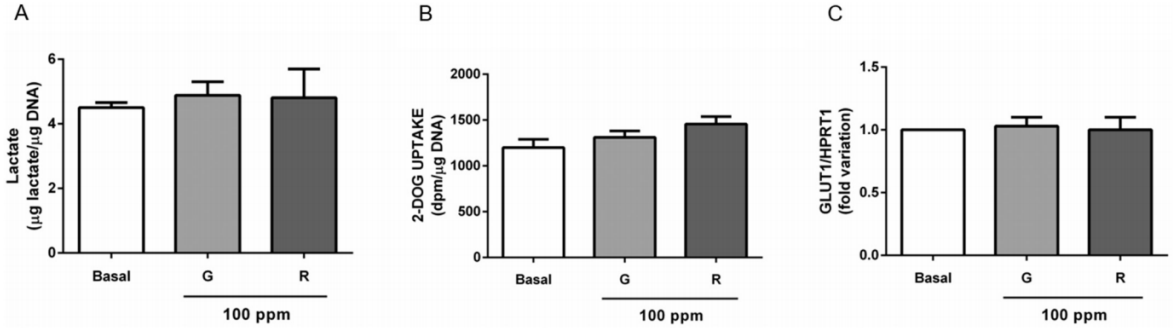


Fig. 2. Effect of G and R on lactate production, glucose uptake and on *Glut1* mRNA levels in SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. (A) Lactate levels were determined in the conditioned media. (B) Glucose uptake assay (2-DOG uptake) was performed after the 48 h incubation period. (C) Total RNA was extracted and RT-qPCR was performed to detect *Glut1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

Effects of glyphosate and Roundup on blood-testis barrier integrity: SC supply germ cells with a microenvironment preserved by the BTB. The main component of the BTB is the presence of tight junctions between neighboring SC. The establishment of these junctions between SC in culture was assessed daily from day 3 to day 8 by measuring TER across the SC monolayer. When SC were plated, junctions begin to assemble and an increase in TER was observed. On day 5, when SC completed barrier assembly, G or R were added to the culture medium. Compared with Control (92.3±3.1), a significant decline in TER was produced by the addition of 100 ppm of G (58.0±1.5) or 10 and 100 ppm of R (67.8±2.8 or 62.2±4.9, respectively)(Fig. 4 and Table 2).

Table 2
Effect of 48-h treatment with G and R on TER.

	TER ($\Omega \cdot cm^2$)
Control	92,3 ± 3,1
G 10 ppm	85,5 ± 4,5
G 100 ppm	58,0 ± 1,5*
R 10 ppm	67,8 ± 2,8*
R 100 ppm	62,2 ± 4,9*

SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G or R on day 5 for 48 h. Results are presented as means ± SD of four independent experiments (*$p < 0.05$).

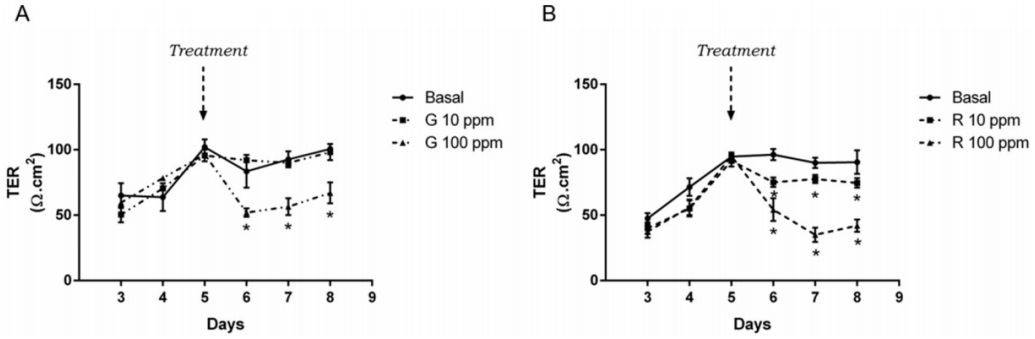


Fig. 4. Effect of G and R on TER across SC.
SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G (A) or R (B) on day 5. TER across SC monolayer was measured from day 3 to 8. Values represent mean ± S.D. of triplicate wells from the same culture of one representative experiment out of four. Asterisks indicate significant differences from basal cultures for each particular day, $p < 0.05$.

Considering that it had been demonstrated that p38-MAPK and ERK1/2 signaling pathways were involved in the disruption of BTB integrity by xenobiotics, the possible alteration of these pathways by G or R treatment was evaluated. R increased P-p38-MAPK and P-ERK1/2 levels. G did not modify P-p38-MAPK and P-ERK1/2 levels at any dose tested. 100 ppm G or R treatment did not modify *claudin11*, neither *occludin* nor *ZO-1* mRNA levels. Claudin11 protein levels were not modify by G or R treatment. In control conditions, claudin11 was detected at the zone of contact between adjacent cells, in a linear and continuous pattern that delineated cell borders in basal conditions. Addition of 100 ppm G or R induced redistribution of claudin11 since immunofluorescence became discontinuous and was redistributed from the cell surface into the cytoplasm.

Effects of glyphosate and Roundup on testosterone regulation of bloodtestis barrier integrity; It is well known that testosterone is the main regulator of BTB function, and that G or R can act as endocrine disruptors. In order to elucidate a possible mechanism responsible for adverse effects of G or R, we decided to evaluate whether herbicides can interfere with androgen action in BTB. Fig. 8A and B shows that 100 ppm G or R treatment did not modify androgen receptor mRNA or protein levels. Fig. 9 shows that, similar to what was observed under basal conditions, 100 ppm G or R treatment induced a redistribution of claudin11 from cell membrane to cytoplasm under the presence of testosterone.

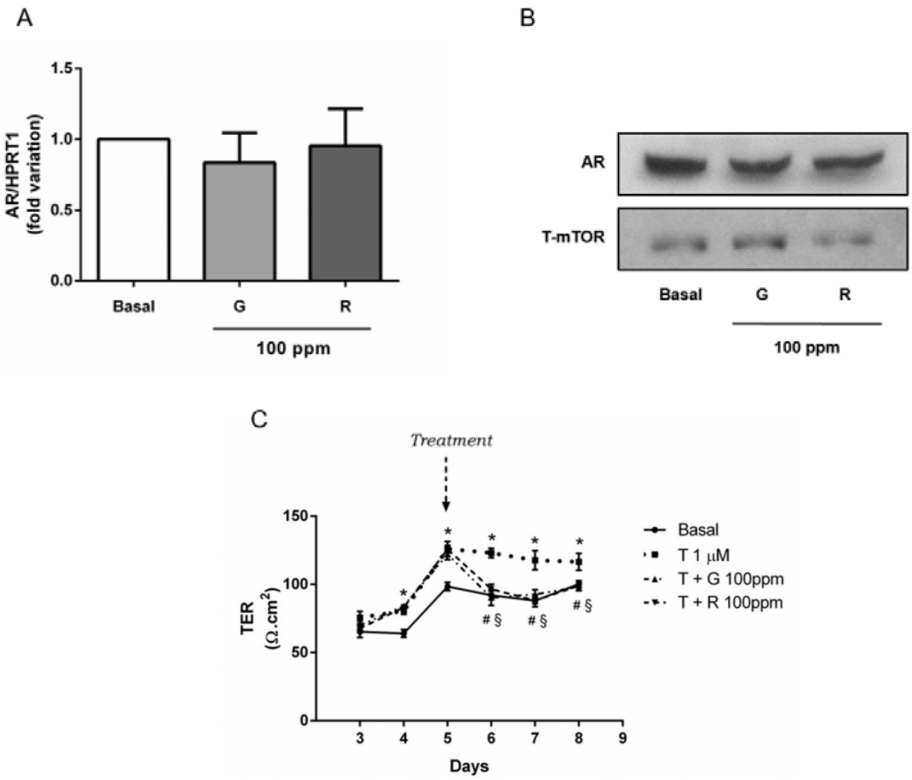


Fig. 8. Effect of G and R on androgen receptor (AR) expression and testosterone regulation of TER across SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. A) Total RNA was extracted and RT-qPCR was performed to detect AR mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal. B) Western blot analysis was performed utilizing antibodies for AR or total Akt (T-Akt). Results are representative of three independent experiments performed/treatment group. C) SC monolayers were maintained under Basal conditions or stimulated with testosterone (T) since day 3. On day 5, SC monolayers were treated with 100 ppm of G or R. TER across SC monolayer was measured from day 3 to 8. Values represent mean ± S.D. of triplicate wells from the same culture of one representative experiment out of three. Symbols indicate significant differences for each particular day: *p < 0.05 T vs Basal; #p < 0.05 T vs T + G; §p < 0.05 T vs T + R.

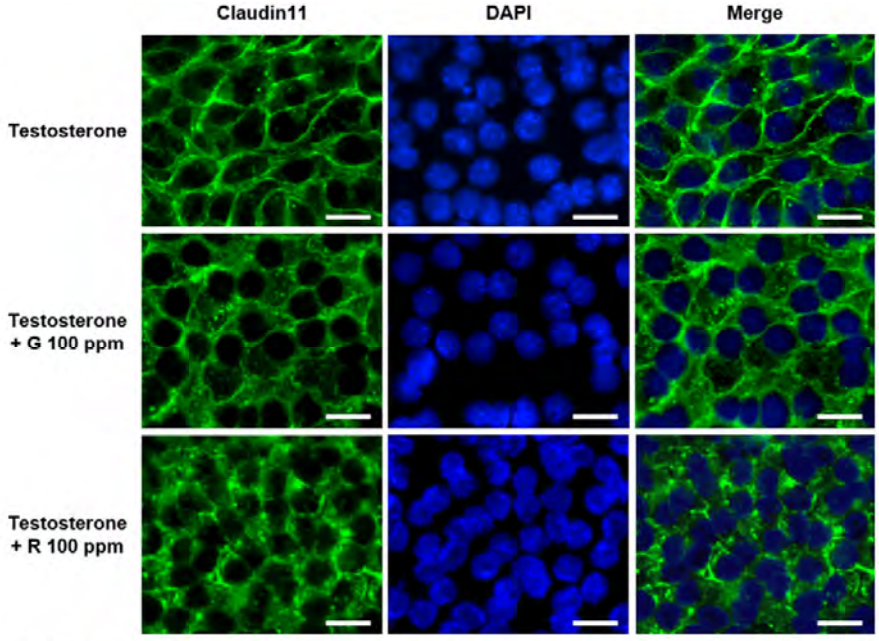


Fig. 9. Effect of G and R on claudin11 localization in the presence of testosterone in SC.
SC were incubated with 100 ppm of G or R in the presence of testosterone for 48 h. Claudin11 was revealed by IF. Bars: 50 μm.

Discussion

Over the last 60 years a progressive decrease in male reproductive function has been observed. Epidemiological and experimental studies suggest that one of the main causes is exposure to environmental toxicants. As previously stated, several studies have shown that G or R can potentially

cause adverse effects in male reproduction. Despite the compelling documented evidence proving the existence of adverse effects on testis function, little is known about direct effects on Sertoli cell function and the possible mechanisms involved. Primary cultures of Sertoli cells are a good and reliable model to assess the direct effects of xenobiotic exposure on this cell type. Regarding G and its commercial formulation R, initial studies on Sertoli cell cultures utilizing doses from 1 to 10,000 ppm of G and R were performed. In these experiments, treatment with 1000 ppm R produced Sertoli cell apoptosis with a maximal effect in 24 h-incubation period. However, in the same study G did not induce apoptosis in isolated Sertoli cells. Other studies, which measured apoptosis and/or cell viability, have also demonstrated that several human cell lines are more sensitive to R than to G. Coincidentally, the results presented herein show that 1000 ppm R decreases cell viability while G does not. These differences on the actions of both agents can be interpreted by the presence of various adjuvants in R. These adjuvants change human cell permeability and amplify toxicity by G, which may explain the differences observed between R and G effects on Sertoli cell viability. As we were focused on analyzing possible effects on Sertoli cell function, in this study we utilized 10 and 100 ppm doses which do not decrease cell viability. As mentioned in the introduction, two essential functions of mature Sertoli cells are the provision of nutrients to germ cells and the maintenance of a favorable microenvironment for spermatogenesis. As for Sertoli cell nutritional function, it has been well documented that Sertoli cell glycolysis provides lactate to satisfy germ cell energy demands while FA oxidation supplies energy to fulfill Sertoli cell energetic demands. Consequently, it can be predicted that an alteration in Sertoli cell metabolism can lead to a perturbation in normal germ cell development. The results obtained in the present investigation show that neither G nor R modify lactate production, glucose uptake and GLUT1 expression in Sertoli cells. Additionally, the results show that G and R do not modify FA oxidation and FAT/CD36 and CPT1 expression, proteins that are essential for FA utilization. Altogether, the results presented herein lead us to conclude that G and R effects on testicular function are not mediated by impairment of Sertoli cell metabolism. Noticeably, other reproductive toxicants, including phthalate esters, nitro-benzene, gossypol and certain heavy metal ions, alter Sertoli cell lactate production and show that toxicant effects strongly depend on the chemical nature of the toxicant. Regarding lipid metabolism, it has been demonstrated that 24 h exposure to 5000 ppm G or R induce lipid droplet accumulation in the Sertoli cell line TM4 cytoplasm. This increase in lipid droplet accumulation was interpreted as storage of potentially deleterious lipophilic formulants in the cytoplasm of TM4 cells and was considered a sign of the cytotoxic effect. An alternative explanation for the above results would be a modification in lipid metabolism, however, the authors did not explore this possibility. We have explored the latter possibility and the results presented herein show that 100 ppm G and R, doses that do not affect Sertoli cell viability, do not alter FA oxidation. It is worth mentioning that Sertoli cells, in addition to the nourishment provided to germinal epithelia, supply germ cells with a suitable microenvironment for successful meiosis and completion of spermatogenesis. This microenvironment is sustained by the BTB, whose main components are the tight junctions between neighboring Sertoli cells. The BTB is highly dynamic and is regulated by an array of intriguingly coordinated signaling pathways and molecules. Several studies have shown that many environmental toxicants, such as cadmium, bisphenol A, fluoride and sulfur dioxide exert their effects by targeting Sertoli and germ cell junctional proteins, as well as the permeability of the BTB. TER is a widely accepted quantitative technique that measures the integrity and permeability of BTB in vitro. The results presented in this investigation show that 100 ppm G and 10 and 100 ppm R decrease TER after 24 and 48 h-treatment. Notable, the disrupting effect of G on the permeability of other barriers had already been observed. It was shown that exposure to 10 mg/ml (10,000 ppm) G reduces TER and increases permeability to mannitol in Caco-2 and IEC-18 intestinal cell lines. In addition, 1 and 10μM G treatment (0.16 and 1.6 ppm) decreases TER and increases permeability to fluorescein in iPSC-derived brain microvascular endothelial cells cell line (BMECs). Results from other authors and our own let us postulate that one of the mechanisms by which G exerts toxic effects is related to disruption of barrier properties in different important organs such as intestine, brain and testis. It has been shown that p38-MAPK and ERK1/2 pathways play central roles in the dynamics of BTB. For example, TGFb3-induced physiological effect on Sertoli cell BTB dynamics is mediated via the p38-MAPK pathway. As for toxicant effects, it has been demonstrated that the polychlorinated biphenyls (PCBs), such as congener Aroclor1254, and the perfluorooctane sulfonate (PFOS) can disrupt the BTB integrity by activating the p38-MAPK pathway. The present investigation shows that R increases P-p38-MAPK and PERK1/2 levels. Therefore, it might be suggested that R decreases TER through a p38-MAPK and ERK1/2 dependent pathways. However, the same reasoning

cannot be applied to the effects of G considering that it lowers TER while it does not modify P-p38-MAPK and P-ERK1/2 levels. Hence, it seems that there is no direct relationship between P-p38- MAPK and P-ERK1/2 levels and TER levels, at least as a consequence of G and R exposure. We then decided to look for further mechanisms that may be involved in the disruption of BTB integrity by exposure to G and R. The next set of experiments was devoted to analyze possible alterations in the expression of some tight junction proteins, such as occludin, claudin11 and ZO-1, in response to G or R. An increased expression of these proteins, at the time when the junctions are assembled as manifested by a stable TER across the Sertoli cell epithelia, was demonstrated. It is worth mentioning that certain agents that perturb BTB permeability, such as cytokines or toxicants, alter the expression of cell junction proteins. The results obtained in the present investigation show that neither occludin nor claudin11 or ZO-1 expression was modified by G or R treatment. Finally, we decided to investigate whether the cellular localization of a tight junction protein such as claudin11 could shed some light on the mechanism underlying the decrease in TER. Claudin11 is a key molecule that provides functional integrity to the BTB. It is located in functional Sertoli cell tight junctions and its intracellular distribution pattern changes when gonadotropins are suppressed, coincident with a dysfunctional barrier. Claudin11 null mice are sterile highlighting the importance of this protein for BTB integrity. It is worth mentioning that in men with testicular disorders such as intraepithelial neoplasia, hypospermatogenesis, spermatogenic arrest, and Sertoli cell only testes, claudin11 is located in the cytoplasm, away from the tight junctions reinforcing the idea that localization of claudin11 in cytoplasm can be considered a sign of BTB dysfunction. In in vitro studies, a direct relationship between TER and localization of claudin11 in membrane junctions has been observed. The results presented herein show that although G and R treatment does not modify claudin11 protein levels, a redistribution of claudin11 from membrane to cytoplasm is observed. This alteration in the distribution of claudin11 may be interpreted as the result of an increase in membrane protein recycling from cell surface to cytoplasm. This last result may explain, at least in part, the effects of G and R in the integrity of BTB and the deleterious effect of these toxicants at testicular level. Disruption of the Sertoli cell permeability barrier by loss of usual distribution patterns of other junctional proteins such as occludin, ZO-1, and Cx43 induced by other toxicants has also been demonstrated and these results indicate that redistribution of these proteins can also contribute to alter barrier permeability. Further studies will be necessary to definitively assign a role to this phenomenon in the alteration of BTB dynamics by G or R. Several lines of evidence obtained from in vivo and in vitro approaches highlight the importance of testosterone in the regulation of BTB assembly and function. In vivo studies showed that reestablishment of testicular androgen levels by hCG or testosterone treatment leads to a restoration of claudin11 localization and BTB function. In vitro studies showed that Sertoli cell permeability barrier formation and claudin11 localization are promoted by androgen treatment. Furthermore, numerous studies describe G or R as endocrine disruptors. On the one hand, it was demonstrated that in vivo treatment of drakes with R decreases the expression of the androgen receptor (AR) in Sertoli cells while in rats, G treatment does not modify AR expression in the same cell type. On the other hand, it was shown that in vivo treatment of rats decreases serum testosterone levels, and furthermore, it was demonstrated that the effect on testosterone levels may be attributed to a decrease in the expression of STAR in Leydig cells. Therefore, it is tempting to speculate that G or R effects on BTB integrity can be partially attributed to the interference with androgen action. The results presented herein show that neither G nor R treatment modified androgen receptor mRNA and protein levels. They also show that the effects of the herbicides on TER and on claudin11 localization in the presence of testosterone were similar to those observed under basal conditions. Thus, G and R are able to disrupt BTB function in the presence of testosterone. Further in vivo experiments will be necessary to determine a possible role of G or R as endocrine disruptors.

Conclusion

In summary, this investigation shows that G and R alter the Sertoli cell junction barrier permeability. This study also shows that, at least in part, the loss of location of claudin11 at the interface between neighboring Sertoli cells might be responsible for the disassemble of the barrier. We postulate that BTB integrity is a sensitive target for the adverse effects of G or R on male reproductive function.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This *in vitro* investigation showed that exposure to G and R at 100 ppm alters Sertoli cell junction barrier permeability, measured by decreased TER, and also decreased testosterone-stimulated TER. This study also showed that, at least in part, the loss of location of claudin11 at the interface between neighboring Sertoli cells might be responsible for the disassembly of the barrier. G or R did not modify androgen receptor mRNA or protein levels, nor did G modify P-p38-MAPK and P-ERK1/2 signalling pathways involved with BTB integrity at any doses tested, or affect the expression of intercellular junction proteins (claudin11, occludin and ZO-1). However, G and R induced redistribution of claudin11 at the zone of contact between cells. Neither G nor R modified lactate production, glucose uptak, GLUT1, FA oxidation, or *FAT/CD36* and *CPT1* expression in SC, thus indicating no effect of G or R on SC nutritional function or metabolism.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not sufficiently characterized, there was no positive control and most of the endpoints were tested at only 2 concentrations preventing any dose response evaluations, with the highest concentration exceeding that which is physiologically relevant.

Reliability criteria for *in vitro* toxicology studies

Publication: Gorga *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)	N	Purity not reported. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup Full II (Monsanto, Argentina)
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		
Test concentrations in physiologically acceptable range (< 1 mM)	Y	3 test concentrations used of which the highest was > 1 mM (1000 µg/mL).
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control reported.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	

Dose-effect relationship reported		Explored but not found (only 2 concentration levels tested for most endpoints)
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 sufficiently characterized and most of the endpoints were tested at only 2 concentrations.		

1. Information on the study

Data point:	CA 5.5
Report author	Hao Y., Xu W. et al
Report year	2019
Report title	Roundup-Induced AMPK/mTOR-Mediated Autophagy in Human A549 Cells
Document No	Journal of agricultural and food chemistry (2019) Vol. 67(41), 11364-11372
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate-based herbicide (GBH) is one of the most widely used pesticides based on a 5-enolpyruvylshikimate-3-phosphate synthase target, which does not exist in vertebrates. Here, the autophagic effects of the most famous commercial GBH Roundup (RDP) on human A549 cells in vitro has been studied. Intracellular biochemical assay indicated opening of mitochondrial permeability transition pore, LC3-II conversion, up-regulation of beclin-1, down-regulation of p62, and the changes in the phosphorylation of AMPK and mTOR induced by RDP in A549 cells. Further experimental results indicated that all the effects induced by RDP were related to its adjuvant polyethoxylated tallow amine, not its herbicidal active ingredient glyphosate isopropylamine salt. All these results showed that RDP has the ability to induce AMPK/mTOR-mediated cell autophagy in human A549 cells.

Materials and methods

Chemicals - Glyphosate isopropylamine salt (≥ 95%) was obtained from Weihai Hanfu Biochemical Pharmaceutical Co., Ltd., Weihai, China.

Cell culture - DMEM supplemented with 1% antibiotics (streptomycin and penicillin) and 10% fetal bovine serum was used to culture human alveolar carcinoma A549 cells (ATCC, CCL-185). The cells were placed in an incubator at 37 °C with a humidified atmosphere of 5% CO_2.

Cell viability - The MTT assay was performed to evaluate the cytotoxicity of glyphosate in A549 cells. 1.0×10^5 cells in 100 μL of fresh DMEM medium were plated in 96-well plates. After incubation for 24 hours, medium was replaced with fresh medium containing glyphosate at 100 μg/mL. Fresh medium served as the control. Three replicates were used per test group. After incubation for 2 hours, the MTT reagent (20 μL/well, 5 mg/mL) was added to form formazan crystals during incubation for 4 hours at 37 °C. The medium was removed and the formazan crystals were dissolved in DMSO (150 μL/well). Optical density (OD) was measured with a microplate reader at 570 nm. Percent cell viability inhibition was calculated as follows: $cell\ inhibition(\%) = (OD_{control} - OD_{treatment})/OD_{control} \times 100$. SPSS version 17.0 was used to calculate IC_{50} values.

Monodansylcadaverine (MDC) staining – MDC staining was used to mark autophagic vacuoles. A549 cells (1×10^5 cells/well) were grown in 24-well plates. The cells were exposed to glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After rinsing twice with PBS at pH 7.4, the cells were incubated with 10 μM MDC in the dark for 20 minutes at 37 °C. After washing with PBS at pH 7.4, the treated cells were photographed and analyzed by fluorescence microscopy at 488 nm at a magnification of 200×. Three photos were taken per group and analyzed to determine the number of autophagosomes.

Visualization of double-membrane autophagosomes - Visualization of the ultrastructure of A549 cells was performed by transmission electron microscopy (TEM). A549 cells were harvested after being treated for 2 hours with glyphosate at 100 μg/mL. Fresh medium served as the control. The cells were then rinsed twice with PBS at pH 7.4 and placed in a 2.5% glutaraldehyde solution and kept overnight at 4 °C. Subsequently, the cells were post-fixed in 1% osmium tetroxide (OsO_4) after rinsing with PBS at pH 7.4. The cells were then dehydrated with an ascending series of ethanol solutions and embedded with Epon 812. Ultrathin sections were stained with 2% uranyl acetate and lead citrate solutions. The images were recorded using a JEOL JEM-2100 transmission electron microscope.

Autophagic flux - Ad-mCherry-GFP-LC3B is an adenovirus that expresses the mCherry-GFP-LC3B fusion protein, which is used to analyze autophagic flux after infection of cells. A549 cells were grown in 3 cm glass bottom cell culture dishes and infected with adenovirus (40 MOI) for 24 hours. After incubation for another 24 hours, the cells were exposed to glyphosate at 100 μg/mL and incubated for 2 hours and then rinsed twice with PBS at pH 7.4. Fresh medium served as the control. The expression of mCherry and green fluorescent protein (GFP) was visualized and analyzed by a Nikon confocal microscope at 488 and 561 nm at a magnification of 100× by taking three photos per group. Diffuse yellow fluorescence indicates no occurrence of autophagy whereas yellow spots indicate autophagy. Autophagic flux was evaluated by the accumulation of mCherry-GFP-LC3B on the autophagosome membrane.

Colocalization of mitochondria and lysosomes - MitoTracker Green was used for mitochondria-specific fluorescent staining of live cells. LysoTracker Red was used for lysosomal-specific fluorescent staining of live cells. The two probes were used for mitochondrial and lysosomal colocalization imaging. A549 cells were treated with glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After treatment and rinsing, the cells were incubated with MitoTracker Green (0.5 μM) and LysoTracker Red (0.5 μM) for 30 minutes. Afterwards, the cells were photographed and analyzed by fluorescence microscopy at 488 and 561 nm at a magnification of 200×. Three photos were taken per group and analyzed. The counterstaining cells were photographed by a Nikon confocal microscope at a magnification of 100× and emission recorded at 488 nm for MitoTracker Green and 561 nm for LysoTracker Red. The related plugins (JACoP, colocalization threshold) of ImageJ v1.8.0 software were used to obtain a relative coefficient of colocalization between Mito and Lyso.

Opening of mitochondrial permeability transition pore (mPTP) - Opening of mPTP was measured by using calcein and $CoCl_2$. A549 cells were grown in 12-well plates and treated with glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After rinsing twice with PBS at pH 7.4, the cells were incubated with 1 mM calcein in the dark for 20 minutes at 37 °C and then exposed to 1 mM $CoCl_2$ for 30 minutes. The control group was not incubated with $CoCl_2$. After rinsing with PBS at pH 7.4, the treated cells were analyzed by fluorescence microscopy at 488 nm at a magnification of 200× by taking three photos per group.

Immunoblotting of proteins linked with autophagy - The relative protein expression levels of LC3, beclin-1, p62, p-AMPK, p-mTOR, and p-p70s6k in A549 cells were determined by immunoblot analysis to explore the underlying mechanisms of induced autophagy. A549 cells were treated with glyphosate at 100 μg/mL for 2 hours. Fresh medium served as the control. After treatment and rinsing the cells were lysed by a mixture of 50 μL immunoprecipitation assay lysis buffer with 0.5 μL protease inhibitor (100 mM). The total protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay Kit. Equal amounts of lysate proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes with the proteins were blocked in Tris-buffered saline containing 5% nonfat dried milk and 0.05% Tween-20 for 2 hours. Blocked membranes were incubated with rabbit polyclonal antibodies for LC3, beclin-1, p62, p-AMPK, p-mTOR, p-p70s6k, and β-actin overnight at 4 °C. After being washed three times in Tris-buffered saline containing 0.05% Tween-20, membranes were incubated with anti-rabbit IgG secondary antibodies for 2 hours. The antibody-bound proteins were detected by Electro-Chemi-Luminescence kit and scanned by the chemiluminescent gel imaging system. The bands' grayscale values were quantified by ImageJ v1.8.0 software.

Adenosine triphosphate (ATP) content - The levels of intracellular ATP were determined by the ATP Assay Kit from Beyotime following the manufacturer's instructions. After treatment with glyphosate at 100 μg/mL or fresh medium as the control for 2 hours, A549 cells were lysed in the lysis buffer for 30 minutes at 4 °C. After centrifugation at 12,000 rpm for 15 minutes, a part of the supernatant was transferred into a light-protected 96-well plate for determination of the activity of ATP. The other part was used to determine the protein concentrations by the Pierce BCA Protein Assay Kit. The ATP concentrations (μmol/L) were converted to the protein concentrations (mg/L).

Statistical Analysis - Three separate replicates were performed for each assay. The statistical analysis process runs under SPSS version 17.0, statistical program (SPSS Inc). Data are presented as the means ± standard deviation (SD). Three independent experiments of MTT assay were analyzed by two-way ANOVA followed by Tukey post hoc testing. Different small alphabets indicate significant differences ($P \leq 0.05$). Others were subjected to one-way analysis of variance (ANOVA) followed by Dunnet's test for determining the differences with control ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$).

Results

Viability - Inhibition of cell viability (as % of control) of A549 cells was 5.36 ± 0.82 for glyphosate at 100 μg/mL after 2 hours of treatment.

Monodansylcadaverine (MDC) staining - MDC staining in A549 cells treated with glyphosate at 100 μg/mL was not significantly different from the control.

Visualization of double-membrane autophagosomes - The visualization of autophagosomes by TEM showed an homogeneous cytoplasm with normal mitochondria in A549 cells treated with glyphosate at 100 μg/mL.

Autophagic flux - In the control and in the cells treated with glyphosate at 100 μg/mL, mCherry-GFP-LC3B is present in the form of diffuse yellow fluorescence which is indicative of the absence of autophagy.

Colocalization of mitochondria and lysosomes - The relative coefficient of colocalization between Mito and Lyso was 0.22 ± 0.01 for the control and 0.21 ± 0.02 for the cells treated with glyphosate at 100 μg/mL.

Opening of the mitochondrial permeability transition pore (mPTP) - When compared with the $CoCl_2$-treated control group, no significant increase in fluorescence was noted in the cells treated with glyphosate at 100 μg/mL which is indicative of the absence of mPTP opening.

Immunoblotting of proteins linked with autophagy - The effect of glyphosate on the autophagy related proteins beclin-1, LC3-II/I, and p62 was studied by immunoblotting. When compared with the control no difference was found in the expression ratio of LC3-II/I as well as protein expression of beclin-1 in cells treated with glyphosate at 100 μg/mL. Immunoblotting was also used to examine the phosphorylation of mTOR, AMPK, and p70s6k. No difference in phosphorylation levels of mTOR, p70s6k, and AMPK could be demonstrated in cells treated with glyphosate at 100 μg/mL when compared to controls. These results indicate that glyphosate does not contribute to the activation of the AMPK/mTOR pathway.

Adenosine triphosphate (ATP) content - When compared with the control, no difference was found in the levels of cellular ATP of cells treated with glyphosate at 100 μg/mL.

Conclusion

All commercial glyphosate formulations are more toxic than glyphosate so that the increased toxicity may be attributed to the adjuvants. The herbicidal active ingredient of Roundup is glyphosate isopropylamine salt and its main adjuvant is polyethoxylated tallow amine (POEA). To explore whether

the herbicidal active ingredient or the adjuvant contributes Roundup's ability of inducing autophagy in human A549 cells, TEM, MDC, immunoblotting, and Ad-mCherry-GFP-LC3B analyses were performed on 4 concentrations of Roundup from 50 to 125 µg glyphosate acid eq./mL and one concentration of glyphosate (100 µg/mL) and POEA (35 µg/mL). This study revealed that the commercial formulation Roundup has the ability to cause autophagy in A549 cells via the AMPK/mTOR signaling pathway. Previous studies showed that compared with glyphosate, commercial glyphosate based herbicides are more toxic, and the toxic effect is highly correlated with the adjuvant POEA. This study indicates that the adjuvant POEA in Roundup contributes to its ability of inducing autophagy in A549 cells, and that the herbicidal active ingredient glyphosate has no contribution.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The effect of glyphosate, POEA and a herbicidal formulation containing POEA as a co-formulant on the AMPK/mTOR signaling pathway was studied in human alveolar carcinoma A549 cells *in vitro*. Only the results of exposure to glyphosate at 100 µg/mL are reported and discussed in this summary. The endpoints selected to study the effect of glyphosate on autophagy are inhibition of viability, monodansylcadaverine (MDC) staining to mark autophagic vacuoles, visualization of double-membrane autophagosomes by TEM, autophagic flux, colocalization of mitochondria and lysosomes, opening of the mitochondrial permeability transition pore (mPTP), expression of proteins involved in the AMPK/mTOR signaling pathway, and ATP content. No effect could be demonstrated of glyphosate on any of these endpoints indicating that glyphosate, in contrast to POEA and Roundup, does not contribute to the activation of the AMPK/mTOR signaling pathway and has thus no role in autophagy.

This publication is relevant for the risk assessment of glyphosate but reliable with restrictions because only one glyphosate concentration was tested and no positive controls were used.

Reliability criteria for *in vitro* toxicology studies

Publication: Hao, Xu *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)	Y	Purity of ≥95%. Source: Weihai Hanfu Biochemical Pharmaceutical Co., Ltd. ,China.
Only glyphosate acid or one of its salts is the tested substance	N	Also GBH (Monsanto, St. Louis, USA) and POEA tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Human alveolar carcinoma A549 cell line.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Glyphosate only at one concentration tested: 100 µg/mL.

Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	N	Only one concentration for glyphosate.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is relevant for the risk assessment of glyphosate but reliable with restrictions because only one glyphosate concentration was tested and no positive controls were used.		

1. Information on the study

Data point:	CA 5.4
Report author	Ilyushina N. A. *et al.*
Report year	2018
Report title	Comparative investigation of genotoxic activities of glyphosate technical products in the micronucleus test in vivo
Document No	Toksikologicheskii Vestnik, (2018) No. 4, pp. 24-28.
Guidelines followed in study	OECD Test Guideline 474
Deviations from current test guideline	Positive control animals were included but the data are not reported. No ratio of PCE to NCE was reported. No evidence of bone marrow exposure Data have been presented per group rather than per animal
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

In this study, the induction of micronucleus formation in vivo in polychromatophilic erythrocytes of bone marrow of CD-I mice was assessed by the action of three different technical glyphosate products on the market in the Russian Federation. It was found that the tested samples of technical products showed different cytogenetic activity, while only one of them caused a statistically significant, dose-dependent increase in the frequency of induction of micronuclei compared to the negative control.

The analysis of the composition of the studied product samples showed that the cytogenetic activity may depend on the content of potentially mutagenic impurities, formaldehyde in particular.

The acquired data provides some additional grounds for lowering the upper limit of formaldehyde content in technical glyphosate products and also indicates the need to assess the genotoxic activity of analogue pesticides entering the market of plant protection products.

Materials and methods

Three glyphosate technical products were used that were manufactured at different factories and that contained the active substance (glyphosate acid) in amounts of 96.6%, 95.8% and 95.7% in technical products I, II and III, respectively.

Chromosomal disturbances were revealed through the use of in vivo micronucleus analysis [OECD Test No. 474: Mammalian Erythrocyte Micronucleus Test. 2016] of the bone marrow erythrocytes in CD-I laboratory mice obtained from the husbandry center at the Andreyevka Branch of the Federal State Budget Research Institution “Biomedical Technology Science Center” of the Russian Federal Medicobiological Agency.

Five mice per group were used. Technical glyphosate was administered intragastrically in 1% starch in doses of 500, 1,000 and 2,000 mg/kg of body weight (the maximum dose according to OECD Test No. 474) twice, twenty-four hours apart, and the animals were euthanized 22 hours following the second administration. At the same time, an excipient (1% starch) was given as a negative control following the same pattern as was used for the technical glyphosate. Cyclophosphamide was used as a positive control, given intragastrically in the amount of 40 mg/kg once, at the same time as the second dose of glyphosate.

Bone marrow preparations from each specimen were studied microscopically (on a Japan-made Nikon

Eclipse Ci-L), counting 4,000 polychromatophilic erythrocytes (PCEs) for measuring micronucleus frequency), and the portion among the erythrocytes as a whole made up by PCEs, counting no fewer than 500 erythrocytes for each animal.

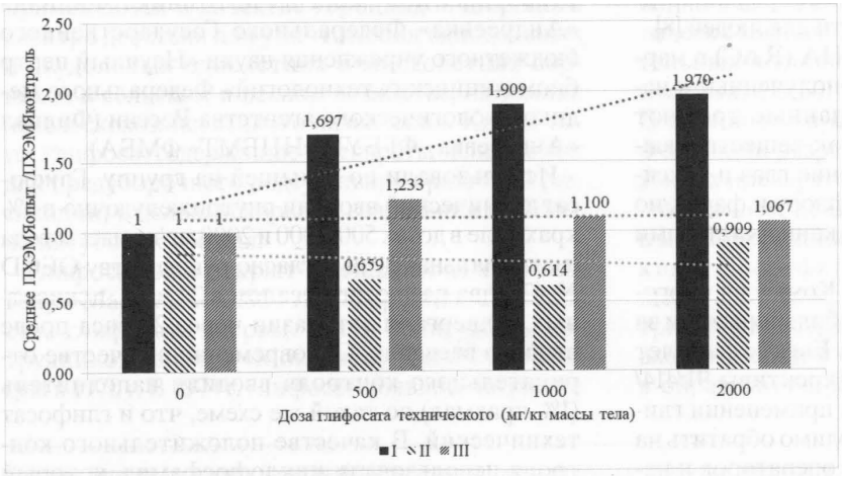
A statistical analysis of the study results was carried out using SPSS Statistics v. 22.0 (IBM, New York, USA). Frequencies of micronuclei in the PCEs were compared by building a generalized log-linear model of the Poisson Regression ($\alpha = 0.05$). The data were also checked for dependencies between micronucleus frequency and the dose of the technical product being tested using the Mantel-Haenszel method.

Results

All the research was carried out in accordance with OECD Manual No. 474 and Methodological Instructions MU-1.2.3365-16. Each experiment used positive and negative controls simultaneously. The frequency of PCEs containing micronuclei in the case of the positive control was in a range from 1.58 to 2.95%, while in the case of the negative control it was from 0.06 to 0.12%. All the values were within the range of the distribution boundaries of historical laboratory controls.

The quantification of the portion of total erythrocytes made up by polychromatophilic erythrocytes show that in none of the analyses was erythropoiesis suppressed, i.e. there were no toxic effects on mouse bone marrow.

Varying effects were revealed during the study of the mutagenic activity of the glyphosate technical products through micronucleus analysis. Technical products II and III did not significantly increase the PCE frequency in mouse bone marrow compared to the simultaneous negative controls (Table 1, Figure 1).



Technical Glyphosate Dose (mg/kg of body weight)

Figure 1. Frequencies of increased occurrence of PCEs compared to the negative control depending on the dose of glyphosate technical products. I, II, III – Reference numbers for different technical products. $PCEMN_{exp}$ denotes the frequency of PCEs with micronuclei in groups of mice that received different glyphosate doses. $PCEMN_{control}$ denotes the frequency of PCEs with micronuclei in groups of mice that received an excipient with 1% starch (negative control).

When administering technical Product I, a low, but statistically significant genotoxic effect was observed at all doses (Table 2). Moreover, a statistically significant linear dependency was observed of the frequency of PCEs with micronuclei upon the dose of the compounds under study ($p= 0.002$) (Table 1).

A comparison was made between the content of ingredients in the study samples based on the available batch analysis certificates for the technical products. Two ingredients were of special interest from the point of view of genotoxicity: nitroso-glyphosate and formaldehyde. According to the provided certificates, Ni-trosoglysophate, was not present in any of the batches of technical products (its content, in accordance with FAO specifications, must not exceed 1 mg/kg). Formaldehyde content in products I, II and III amounted to 0.13%, 0.024% and 0.06%, respectively. A formaldehyde level in the glyphosate product of 0.13% is the maximum permissible concentration in glyphosate technical products in accordance with the FAO specifications[14]. However, it should be noted that, according to Annex I of Directive 91/414/EEC, as amended in 2017, the partial content of formaldehyde in glyphosate must not exceed 1 mg/kg [10].

A comparison of the results we have obtained on the mutagenic activity and formaldehyde concentrations in glyphosate technical samples showed that statistically significant genotoxic effects and a linear dependence of the frequency of PCEs with micronuclei upon the dose of the compound under study were only observed in the case of the product with 0.13% formaldehyde. Lower concentrations of this ingredient did not induce the statistically significant formation of PCEs with micronuclei in bone marrow cells.

Thus, the observed mutagenic effects were most likely not related to the active ingredient, but rather to the formaldehyde contained as an ingredient in the products.

Table 1: Probable profile (95%) of confidence intervals for the frequency of polychromatophilic erythrocytes with micronuclei (MN) under the effects of different doses of glyphosate technical products relative to the negative control

Glysophate dose (mg/kg of body weight)	Sample I		Sample II		Sample III	
	Number of PCEs with MN per 40,000 PCEs	95% confidence intervals	Number of PCEs with MN per 40,000 PCEs	95% confidence intervals	Number of PCEs with MN per 40,000 PCEs	95% confidence intervals
0	33	1	44	1	30	1
500	56	1.111 1.697 2.634	29	0.408 0.659 1.048	37	0.763 1.233 2.009
1000	63	1.263 1.909 2.941	27	0.376 0.614 0.984	33	0.671 1.100 1.812
2000	65	1.306 1.970 3.028	40	0.591 0.909 1.395	32	0.647 1.067 1.762
Assessment of linear trend using the Mantel-Haenszel method	1 (146)		II (189)		III (341)	
	Value	Asymptomatic significance p (one-dimensional)	Value	Asymptomatic significance p (one-dimensional)	Value	Asymptomatic significance p (one-dimensional)
	9.791	0.002	0.28	0.597	0.06	0.938

The results obtained are in agreement with data from the literature. In the majority of studies conducted using the Ames test, glyphosate did not induce gene mutations in bacteria [Li A.P., Long T.J., 1988]. In both *in vivo* and *in vitro* micronucleus analyses carried out on different subjects, contradictory data were obtained. An increased frequency of the appearance of micronuclei was noted in several studies on cultivated human buccal epithelial cells [Koller V.J. et al, 2012], in mouse bone marrow [Manas F., 2009], and in human lymphocytes having undergone been affected by glyphosate-containing preparations during crop spraying [Bolognesi C. eta la, 2009]. However, the majority of the studies gave negative results in terms of micronucleus analyses [Kier L.D., 2013]. However, none of the studies analyzed the content of possible mutagenic ingredients in technical products and glyphosate-containing preparations and their contribution to the observed effects.

Conclusion

The comparative investigation of the genotoxic activity of various glyphosate technical products through the analysis of micronuclei in mouse bone marrow erythrocytes showed that cytogenetic activity may depend on the content in the product of potentially mutagenic ingredients, and in particular of formaldehyde. The contradictory results obtained in different studies of the mutagenic activity of glyphosate and preparations containing it may be connected to the different concentrations of ingredients that may exhibit effects of intracellular genetic structures.

The obtained data comprise additional substantiation for reducing the upper limit for formaldehyde content in glyphosate technical products, and also provide evidence of the need to assess the genotoxic activity of analogous pesticides entering the Russian Federation's market.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes the results of three different technical batches of glyphosate tested in a mouse micronucleus test. The study followings the recommendations of OECD 474, with some deficiencies, mostly regarding the reporting of data rather than test methodology. However, the reliability of the reported conclusions are unknown due to a lack of clarity and accuracy of the reported data. Individual animal data and group mean±SD frequency of micronucleated (MN) PCE have not been reported. Instead the results per group appear to be presented as the total number of MN PCE found per 40,000 PCE, together with the frequency of MN PCE values, expressed as relative to control, for the group with calculated 95% upper and lower limits (no explanation for how these data awere derived is provided). The text describes the vehicle control MN PCE frequencies as ranging between 0.06% and 0.12% (within HCD) but similar detail is not provided for the treated groups. Consequently, there is no indication of animal variability within the groups and it is unknown if any of the treated animals fall outside of HCD. Furthermore, the total number of MN PCE is described as being per 40,000 PCE, however, only 4000 PCE were scored per animal and with 5 animals per group this would result in total of 20,000 PCE per group.

The authors postulate that the positive results observed for technical batch I are likely to be due to the presence of 0.13% formaldehyde in the material, although they provide no data to support their hypothesis.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the 3 glyphosate batches was not revealed although the concentrations of the most important impurities were given for each batch. Although reference was made to OECD test guideline 474 too little detail was given on the conduct of the MN assay to conclude to reliability without restrictions and the data are inadequately reported.

Reliability criteria for *in vivo* toxicology studies

Publication: Ilyushina *et al*., 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	OECD test guideline 474 according to the authors.
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	Technical glyphosate from 3 sources with purity of 96.6%, 95.8%, and 95.7%. Sources were not reported but the concentration of impurities (nitroso-glyphosate and formaldehyde) was given.
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	CD-1 mouse.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	0, 500, 1,000, and 2,000 mg/kg bw twice, twenty-four hours apart.
Positive control	Y	Cyclophosphamide.
Number of animals used per dose level reported	Y	5/dose group.
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	Y	Referred to but not presented.
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 source of the 3 glyphosate batches was not revealed although the concentrations of the most important impurities were given for each batch. Although reference was made to OECD test guideline 474 too little detail was given on the conduct of the MN assay to conclude to reliability without restrictions, and the data are inadequately reported.		

1. Information on the study

Data point:	CA 5.4
Report author	Ilyushina N. *et al.*
Report year	2019
Report title	Maximum tolerated doses and erythropoiesis effects in the mouse bone marrow by 79 pesticides' technical materials assessed with the micronucleus assay
Document No	Toxicology Reports (2019) Vol. 6, 105-110
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Effects of technical materials of pesticide active ingredients, belonging to various chemical classes, on erythropoiesis in mouse bone marrow were studied as part of the research on the pesticide mutagenic activity in micronucleus test. The purpose of the present study was to estimate the toxic action of the test substances on the target organ and the validity of the results of the micronucleus assay under conditions of erythropoiesis suppression.

Materials and methods

Chemicals - Four glyphosate batches were tested with a purity of respectively 95.7, 98.3, 95.1, and 95.8%.

Animals – CD-1 mice were purchased from "Andreevka" Branch of the Federal Government Budgetary Establishment of Science "Scientific Center of Biomedical Technologies" of the Federal Bio-Medical Agency of the Russian Federation. The acclimation period was 7 days. Mice had access to drinking water and feed ad libitum, and were maintained under a 12:12-hour light/dark photoperiod at 22 – 22.5 °C and 36 - 40% humidity.

Mammalian Erythrocyte Micronucleus Test (OECD TG 474) - At least 5 groups of minimum 5 mice per sex were used. Each assay included a positive control group (40 mg/kg bw cyclophosphamide), a negative control group (1% potato starch in water), and 3 treatment groups. Glyphosate was administered orally by gavage once a day for 2 subsequent days (24 hours apart) at a volume of 10 mL/kg bw. The maximum dose in the main experiment was either 2,000 mg/kg bw or the MTD as determined in a preliminary dose-finding experiment. Mice were sacrificed 22 hours after the second administration by cervical dislocation, then femurs were removed and bone marrow was harvested. To assess the effect of glyphosate on erythropoiesis, the ratio of polychromatic erythrocytes (PCEs) to the sum of PCEs and normochromatic erythrocytes (NCEs) was determined by counting at least 500 cells (PCEs + NCEs) per animal (at least 250 cells for each slide) under a Nikon Eclipse Ci-L microscope. At least 4,000 PCEs were counted per animal by two different researchers.

Statistical analysis - Statistical analysis was performed using SPSS Statistics v. 22.0 software. The statistical significance of the difference in the proportion of PCEs/(PCEs + NCEs) between the highest dose group and the concurrent negative control group was evaluated using the independent samples t-test for each study.

Results

It should be noted that the negative control values slightly varied from experiment to experiment, and that the historical negative controls of the laboratory were 0,50 ± 0,06 and 0,52 ± 0,06 PCEs/(PCEs+NCEs) females and males, respectively. Cyclophosphamide did not cause a significant decrease in the proportion PCEs/(PCEs+NCEs) in comparison with the negative control. The 4 glyphosate batches tested at the limit dose of 2,000 mg/kg bw in CD-1 mice according to the protocol of the *in vivo* micronucleus assay compliant with OECD test guideline 474 did not reveal any effect on erythropoiesis in the bone marrow.

Conclusion

Glyphosate tested at the limit dose of 2,000 mg/kg bw in mice in the *in vivo* micronucleus assay did not show any effect on erythropoiesis in the bone marrow.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Along with 51 other pesticides 4 batches of glyphosate with purities ranging from 95.1 to 98.3% were investigated for their effect on erythropoiesis in mice. To assess the toxicity of glyphosate on the bone marrow the *in vivo* micronucleus test in the mouse according to OECD test guideline 474 was conducted at the limit dose of 2,000 mg/kg bw. No effect of glyphosate on erythropoiesis was found.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the glyphosate batches used was not reported and no suitable positive control was used in the micronucleus test to assess bone marrow toxicity. The test conducted was in compliance with OECD test guideline 474.

Reliability criteria for *in vivo* toxicology studies

Publication: Ilyushina *et al*., 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	OECD TG 474, in vivo MN assay only used with the purpose to assess toxicity to erythropoiesis in the bone marrow. The positive control used (cyclophosphamide) is not suitable as a positive control for bone marrow toxicity.
Study performed according to GLP	N	Not stated.
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?	4 batches were tested with purity of 95.7, 98.3, 95.1, and 95.8%. Source was not mentioned.
Only glyphosate acid or one of its salts is the tested substance	N	51 other pesticides

		were tested as well.
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	Only limit dose of 2,000 mg/kg bw was considered.
Number of animals used per dose level reported	Y	At least 5 groups of minimum 5 mice per sex.
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?	Only effect on erythropoiesis was reported.
Statistical methods described	Y	
Historical control data of the laboratory reported	Y	
Dose-effect relationship reported	N	Not possible since only one dose was used.
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 source of the glyphosate batches used was not reported and no suitable positive control was used in the micronucleus test to assess bone marrow toxicity. The test conducted was in compliance with OECD test guideline 474.		

1. Information on the study

Data point:	CA 5.6
Report author	Johansson H. K. L. *et al.*
Report year	2018
Report title	Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis
Document No	Reproductive Toxicology (2018) Vol. 82, 25-31
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

Glyphosate has been suggested to be an endocrine disrupting chemical capable of disrupting male reproduction. There are conflicting data, however, with studies reporting effects from exposure to either glyphosate alone or to herbicide formulations, making comparisons difficult. Rat testis histopathology and androgen function following two weeks exposure to either glyphosate at 2.5 and 25 mg/kg bw/day (5x and 50x Acceptable Daily Intake, ADI, respectively), or equivalent high dose of glyphosate in a herbicide formulation; Glyfonova, were assessed.

Materials and methods

Chemicals - Glyphosate (purity ≥ 96%) was purchased from Sigma-Aldrich, St Louis, USA.

Animals - Four-week old male Sprague-Dawley rats were randomly caged in pairs and acclimatized for 7 days. Cages were subsequently distributed into four exposure groups based on animal weight, but following exposure only one animal per cage was used for analyses. Ten animals per dose group were treated with glyphosate orally by gavage at 2.5 and 25 mg/kg bw. Water was used as the control. After 2 weeks of exposure, the animals were decapitated under CO_2/O_2 sedation and the testes collected. From each of 10 males per dose group, one testis, selected at random, was snap frozen in liquid nitrogen and the other was fixed in 10% formalin.

Testosterone assay - One half of each testis was used for hormone extraction. The testes were cut into two equally sized pieces. After removal of the testis cap the inner tissue (seminiferous tubules and interstitial cells) was transferred immediately to a glass vial containing 0.5 mL sterile water and reweighed to determine total tissue weight per sample. Subsequently, 2.5 mL heptane was added and the sample homogenized by manual disruption and then frozen solid. The heptane fraction was transferred and the process repeated to yield a second 2.5 mL heptane fraction that was pooled together with the first fraction. The extracts were dried under nitrogen. Before hormone analyses, the dry extracts were dissolved in EIA buffer at 4 °C overnight, then vortexed and placed in a 42 °C water bath for 10 minutes. Intra-testicular testosterone assays were performed using the Testosterone ELISA kit according to the manufacturer's instructions. Reads were obtained using 96-well plates and absorbance read at 405 nm using a microplate reader. Each sample was assayed in duplicates and result means presented as pg testosterone/g testis.

Histology - Testes were fixed in 10% formalin, processed for paraffin embedding and sectioned at 5 μm for histological assessments. Hematoxylin & Eosin (H&E) staining was performed following standard protocols.

Immunohistochemistry - Immunohistochemistry with peroxidase was carried out on sections of formalin-fixed testes. Sections were dewaxed in petroleum and rehydrated by immersion in ethanol

solutions and finally water. Antigen retrieval was carried out in 0.01M citrate buffer (pH 6) in a microwave and then allowed to cool at room temperature. Samples were washed in PBS and blocked with 1% bovine serum albumin (BSA) and then incubated overnight at 4 °C with primary antibody. The following day, samples were washed in PBS, blocked with 3% H_2O_2 for 10 minutes and again washed in PBS. The samples were incubated with EnVision+System (Dako) for 30 minutes, washed, then with Liquid DAB+System (Dako) for 15 minutes and washed again. To visualize all cell nuclei, sections were counterstained with Meyers' hematoxylin. Following washing and rehydration, samples were mounted with Eukitt. The primary antibody used was against the Androgen receptor.

Immunofluorescence – Five µm sections (4/group) were dewaxed in petroleum and washed in ethanol and dehydrated. Antigen retrieval was done by heat treatment in Tris-EDTA buffer (pH 9) and then cooled at room temp before washing in PBS and blocking in 5% bovine serum albumin (BSA). The samples were incubated with primary antibodies overnight at 4 °C. The next day, slides were brought to room temperature and incubated with secondary antibodies for 1 hour in the dark. Samples were then counterstained with 4,6-diamidino-2-phenylindole (DAPI). The primary antibodies were goat anti-HSD3β, rabbit anti-DDX4, goat anti-CYP11A1 and rabbit anti-STAR. Secondary antibodies were donkey anti-goat AlexaFluor-488 and donkey anti-rabbit AlexaFluor-568.

RNA extraction, cDNA synthesis and quantitative RT-PCR - Total RNA was extracted from a ~100 mg cross-sectional piece from each testis (n=10/group), of which 500 ng RNA was used for each cDNA synthesis. TaqMan Gene Expression Assays were Ar (Rn00560747), Ddx4 (Rn01489814), Cyp11a1, Cyp17a1, Insl3 (Rn00586632), Pcna (Rn01514538), Star and Hsd3b1 (Rn017747410). RT-qPCR assays were run on a QuantStudio 7 Flex Real-Time PCR System in a 384-well format using 3 µL diluted (1:20) cDNA as template in each 20 µL reaction. Relative transcript abundance was calculated by the comparative Ct-method with the geometric mean of the reference genes Sdha (Rn00590475) and Rpl13a (Rn00821946).

Apoptosis - One section per testis (n=5/group) was stained with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, Apoptag®Peroxidase in Situ Apoptosis Detection Kit) according to the manufacturer's instructions with one exception i.e. pre-dilution (1:80) of the TdT enzyme in MilliQ water before dilution with reaction buffer. Color was developed for 15 minutes using DAB+substrate. Lastly, the nuclei were stained using Mayer Hematoxylin prior to mounting using Eukitt. All positively stained cells within the seminiferous tubules on a cross-section were counted. Cells that were clearly stained as a result of cell division rather than apoptosis were excluded, as were stained cells at the outer border of the tissue sections. The relative percentage of seminiferous epithelium to whole testis was calculated using a 15 point-grid count on three separate fields of view from each sample. Second, whole testis section areas were calculated in Adobe Photoshop CC 2017 using the measurement tool. Third, apoptotic cells were defined relative to percentage seminiferous epithelium to whole section. Data and statistical analyses are presented as number of apoptotic cells per seminiferous tubuli area.

Statistical analysis - Data were tested for normal distribution and homogeneity of variance and logarithmic transformation was applied if required. ANOVA with Dunnett's post-test was applied and $p < 0.05$ was considered statistically significant. Statistical software GraphPad Prism 5 was used for analysis.

Results

Testosterone levels - No statistically significant increase in testicular testosterone levels was observed in rats treated orally at 2.5 and 25 mg/kg bw/day for 2 weeks.

Quantitative gene and protein expression analysis - In the testis from animals orally exposed to glyphosate at 2.5 or 25 mg/kg bw/day for 2 weeks no significant differences in gene expression were observed for the Leydig cell specific genes Cyp11a1, Cyp17a1, Insl3, Hsd3β1 and Star and the somatic marker gene Ar or germ cell marker gene Ddx4.

Testis histopathology - No adverse histopathology in any of the glyphosate exposed groups was observed when compared with controls. The seminiferous tubules were intact and displayed active spermatogenesis with comparable spermatogenic cycling between examined specimens (qualitative assessment). Neither were there any differences between exposed and control animals with regard to

missing germ cell layers or multinucleated germ cells. The interstitial space was comparable between glyphosate treated groups and controls.

Qualitative protein expression analysis - The Leydig cell-specific steroidogenesis factors CYP11A1 and STAR were both expressed at comparative levels between the glyphosate treated animals and the controls. Also no difference in expression and distribution was noted between glyphosate treated groups and controls for the steroidogenic enzyme HSD3B1 and the germ cell-specific factor DDX4.

Apoptosis - The number of apoptotic cells was comparable between control testes and all glyphosate treated groups (5 per group).

Discussion and conclusions

Glyphosate at 2.5 mg/kg bw/day (5-fold ADI) and 25 mg/kg bw/day (50-fold ADI) did not show significant effects on intra-testicular testosterone levels, testicular gene and protein expression, testicular histopathology and apoptosis in the testes of rats treated orally for 2 weeks. This suggests that the active compound glyphosate may not contribute to previously reported endocrine disrupting effects, but rather other constituents in the commercial formulated products. This is in agreement with conclusions from the most recent expert opinion review on glyphosate commissioned by the European Food Safety Authority which conclude that there is no evidence of endocrine mode of action of glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The effects of glyphosate on intra-testicular testosterone levels, expression of Leydig cell specific genes Cyp11a1, Cyp17a1, Insl3, Hsd3β1 and Star and expression of somatic marker gene Ar or germ cell marker gene Ddx4, expression of Leydig cell-specific steroidogenesis factors CYP11A1 and STAR, testicular histopathology and apoptosis were investigated in male rats treated orally at 0, 2.5 and 25 mg/kg bw/day for 2 weeks. No effects were found on either of the testicular parameters tested suggesting that glyphosate does not contribute to endocrine disrupting effects of the male reproductive system.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only two dose levels were used to explore the dose-effect relationship for the endpoints assessed.

Reliability criteria for *in vivo* toxicology studies

Publication: Johansson *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)	Y	Purity ≥ 96%. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance		Also formulations were tested: Glyfonova 450 Plus, FMC corporation.
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	Oral by gavage.
Dose levels reported	Y	Only 2 dose levels for glyphosate (2.5 and 25 mg/kg bw)
Number of animals used per dose level reported	Y	10 animals/group.
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	Y	Only 2 dose levels tested.
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 only two dose levels were used to explore the dose-effect relationship for the endpoints assessed.		

1. Information on the study

Data point:	CA 5.4
Report author	Kasuba V. *et al.*
Report year	2017
Report title	Effects of low doses of glyphosate on DNA damage, cell proliferation and oxidative stress in the HepG2 cell line
Document No	Environ Sci Pollut Res (2017) Vol. 24, 19267–19281
Guidelines followed in study	Not mentioned
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

The toxic effects of glyphosate in vitro on HepG2 cells exposed for 4 and 24 h to low glyphosate concentrations likely to be encountered in occupational and residential exposures [the acceptable daily intake (ADI; 0.5 μg/mL), residential exposure level (REL; 2.91 μg/mL) and occupational exposure level (OEL; 3.5 μg/mL)] were studied. The assessments were performed using biomarkers of oxidative stress, CCK-8 colorimetric assay for cell proliferation, alkaline comet assay and cytokinesis-block micronucleus (CBMN) cytome assay. The results obtained indicated effects on cell proliferation, both at 4 and 24 h. The levels of primary DNA damage after 4-h exposure were lower in treated vs. control samples, but were not significantly changed after 24 h. Using the CBMN assay, a significantly higher number of MN and nuclear buds at ADI and REL after 4 h and a lower number of MN after 24 h were found. The obtained results revealed significant oxidative damage. Four-hour exposure resulted in significant decrease at ADI [lipid peroxidation and glutathione peroxidase (GSH-Px)] and OEL [lipid peroxidation and level of total antioxidant capacity (TAC)], and 24-h exposure in significant decrease at OEL (TAC and GSH-Px). No significant effects were observed for the level of reactive oxygen species (ROS) and glutathione (GSH) for both treatment, and for 24 h for lipid peroxidation. Taken together, the elevated levels of cytogenetic damage found by the CBMN assay and the mechanisms of primary DNA damage should be further clarified, considering that the comet assay results indicate possible cross-linking or DNA adduct formation.

Materials and Methods

Chemicals - Glyphosate was purchased as analytical standard purity grade (≤100%) as Pestanal®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany.

Cell line - HepG2 cell line (ATCC® HB8065™) was purchased from the American Type Culture Collection (Rockville, MD, USA), cultivated in EMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U/mL) and maintained in a humidified atmosphere (95% relative humidity) with 5% CO_2 at 37 °C. Testing was performed when the duplication time has been established to be constant, and the passage step was more than 3 but less than 10 cell passages.

Treatment conditions - The cells in culture were exposed to glyphosate concentrations of 0.5 μg/mL, 2.91 μg/mL and 3.5 μg/mL in PBS corresponding with the systemic concentrations at the acceptable daily intake (ADI), the residential exposure level (REL) and the occupational exposure level (OEL). The calculation of the concentrations to be tested was based on the average male human body weight of 60 kg and the total volume of 36 L of extracellular liquid. HepG2 cells were grown until 80% confluence, trypsinized and transferred in cell culture flasks for the micronucleus and comet assays and in 96 well plates for the cell proliferation assay and the determination of markers of oxidative stress. Prior to treatment, the culture medium was removed, cells were washed with PBS and fresh complete medium

with glyphosate was added. Cells were treated for 4 or 24 hours at 37 °C in a humidified atmosphere (95% relative humidity) with 5% CO_2. Negative and positive controls were tested in parallel.

Cell proliferation assay - Proliferation of HepG2 cells after 4 and 24 hours of exposure to glyphosate was studied by means of the CCK-8 colorimetric assay, based on the use of Cell Counting Kit-8. This kit uses 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, sodium salt (WST-8) that is bioreduced by cellular dehydrogenases to an orange formazan product, soluble in tissue culture medium in the presence of the electron carrier, 1-methoxy PMS. The amount of formazan produced is directly proportional to the number of living cells. At the end of treatment cells were washed with PBS and 10 µL of WST-8 solution added. After 4 and 24 hours of incubation at 37 °C in a CO_2 incubator, the optical density at 450 nm was determined for each well using a Victor3™ Multilabel Plate Reader. Data were expressed as a percentage absorbance compared to relevant negative controls. The positive control was 10% DMSO in the complete nutrient medium. The experiment was done twice independently with 4 repeated measurements in each.

Alkaline comet assay - After 4 or 24 hours of treatment, cells were washed twice with PBS, detached by trypsinization, centrifuged and resuspended in complete EMEM. 7 µL of single cell suspension at 10^4 cells/mL was mixed with low melting point agarose and layered on slides. Duplicate slides were prepared for each time period and concentration together with negative and positive controls. Hydrogen peroxide was used as the positive control. Following preparation of all microgels, they were immersed in fresh cold lysis solution for 24 hours. Afterwards, slides were washed with bidistilled water and denatured in freshly prepared cold denaturation/electrophoresis buffer. After 20 min at 4 °C, cells were placed in the electrophoretic chamber and subjected to electrophoresis. Afterwards the slides were neutralized, dehydrated and stained with ethidium bromide (20 µg/mL) for image analysis with the fluorescent microscope Olympus BX51 (×200 magnification) using the Comet Assay IV software. A total of 100 nucleoids (50 per slide) were measured per each experimental point. The level of DNA damage was estimated based on tail intensity (TI), indicating %DNA in the comet tail. The experiment was performed once with two replicates that were compared, and if no statistically significant difference was found they were combined.

Cytochalasin B-blocked micronucleus cytome assay (CBMN cytome assay) - Cells were seeded at 10^4 cells/mL in complete EMEM medium. After 4 or 24 hours of treatment with glyphosate, cells were washed twice with PBS and fresh complete medium was added. At the 44^{th} hour, cytochalasin B (3 µg/mL) was added and the cell cultures harvested 24 hours thereafter. The positive control, cyclophosphamide, was incubated simultaneously. After harvesting, the medium was discarded, and cells were washed twice with PBS, detached by trypsinization, rinsed and resuspended in complete EMEM medium. Cell suspensions in complete EMEM were centrifuged and the pellet resuspended in PBS, centrifuged and the pellet fixed and put on slides for staining with 2% Giemsa stain and air-dried. The experiment was performed once with two replicates. Micronuclei (MNi), nucleoplasmic bridges (NPBs), nuclear buds (NBUDs) and apoptotic and necrotic cells were scored in binucleated (BN) cells. A total of 2000 BN cells per each experimental point were scored to determine the parameters of the CBMN Cytome assay.

Lipid peroxidation - Malondialdehyde (MDA), and endproduct of lipid peroxidation, was measured using the thiobarbituric acid reactive substance (TBARS) assay with some modification. 0.5 mL of sample was added to 0.5 mL of thiobarbituric-trichloroacetic acid (TBA-TCA) reagent and heated at 90 °C for 30 min and then cooled in an ice bath and centrifuged. Absorbances were measured with a microplate spectrophotometric system (Victor3™ Multilabel Plate Reader) at 530 nm. Two independent experiments were performed and each sample was measured in duplicate. The TBARS concentration in unknown samples was calculated using a standard curve constructed with 1,1,3,3- tetrametoxypropane (0.3–6.07 µM) and expressed as µM.

Total antioxidant capacity - Total antioxidant capacity (TAC) was investigated using the ferric-reducing ability of plasma (FRAP) assay, based on the reduction of Fe3+-TPTZ complex under acidic conditions. The FRAP assay used in this study was slightly modified. 100 µL of cell suspension (10^4 cells in total) was added to 1.0 mL of FRAP reagent. Absorbance was measured after 4 minutes of incubation at 593

nm using the FRAP working solution as a blank. Two independent experiments were performed and each sample was measured in duplicate. The results were calculated on a basis of a standard curve using $Fe_2SO_4 \cdot 7H_2O$ (0.05-4.0 mM).

ROS detection - The amount of intercellular reactive oxygen species (ROS) was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is deacetylated by cellular esterases to form a non-fluorescent compound which is then oxidized in the presence of hydroxyl, peroxyl and other ROS to the fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). All measurements were performed in quadruplicate in dark-sided 96-well microplates in which each well was added 100 µL of cell suspension containing 10^4 cells. Cells were grown in the same medium and conditions as for the cell proliferation assay and treated with the same concentrations of glyphosate for 4 and 24 hours. After treatment the cells were washed with PBS and 100 µL of 50 µM DCFH-DA dye diluted in PBS was added and kept in contact with the cells for 30 minutes at 37 °C in a CO_2 incubator. The control for dye autofluorescence was prepared without addition of dye. Negative (non-treated) cell controls in EMEM were included in each experiment. The positive control was a solution of 100 mM H_2O_2. Measurements were made using a Victor3™ Multilabel Plate Reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Data were expressed as fluorescence arbitrary units (AUs) and later transformed into percentages compared to control values.

Quantification of glutathione - The fluorogenic bimane probe, monochlorobimane (MBCl), reacts specifically with glutathione (GSH) and forms a fluorescent product equal to the amount of GSH that can be measured fluorometrically. Cell cultures in 96-well plates were prepared with 100 µL of cell suspension containing 10^4 cells and each well was treated with glyphosate for 4 and 24 hours. After washing with PBS, cells were incubated with 100 µL of 50 µM MBCl in PBS for 20 minutes at 37 °C in a CO_2 incubator. Measurements were made using a Victor3™ Multilabel Plate Reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Negative cell controls were included in each experiment. Data were expressed as fluorescence AUs and later transformed into percentages compared to control values. All of the measurements were performed in quadruplicate.

Glutathione peroxidase activity - Glutathione peroxidase (GSH-Px) activity in HepG2 cells was measured according to the European standardized method. To increase assay sensitivity for the measurement of GSH-Px, samples were prediluted 60 times instead of a recommended 110-fold dilution as used for blood samples. The amount of glutathione oxidized by *t*-butyl hydroperoxide was determined by following the decrease in β-NADPH concentration at 340 nm. Two independent experiments were performed and each sample was measured in duplicate. One unit of GSH-Px was expressed as the amount of enzyme that oxidizes 1 µmol β-NADPH/min at 37 °C. Activity of GSH-Px was expressed in U/g total protein. The protein concentration of cell lysates was measured using a Total Protein Kit, Micro. Samples, standards and blanks were tested in triplicate, and absorbance was measured in 96-well plates at 570 nm**.**

Statistical analysis - Statistical analysis was performed using StatSoft Dell Statistica 13 package program. Descriptive statistics were used to determine the basic statistical parameters (mean, standard error and deviation, median and minimum and maximum values). Data gathered with the Comet Assay IV software were logarithmically transformed prior to statistical evaluation, with the aim of normalizing distribution and equalizing variances. The intra- and intergroup comparisons between samples were performed using one-way analysis of variance (ANOVA) with post hoc Scheffé's test. Comparisons between values obtained with the CBMN Cytome assay and HepG2 proliferation kinetics measured with CBMN were made by Pearson's $\chi 2$ test for two-by-two contingency tables. For the biochemical and cell proliferation assays descriptive statistics and *t* test for comparisons between independent samples were used. The level of statistical significance was set at $p < 0.05$.

Results

Cell proliferation measured by the CCK-8 assay - Although without statistical significance a slight increase of cell proliferation was observed in HepG2 cells after a 4-hour treatment of about 9% at the ADI and almost 8% at the REL. The values recorded for the OEL-treated cells were not different from

control. After 24 hours of treatment, HepG2 cells showed a not statistically significant increase in cell proliferation of 3% at the REL and of 1% at the OEL when compared to the control. Proliferation of ADI-treated cells did not differ from control.

Primary DNA damage measured by the alkaline comet assay - After 4 hours of exposure, the TI was statistically significantly decreased at all glyphosate concentrations. After 24 hours of exposure, the TI at ADI, OEL and REL were not statistically significantly different from control. Throughout comet measurements, apoptotic and necrotic nucleoids were also counted but there were no significant deviations from control.

Cytogenetic damage measured using CBMN cytome assay - After 4 hours of exposure, a not statistically significant increase in MN was found in HepG2 cells treated with glyphosate at all 3 concentrations. In HepG2 cells treated with glyphosate at ADI and REL, a statistically significant increase in nuclear buds (NBUD) frequency was observed. The incidence of nucleoplasmic bridges (NPB) was highest in cells treated with glyphosate at ADI (6 per 2000 BN cells). Only 2 apoptotic cells per 2000 cells were found in the sample treated with glyphosate at OEL. The nuclear division index (NDI) in glyphosate-treated cells was different from that of the control. In the OEL-treated sample, there were statistically significant changes in the distribution of M1–M4 cells and OEL treatment resulted with more cells in the M2 phase. The same effect was observed when ADI-treated and OEL-treated samples were compared, as well as when REL-treated and OEL-treated samples were compared. After 24 hours of exposure, a lower number of micronucleated BN cells and a lower frequency of BN MN was found at all 3 glyphosate concentrations when compared to the control. Also the frequency of BN NBUDS was statistically significantly lower at all 3 concentrations. Control and glyphosate-treated cells did not significantly differ in the number of NPBs. At ADI 7 and at REL 5 apoptotic cells per 2000 BN cells were found. There was no statistically significant difference in NDI values between glyphosate-treated samples and control. A slightly changed incidence of M2 and M3 cells was found in treated samples as compared to control.

Lipid peroxidation - After 4 hours of exposure, TBARS concentrations in samples treated with glyphosate at ADI and OEL were statistically significantly lower than control values. Treatment of HepG2 cells for 24 hours with glyphosate resulted in lower TBARS concentrations at REL, but the difference was not statistically significant.

GSH level - The level of GSH was not statistically significantly different from the control value for all concentrations and exposure times. After 4 hours of treatment, the ADI-treated sample showed a 4% higher level of GSH than the control whereas REL and OEL-treated cells showed GSH levels similar to control values. The 24-hour ADI-treated sample had the lowest GSH concentration, which was 5% lower than the levels measured in the control samples, and REL- and OEL-treated cells showed GSH levels similar to control values.

GSH-Px activity - GSH-Px activity was statistically significantly decreased in HepG2 cells exposed to glyphosate at the ADI concentration after 4 hours. After 24 hours of treatment, a statistically significant decrease in the GSH-Px activity was observed at the OEL concentration.

Discussion

The present study evaluated how low-dose exposure for 4 and 24 hours affected cytotoxic, genotoxic and biochemical parameters in the human hepatoma cell line HepG2. Glyphosate increased not statistically significantly cell proliferation in HepG2 cells after 4 and 24 hours of exposure. The effect was more pronounced after 4 hours (8–9% as compared to control), than after 24 hours (3% as compared to control). The results of this study have demonstrated that exposure to glyphosate for 4 hours led to a slightly increased cytogenetic damage in terms of MN. Despite the non-significant increase in MN frequencies at ADI and REL, significant increases in nuclear bud frequency were found after 4 hours of exposure. Since nuclear budding represents a mechanism of MN formation, these results indicate that even a low dose of glyphosate as the ADI influences the level of DNA damage and cell stability. After 24 hours of exposure, all of the treated cells showed a lower frequency of micronuclei, nuclear buds

and nucleoplasmic bridges than the control. The results obtained suggest that glyphosate exerts the highest DNA-damaging potential after 48 or even after 72 hours of treatment. Considering that the shorter exposure lasted only 4 hours, while the total cell culture lasted for 72 hours before harvesting, there was an opportunity to better examine the effects of low-dose exposure on DNA damage. On the contrary, in the case of an exposure of 24 hours it seems that this effect only started to be slightly visible. Serum in the cell culture can mask the development of DNA damage observed in micronucleus assay results. This can be the explanation, why the standard time period used in this experiment did not demonstrate an effect, while in the comet assay the primary DNA damage demonstrated higher damage levels.

Conclusion

All cytogenetic, molecular and biochemical methods used in this study indicate that glyphosate applied at low concentrations, possesses a toxic potential towards HepG2 cells, which has to be further explained. It seems that the theory of different effects of low-dose vs. high-dose exposure, and more deleterious effects at low doses, is true. Although at ADI, REL and OEL, no drastically different levels of oxidative damage were seen, the elevated level of permanent DNA damage found with the micronucleus assay calls for concern, especially if leading to adduct formation, as shown by comet assay results. Based on the obtained results, it cannot be said without doubt whether glyphosate acts as an aneugen or a clastogen, but there are indications from previous studies that the aneugenic effect plays an important role in the formation of micronuclei. This study did not evaluate the effects of glyphosate on cytoskeleton and proteins, but this would be a direction for future evaluations of glyphosate toxicity, together with the clarification of its effects on cell membrane level, especially in different phases of the cell cycle.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to examine the effects of acute exposure (4 and 24 hours) of human hepatoma HepG2 cells to glyphosate at concentrations equivalent to the systemic concentrations at the ADI, REL and OEL. The endpoints investigated in this study are cell proliferation, DNA damage, MN formation and oxidative stress. A non-statistically significant increase in cell proliferation was seen in the CCK-8 test with no dose-effect relationship. The comet assay showed a statistically significant decrease in tail intensity after 4 hours with no difference from control after 24 hours. In the CBMN cytome assay a non-statistically significant increase in BN MN frequency was seen after 4 hours without a dose-effect relationship. After 24 hours, a decrease instead of an increase in BN MN frequency was reported. The nuclear bud frequency was statistically significantly elevated after 4 hours of exposure but was statistically significantly lower than control after 24 hours of exposure. The indicator tests for oxidative stress did not show a substance related effect.

This publication is considered relevant but reliable with restrictions because the cytogenetic damage found *in vitro* at a systemic concentration corresponding with the ADI (0.5 µg/mL which should have been 0.17 µg/mL) was not confirmed in *in vivo* regulatory MN studies with doses up to 2000 mg/kg bw corresponding with a systemic concentration of about 50 µg/mL.

Reliability criteria for *in vitro* toxicology studies

Publication: Kasuba *et al.,* 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines		
Study performed according to GLP		
Study completely described and conducted following scientifically acceptable standards		
Test substance		

Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of ≤100% as Pestanal®. Source: Sigma-Aldrich Laborchemikalien GmbH, Germany.
Only glyphosate acid or one of its salts is the tested substance		As "Pestanal"
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	Culture concentration not in correspondence with ADI of 0.5 mg/kg bw.
Metabolic activation system clearly and completely described	N	HepG2 cells used
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	N	Very low concentrations used
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	?	No significant effects were observed for the level of ROS and GSH for 4h and 24h incubation, and for 24 h for lipid peroxidation. Elevated levels of cytogenetic damage were found in the CBMN assay and the comet assay results indicate possible cross-linking or DNA adduct formation. These data were obtained at in vitro test concentrations that correspond with an external dose of 0.5 mg/kg bw/day (ADI) whereas there are regulatory studies with no effects at doses up to 2,000 mg/kg bw/day
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant but reliable with restrictions because the cytogenetic damage found in vitro at a systemic concentration corresponding with the ADI (0.5 µg/mL which should have been 0.17 µg/mL) was not confirmed in *in vivo* regulatory MN studies with doses up to 2000 mg/kg bw corresponding with a systemic concentration of about 50 µg/mL.		

1. Information on the study

Data point:	CA 5.4
Report author	Koller V. J. *et al.*
Report year	2012
Report title	Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells
Document No	Arch Toxicol (2012) Vol. 86, 805–813
Guidelines followed in study	SCGE assays were performed according to the guidelines described by Tice et al. (2000) and the CBMN Cytome assay according to Fenech (2007). The corresponding Guidance Documents are OECD 489 and OECD 487
Deviations from current test guideline	Cell type (buccal cells)
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

Aim of this study was to investigate the cytotoxic and genotoxic properties of glyphosate (G) and Roundup (R) (UltraMax) in a buccal epithelial cell line (TR146), as workers are exposed via inhalation to the herbicide. R induced acute cytotoxic effects at concentrations [40 mg/l after 20 min, which were due to membrane damage and impairment of mitochondrial functions. With G, increased release of extracellular lactate dehydrogenase indicative for membrane damage was observed at doses [80 mg/l. Both G and R induced DNA migration in single-cell gel electrophoresis assays at doses [20 mg/l. Furthermore, an increase of nuclear aberrations that reflect DNA damage was observed. The frequencies of micronuclei and nuclear buds were elevated after 20-min exposure to 10 -20 mg/l, while nucleoplasmatic bridges were only enhanced by R at the highest dose (20 mg/l). R was under all conditions more active than its active principle (G).

Materials and Methods

Chemicals - Glyphosate (purity 95% w/w) and Roundup (Roundup Ultra Max, 450 g/L glyphosate acid) were obtained from Monsanto Europe S.A.

Storage and cultivation of the indicator cells - The human cell line TR146 was cultured under standard conditions in DMEM supplemented with 10% heat-inactivated fetal calf serum. The medium was changed every 2–3 days. When the cultures reached confluency, the cells were washed with Dulbecco's PBS, detached with TrypLE Express, centrifuged, and sub-cultured. TR146 cells express ultrastructural characteristics of normal human buccal epithelial cells, e.g. intermediate filaments, microvilli-like processes, and lack of complete keratinization.

Cytotoxicity assays - For cytotoxicity experiments, $5x10^4$ indicator cells were seeded in 96-well plates and exposed to concentrations of glyphosate and Roundup ranging from 0 to 200 mg glyphosate equivalents/L for 20 minutes. Toxicity due to damage of the cell membrane was determined with the extracellular LDHe assay that is based on the measurement of the oxidation of NADH to NAD. Alterations of mitochondrial functions were studied in XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) assays that measure succinate dehydrogenase activity of viable cells. The SRB test was used to monitor total protein synthesis as a marker of cell proliferation. SRB binds to cellular proteins and can be quantified after solubilization. The neutral red assay was conducted to evaluate membrane integrity and lysosomal activity of the cells. All experiments are based on spectrophotometric measurements and were evaluated with an automated microplate reader. In all experiments, three measurements were performed per dose and repeated at least once.

Single-cell gel electrophoresis (SCGE) assay - SCGE assays were performed to determine the effect of glyphosate and Roundup on DNA stability. The concentrations of glyphosate and Roundup used in this assay ranged from 0 to 2000 mg glyphosate equivalents/L. TR146 cells were seeded in 24-well plates and allowed to attach. Thereafter, the culture medium was replaced by 400 µL of different concentrations of glyphosate dissolved in serum-free medium. After incubation in the dark at 37ºC and 5% CO_2 for 20 minutes, the medium was discarded and the cells washed twice with DPBS and detached. After two washing steps with DMEM and centrifugation, the pellets were resuspended in low melting point agarose, spread onto slides precoated with normal melting point agarose and lysed in the dark at 4ºC for at least 1 hour. After 20 minutes unwinding in an alkaline electrophoresis solution electrophoresis was carried out. Air-dried slides were stained with ethidium bromide and the percentage of tail DNA was measured using a computer-aided image analysis system (Comet IV, Perceptive Instruments Ltd., Haverhill, UK). For each experimental point, 3 cultures were made in parallel and from each culture, one slide was prepared and 50 randomly distributed cells evaluated.

Cytokinesis-block micronucleus (CBMN) cytome assay - The CBMN assay using the cytochalasin B technique was performed to determine the effect of glyphosate and Roundup on chromosome integrity. The concentrations of glyphosate and Roundup used in this assay ranged from 0 to 20 mg glyphosate eq./L. Into 6-well plates, 4.5 x 10^5 cells were seeded and allowed to attach overnight. After treatment for 20 minutes with the test compounds or 100 µg/mL methyl methanesulfonate as the positive control, the cells were washed twice with PBS and cultured in DMEM containing 10% FCS and cytochalasin B for 48 hours. Subsequently, the cells were washed twice with DPBS and harvested. The slides were made by cytocentrifugation and subsequently air-dried, fixed, and stained with Diff Quick stain. The total number of micronuclei (MNi) in binucleated cells (BN) as well as the number of binucleated cells with micronuclei (BN-MNi), nuclear buds (NB), nucleoplasmic bridges (NPB), and apoptotic and necrotic cells was determined. Cells that divided after addition of cytochalasin B were recognized as binucleated. The nuclear division index (NDI) was determined in 500 cells. For each experimental point, TR146 cultures were prepared in triplicate. From each culture, >1,000 binucleated cells were evaluated under 400-fold magnification (Nikon Photophot-FXA, Tokyo, Japan).

Statistical analysis - Data analyses of the cytotoxicity and SCGE assays were performed with the GraphPad Prism 5 Project software system. Results are reported as means ± standard deviations (SD). The results were analyzed using one-way ANOVA and Dunnett's test and p values ≤0.05 were considered as statistically significant. The chi-square test with Yate's correction was used for the evaluation of the CBMN experiments and p values ≤0.05 were considered statistically significant.

Results

Cytotoxicity - Clear differences have been found between the effects of glyphosate and Roundup. Roundup was in all cases more cytotoxic than the active ingredient. In the LDHe test Roundup NADH consumption was statistically significantly increased from 10 mg glyphosate eq./L on whereas this was the case with glyphosate from 80 mg/L. In the XTT test, a statistically significant decrease in cellular integrity was seen with Roundup from concentrations of 40 mg glyphosate eq./L on while there was no effect with glyphosate up to 200 mg/L. In the SRB test there was a statistically significant decrease in cellular integrity from 100 mg glyphosate eq./L on whereas there was no effect with glyphosate up to 200 mg/L. The same difference in cytotoxicity was evident in the NR assay where Roundup produced a statistically significant decrease in cell integrity from 100 mg glyphosate eq./L on whereas there was no effect with glyphosate. The LC_{50} values for the cytotoxicity of Roundup were about 100 mg glyphosate eq./L for the XXT test, 140 mg/L for the NR assay and 150 mg/L for the SRB assay.

SCGE assay - In the SCGE assay, glyphosate produced at statistically significant increase in tail intensity from 20 mg/L on without a dose-effect relationship from 40 to 2,000 mg/L. A statistically significant and dose dependent increase in tail intensity was observed with Roundup from 20 mg glyphosate eq./L up to 200 mg glyphosate eq./L, a dose level with 0% cell integrity.

CBMN assay - The endpoints recorded in this assay were the frequency of binucleated (BN) cells with micronuclei (MNi), the total number of MNi, nuclear buds (NB) and nucleoplasmic bridges (NPB). After addition of cytochalasin B for 48 h, more than 75% of the cells were binucleated (BN) and the nuclear division index (NDI) in untreated cultures was 1.89 ± 0.09. Treatment of the cells with 100 µg/mL of the positive control MMS for 20 minutes produced a significant induction of BN cells with MNi, total number of MNi, NB and NPB. Exposure of the cells to glyphosate and Roundup at 10, 15 and 20 mg glyphosate eq./L for 20 minutes led to a statistically significant and dose-dependent increase of the frequency of MN, BN-MN, NB and NPB. The number of necrotic cells was statistically significantly increased for glyphosate and Roundup at 20 mg/L. The number of apoptotic cells was statistically significantly increased with glyphosate at 20 mg/L but not with Roundup.

Discussion

The results show that Roundup but not glyphosate causes pronounced cytotoxic effects in human-derived buccal epithelial cells. The genotoxicity tests show that glyphosate as well as its formulation induce DNA strand breaks as well as nuclear anomalies that reflect DNA instability including chromosomal damage. Glyphosate did not induce effects in the NR, SRB, and in the XTT assay up to concentrations of 200 mg/L, while a clear effect was seen in the LHDe assay from 80 mg/L on. On the contrary, statistically significant cytotoxic effects were observed with Roundup in all four assays, and significant changes were seen in the LDHe assay from 10 mg glyphosate eq./L and in the XTT test from 40 mg glyphosate eq./L on. Comparisons of the sensitivity of the different toxicity tests indicate that Roundup causes membrane damage at lower concentrations than the inhibition of mitochondrial functions in embryonic and placental-derived cells (JEG3), while umbilical vein cord endothelial cells (HUVEC) were equally sensitive to both endpoints. High concentrations of glyphosate affected in these experiments primarily mitochondrial functions while no effects were seen in an assay detecting changes of cell membrane functions.

The results of the SCGE assays show that glyphosate as well as Roundup induce comet formation under alkaline conditions. The effects increased as a function of the exposure and DNA migration was also observed under conditions that did not affect the cellular integrity. Also in earlier investigations with human-derived cells, SCGE assays were used to monitor the DNA-damaging effects of glyphosate. Significant induction of DNA migration was seen in human hepatoma cells (HepG2) with Roundup 400 with glyphosate levels of more than 5 mg/L after 24 hours of treatment. Other cell lines such as normal human fibroblasts (GM38) and the human fibrosarcoma line HT1080 were less sensitive in long-term (72 h) exposure studies. Taken together, these effects show that distinct differences exist in the sensitivity of cells from different organs and indicate that drug-metabolizing enzymes, which are represented in the enzyme activation mix and in HepG2 cells, increase the DNA-damaging properties of glyphosate. The most relevant finding of the present study is the observation of a statistically significant, dose-dependent induction of MNi, BN-MNi, and NB reflecting the genomic damage by glyphosate and its formulation. The most sensitive endpoint was MNi induction. Treatment of the cells with highest level (20 mg glyphosate eq./L) of Roundup caused a 3-fold increase over the background. A weaker effect was seen with the corresponding concentration of glyphosate. NPB was a less responsive endpoint with a statistically significant increase with glyphosate and Roundup at all dose levels. These results indicate that the damage seen in the SCGE assays is not completely repaired but leads to persisting alterations of the genetic material. MNi reflect numerical as well as structural chromosomal aberrations, while NB are formed as a consequence of gene amplification or expulsion of intact chromosomes or fragments; NPB are caused by formation of dicentric chromosomes.

The findings of the present study suggest that buccal epithelial cells are more sensitive toward the cytotoxic and DNA-damaging effects of glyphosate and Roundup than cells from the hematopoietic system. The Roundup formulation that was tested contains 450 g/L of glyphosate and should be diluted according to the instructions of the manufacturer to 1–3% before use (final concentration 4,500–13,500 mg/L). The fact that we found significant acute and genotoxic effects at levels between 10 and 20 mg/L after 20 min indicates that short contact with a 225–1,350-fold dilution of the formulation may cause adverse effects in cells from the oral cavity (and possibly also in other respiratory epithelia).

3. Assessment and conclusion

Assessment and conclusion by applicant:

The objective of the present study was to find out whether exposure of human-derived buccal epithelial TR146 cells to glyphosate and Roundup causes adverse effects. In cytotoxicity experiments, four different endpoints were used, which reflect different modes of action. To assess the effect of glyphosate and Roundup on DNA stability, single cell gel electrophoresis assays (SCGE) were conducted under standard alkaline conditions reflecting the formation of single- and double-strand breaks. Additionally cytokinesis-block MN cytome assays have been conducted in which different nuclear anomalies were measured. This study demonstrated that there is a big difference in cytotoxicity between glyphosate and Roundup. This is not surprising since the surfactants present in glyphosate formulations decrease the integrity of cell and mitochondrial membranes causing toxicity and ensuing DNA instability. Glyphosate was found to significantly increase tail intensity as of 20 mg/L but without any further increase with dose from 40 to 2000 mg/L. Roundup increased in a dose dependent manner the tail intensity from 20 mg glyphosate eq./L up to 200 mg glyphosate eq./L with increasing cytotoxicity and 0% cell integrity at 200 glyphosate eq. mg/L. This indicates that there is a relationship between the cytoxicity of Roundup and DNA instability. This study has demonstrated a greater sensitivity of buccal epithelial cells for glyphosate and its formulations than hematopoietic cells where no effects have been noted in *in vivo* MN tests with doses up to 2,000 mg/kg bw. Since there is no direct exposure of the buccal epithelium with the Roundup formulation (unless it is swallowed) during application and the inhalation of aerosol of the spray dilution during application is negligible (Jauhiainen A *et al*. (1991) Am. Ind. Hyg. Assoc. J. 52, 61–64) the likelihood of DNA damage in epithelial cells of the GI and the respiratory tract remains very low.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria of an *in vitro* toxicology study.

Reliability criteria for *in vitro* toxicology studies

Publication: Koller *et al.*, 2012.	**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: Monsanto Europe S.A.
Only glyphosate acid or one of its salts is the tested substance	Y	Also formulation (Roundup Ultra Max) was 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	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	10-1000 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	Roundup, but not glyphosate causes pronounced cytotoxic effects in human-derived buccal epithelial cells. The alkaline comet test results show that glyphosate as well as Roundup induce comet formation that reflect strand breaks and apurinic sites. 20 µg/mL glyphosate in Roundup caused a 3-fold increase over the background, with glyphosate alone a weaker effect was seen.
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 of an *in vitro* toxicology study.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	Kongtip P. *et al.*
Report year	2017
Report title	Glyphosate and Paraquat in Maternal and Fetal Serums in Thai Women
Document No	Journal of Agromedicine 2017, vol. 22 no. 3, 282-289
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	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 longitudinal study measured the glyphosate concentrations found in maternal and umbilical cord serum in 82 pregnant women who gave birth in three provinces of Thailand. Through questionnaires and biological samples collected at childbirth, factors such as personal characteristics, family members occupation, agricultural activities, and herbicide use in agricultural work were evaluated as predictors of glyphosate levels in the pregnant women. Statistical analysis used univariate and binary multiple logistic regression, where the outcome was the probability of exposure to glyphosate above the limit of detection associated with occupation and household factors. The glyphosate concentrations in the pregnant women's serum at childbirth (median: 17.5, range: 0.2–189.1 ng/mL) were significantly higher ($P < .007$) than those in the umbilical cord serum (median: 0.2, range: 0.2–94.9 ng/mL). Women with glyphosate levels >limit of Detection (LOD) in serum at childbirth were 11.9 times more likely to report work as an agriculturist ($P < .001$), 3.7 times more likely to live near agricultural areas ($P = .006$), and 5.9 times more likely to have a family member who worked in agriculture ($P < .001$).

Materials and Methods

Subjects for a pilot birth cohort were recruited from pregnant women who came for prenatal care at three hospitals in Thailand: Amnatchareon Hospital in Amnatchareon Province in the northeast, Sawanpracharak Hospital in Nakhorn Sawan Province in the lower north, and Paholpolpayuhasena Hospital in Karnjanaburi Province in the west of Thailand, from May to December 2011. To be recruited, the women had to be in their 7th month of pregnancy, 19–35 years of age, not have diabetes or hypertension, and plan to give birth and have follow-up infant care at the recruiting hospital. The 82 subjects for this study included 81 full-term normal birth neonate and 1 full-term cesarean birth neonate.

During their 7th month of pregnancy, the women were interviewed about their general health, diet, and work exposures, including agricultural work, as well as about use of pesticides at home and work. Several questionnaires, each with several sections, constituted the data collection. The questionnaire was based on the type of information collected in previous studies of agricultural workers and modified for the conditions of agriculture and types of pesticide exposures experienced in Thailand. The questionnaires were reviewed by staff at each hospital, then piloted and revised based on comments. One questionnaire collected data on demographics and the mother's general health, whereas another collected birth data. The pesticide exposure questionnaire consisted of six sections; the first section contained 12 items related to pesticide use in the home or outside the home, as well as sources of drinking water. The second section contained 9 items about the woman's work history outside the home. The third section, with 10 items, covered agricultural activities if conducted by the woman. The fourth section, with 3 items, covered agricultural activities conducted by the woman during pregnancy. The fifth section, with 6 items, asked about the agricultural work of family members. The sixth section, with

30 items, collected detailed information only from those who were agricultural workers or had family members who were agricultural workers and included detailed information on the mixing and spraying of pesticides. To summarize, the pesticide exposure questionnaire had 40 items for all subjects and 30 items only for those who were self-identified as agricultural workers or who had family members who were agricultural workers. For comparisons with agriculturists, women with other occupations were used as the control group. The nurses at the prenatal clinics in the three study hospitals were trained to recruit and interview subjects. The maternal and umbilical cord serum was collected during delivery by the delivery nurses and was frozen at -45°C until analysis. This study was reviewed and approved by the Ethics Committee on Human Rights Related to Human Experimentation, Mahidol University, and the University of Massachusetts Lowell Institutional Review Board.

Glyphosate was obtained from Sigma-Aldrich Inc., Singapore. Acetonitrile, trimethylamine, methylene chloride, dichloromethane (all high-performance liquid chromatography [HPLC] grade), and sodium dodecyl sulfate (SDS) (gas chromatography [GC] grade) were purchased from Apex Chemical, Bangkok, Thailand. Other chemicals were of analytical grade. Analysis was performed with an HPLC system (Agilent 1200 Series) with a fluorescence detector for glyphosate. The serum sample was analyzed on Luna 5 μm C18 (150 × 4.6 mm) column (Phenomenex, Torrance, CA, USA) with guard column at 45°C. A calibration curve was developed using hospital serum samples of non-subjects tested for the non-presence of glyphosate and then spiked with glyphosate to yield final concentrations of 12.5, 25, 50, 100, 150, and 200 ng/mL (n = 3 replicates). Samples of glyphosate were prepared using 250-μL of serum and 30 μL of derivatized glyphosate and injected into the HPLC. Evaluation of the detection limit was performed following the National Institute for Occupational Safety and Health method. For determination of accuracy and precision, concentrations of hospital serum samples of non-subjects tested for the non-presence of pesticide were used to prepare concentrations of 50, 100, and 150 ng of glyphosate/mL. Three replicates of each concentration were analyzed on three separate days. The calibration curve for glyphosate was linear over the concentration range of 12.5–200 ng glyphosate/mL serum with the correlation coefficient of 0.998. The detection limit for the analysis of glyphosate in serum was0.4 ng/mL. Values below the detection limit for serum glyphosate were used as the detection limit divided by 2 since the data were highly skewed. The recovery of the method ranged from 94.33% to 99.03% with a relative standard deviation (RSD) of <3% for glyphosate concentrations of 50–150 ng/mL.

The descriptive statistics were calculated using SPSS (SPSS version 18; PASW Statistics Base 18, Bangkok, Thailand). Since exposures were highly skewed due to the large percentage of values below the limit of detection (LOD), concentrations were reported as the median and range. For comparison of the paired mother and cord blood serum concentrations, only pairs with both measurements above the LOD levels were used with the Wilcoxon signed-rank test. Binary logistic regression was used to evaluate whether various factors were significantly associated with the probability of having glyphosate concentrations over the LOD. Several a priori factors were evaluated for inclusion as potentially significant covariates, such as maternal location/province and educational level of the mother, but these were found not to be significantly associated with the probability of having glyphosate concentrations over the LOD. Thus, occupational factors were evaluated in single-factor models and reported as unadjusted odds ratios (ORs). In models examining secondary exposure factors, maternal occupation was included as a covariate in the model to control for the influence of maternal agricultural work on serum levels.

Results

The average age of the 82 women who gave birth during the study was 26 years old (range: 19–34 years), with most having completed secondary school (41%), although 26% only completed primary school. Of the 82 women, 39% of the women described their occupation as agriculturist/farmer, whereas 21% listed themselves as a housewife, and about 13% were employees or owned their own business (often these are small retail stands with food or other items for sale). Of the 82 pregnant women who gave birth during the study, cord blood was only collected from 75 newborns, all born full term (37–41 weeks).

The percentage of maternal samples of glyphosate that were at or below the LOD was 46.3%, whereas for cord serum 50.7% were ≤LOD. Comparison of the glyphosate concentrations in paired serum

samples of mother and cord blood that were both >LOD (n = 36) found that they were significantly different (P < .001), with the mother's serum levels the higher of the two. With regard to occupational factors predicting glyphosate exposures, the odds of having a detectable level of glyphosate in serum were 11.9 higher (95% confidence interval [CI] 3.6-39.5) than the LOD among women who worked in the fields compared with those who did not. Pregnant women who worked in agricultural fields during the first, second, or third trimester of pregnancy also had significantly elevated ORs of 13.5 (95% CI 2.8-64.3), 7.7 (95% CI 2.0-29.8), and 12.4 (95% CI 1.5-102.7), respectively, of having serum levels >LOD compared with those women who never worked in the fields. Likewise, pregnant women who reported picking crops during pregnancy had a significantly elevated OR (5.4; 95% CI 1.4-20.8)), whereas those who reported the agricultural activity of digging in farm soil or controlling weeds during pregnancy did not have elevated ORs.

With regard to secondary factors, pregnant women who lived near agricultural fields (<0.5 km) were significantly more likely to have serum glyphosate levels >LOD than those women living far from agricultural fields (OR=3.7, 95% CI 1.5-9.2). Note that there was no significant relationship between home location and agricultural occupation; thus, the adjusted OR did not reflect collinearity or confounding. Those pregnant women who reported a family member who was an agriculturist/farmer living in the same house were significantly more likely to have elevated serum glyphosate than those who did not (OR=5.9; 95% CI 2.2-16.1); however, when adjusted for occupation, the OR became non-significant because of the high correlation between having a family member who was a farmer in the same house and maternal occupation as a farmer.

The most striking finding of this study was that glyphosate concentrations over the LOD were significantly more likely to be found in the serum of pregnant women collected at childbirth if their occupation was an agriculturist/farmer or conducted various agricultural tasks or if they lived near agricultural fields sprayed with pesticides (unspecified), when compared with women who had no agricultural exposures. Researchers measured glyphosate levels in urine among farmers in the United States and found that farmers who had skin contact with pesticides or did not use rubber gloves had significantly higher glyphosate concentrations in their urine. Among those who did not wear rubber gloves, appreciable differences were found in the urinary glyphosate levels between those who repaired equipment during applications or spilled during mixing/loading or application. This supports the notion that dermal contact is an important route of exposure for this herbicide. Researchers also reported urinary glyphosate in one of the children who lived 1.5 km away from the farm where their father sprayed glyphosate in France. However, in a US study, very few of the spouses (4%) and children (12%) who lived in a home within 1 mile of the sprayed farm fields had measurable urinary glyphosate on the day of spraying, and an even lower percentage had measurable levels on days 1–3 post application. Among the applicators, 60% had measurable glyphosate on the spraying day, with a decrease to 27% on the third day post application. This raises the question of the half-life of glyphosate and how the Thai agricultural women giving birth had measurable levels of glyphosate in their serum. Although these women had regular, repeated exposures to glyphosate, even during their final trimester of pregnancy, it is unlikely that they were exposed in the field on the day prior to giving birth. It is possible that these serum levels were caused by the drift of glyphosate spray, skin contact with the pesticide contaminated family member working in the fields, contamination of the home through clothing (take home exposure), or through poor storage of pesticides near the home. Alternatively, little is known about the metabolism and storage of pesticides during pregnancy and the impact on pesticide half-life. The median (range) glyphosate concentrations of maternal and umbilical cord serums in this current study were 17.5 (0.2–189.1) and 0.2 (0.2–94.9) ng/mL, respectively. The maternal serums of glyphosate at birth were significantly higher than those in cord blood serums. It is not known why maternal serum levels were higher than cord serum samples. At this time, only one study has looked at serum levels of pesticides in pregnant and non-pregnant Canadian women who were not agriculturists or living with a spouse who worked with pesticides. They found no detectable levels of glyphosate in the pregnant women before birth or in their cord blood samples. However, 5% (2/39) of the non-pregnant women in that study had measurable glyphosate serum levels (including one with a level of 93.6 ng/mL). Potential exposures in that study were assumed to be through consumption of pesticides associated with genetically modified foods. In our study, we found measurable glyphosate levels (>LOD) in 14.7% of the maternal serum samples of women who were not agriculturists/farmers by occupation. This is concerning, because researchers studied the effect of glyphosate exposures on the risk of miscarriage among women living

on farms in Ontario, Canada, and found that women who were exposed to glyphosate before conception (from 3 months before up to conception) had a higher risk of spontaneous abortion.

Conclusion

This study suggests that agricultural activities do increase maternal serum levels of glyphosate even in samples taken on the day of birth. In the case of glyphosate, living near farmland where pesticides are sprayed can also significantly increase the risk of serum levels >LOD at birth. These results suggest that a study evaluating the long-term health of children exposed to herbicides during gestation should be considered. Given that herbicides make up the largest volume of pesticide imports in Thailand, and that imports continue to increase, further regulation of the sale and use of pesticides may help safeguard the health of Thai children.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study suggests that agricultural activities increase maternal serum levels of glyphosate, even in samples taken on the day of birth. Living near farmland where pesticides are sprayed can also significantly increase the risk of serum levels >LOD at birth. Limitations of this study include a small sample size (N=82) and large percentages of maternal samples cord serum that were at or below the LOD for glyphosate (46.3% and 50.7%, respectively).

This publication is considered relevant to the risk assessment of glyphosate but reliable with restrictions because the analytical method used for glyphosate could have been described in more detail.

Reliability criteria of exposure studies

Publication: Kongtip *et al*., 2017	**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.	NA	Subjects of a pilot birth cohort being monitored for glyphosate and paraquat.
Exposure to formulations with glyphosate combined with other a.i.	NA	
Exposure to various formulations of pesticides	NA	
Study		
Study design clearly described	Y	
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	

Analytical method described in detail	Y	Analytical method for glyphosate could be described in more detail (derivatization).
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant to the risk assessment of glyphosate but reliable with restrictions because the analytical method used for glyphosate could have been described in more detail.		

1. Information on the study

Data point:	CA 5.3
Report author	Kumar S. *et al.*
Report year	2014
Report title	Glyphosate-rich air samples induce IL-33, TSLP and generate IL-13 dependent airway inflammation
Document No	Toxicology 325 (2014) 42-51
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 aim of this study was to explore the mechanisms of glyphosate induced pulmonary pathology by utilizing murine models and real environmental samples. C57BL/6, TLR4–/–, and IL-13–/– mice inhaled extracts of glyphosate-rich air samples collected on farms during spraying of herbicides or inhaled different doses of glyphosate and ovalbumin. The cellular response, humoral response, and lung function of exposed mice were evaluated. Exposure to glyphosate-rich air samples as well as glyphosate alone to the lungs increased: eosinophil and neutrophil counts, mast cell degranulation, and production of IL-33, TSLP, IL-13, and IL-5. In contrast, in vivo systemic IL-4 production was not increased. Co-administration of ovalbumin with glyphosate did not substantially change the inflammatory immune response. However, IL-13-deficiency resulted in diminished inflammatory response but did not have a significant effect on airway resistance upon methacholine challenge after 7 or 21 days of glyphosate exposure. Glyphosate-rich farm air samples as well as glyphosate alone were found to induce pulmonary IL-13-dependent inflammation and promote Th2 type cytokines, but not IL-4 for glyphosate alone

Materials and methods

Mice

C57BL/6 female (6–9 weeks) mice were purchased from Jackson Laboratory (Sacramento, CA). TLR4–/– mice (backcrossed 10 generations) were received from Cincinnati Children's Hospital Medical Center (CCHMC). Both strains were subsequently bred in house. Female mice of wild type and IL-13–/– BALB/c background were received from the laboratory of Dr. Fred Finkelman, CCHMC. Mice were housed in individually ventilated cages in a pathogen free facility at the Department of Environmental Health, University of Cincinnati (UC) following the UC Institutional Animal Care and Use Committee (IACUC) guidelines and all experiments were conducted following a UC IACUC – approved protocol.

Antibodies and reagents

The following antibodies for flow cytometry were purchased: Ly-6G (Gr-1) eFluor1 450 (RB6-8C5; Isotype Rat IgG2b) from eBioscience (San Diego, CA). CD16/CD32 (2.4G2; Isotype Rat IgG2b) and SiglecF-PE (E50-2440; Isotype Rat IgG2a) were purchased from BD PharMingen (San Jose, CA). A kit for measuring serum levels of MMCP1 was purchased from eBioscience.

Collection of farm air samples during summer pesticide spray seasons

Air samples were collected by three sets of total inhalable aerosol samplers (Button Inhalable Aerosol Sampler, SKC Inc., Eighty Four, PA) operated in parallel on three farms in Butler County, Ohio during

summer glyphosate spray seasons. Samplers were installed at 1.5 m height at the edge of the field downwind from the spraying locations (sizes: approx. 5000 - 10,000 m^2). The sampling period was approximately 24 h starting from glyphosate spraying and air samples were collected at a flow rate of approximately 4 l/min on glass fiber filters. The filters from one set of samplers containing aerosolized glyphosate were eluted using PBS and the suspensions were filtered. A stock solution was prepared by pooling the samples collected from three farms (from now on referred as 'Real Env.') and used for intranasal treatment of mice. The filters from the other two sets of samplers were analyzed for glyphosate and endotoxin to estimate the levels of glyphosate and endotoxin in 'Real Env.' samples.

Analysis of glyphosate in filter extracts

Glyphosate residues from filters were extracted using KH2PO4 buffer/1 M NaOH in an automatic shaker followed by freeze-drying. The freeze-dried samples were dissolved with deionized water and filtered through 0.45 mM Millipore filter. Glyphosate levels in the suspensions were determined by Abraxis ELISA Kit at 450 nm. The average amount of glyphosate per filter was 17.33 µg, which correspond to average airborne concentration of 22.59 ng/m^3.

Analysis of endotoxin in filter extracts

Endotoxin in filter extracts were analyzed using the Limulus amebocyte lysate assay (Pyrochrome LAL; Associates of Cape Cod Inc., Falmouth, MA), as described previously. The samples were spiked with endotoxin standard of 0.50 EU/ml to assure that there was no inhibition or enhancement between the filter extracts and the reagents. The average amount of endotoxin per filter was 24.49 EU, which correspond to average airborne concentration of 4.87 EU/m^3.

Treatment of mice with farm-derived air samples, glyphosate and sensitization with OVA

PBS suspended farm air sample ('Real Env.'; estimated amount of glyphosate: 8.66 µg/mL) and reagent grade glyphosate (Sigma – Aldrich, St. Louis, MO) (100 ng, 1 µg or 100 µg) were delivered (in 30 µL) to the nose of anesthetized mice which were witnessed to aspirate the solution. Treatments were administered either: daily for 7 days or 3 times a week for 3 weeks. Same exposure schedule was followed for OVA alone (100 µg) and for OVA (100 µg) plus different dose of farm air sample and glyphosate. Mice were sacrificed 24 h after final airway treatment with sodium pentobarbital.

Histological analysis of lung

Formalin-fixed paraffin embedded lung sections (5 µm thick) were prepared for H&E and chloroacetate esterase (CAE) staining. The entire histological slide from each mouse was examined in blinded fashion and given a global categorical severity score based on infiltration of cells into parenchymal, peribronchial, and perivascular regions of lungs.

Immunohistochemical staining

To analyze IL-33 and TSLP expression in the lungs section, the following antibodies were used for immunostaining: mouse IL-33 (0.2 mg/mL, AF3626, R&D Systems, Minneapolis, MN); mouse TSLP biotinylated (0.2 mg/ml, BAF555, R&D Systems) and respective isotype controls (R&D Systems). IL-33 and TSLP antibody–antigen complex were detected using Cy3 donkey anti-goat IgG (1:10,000) (Invitrogen/Molecular probes, Grand Island, NY). Slides were counterstained with DAPI (Vector Labs, Burlingame, CA). Images were obtained using a Nikon A1R Si microscope.

Isolation of lung inflammatory cells

Lungs were perfused with PBS, removed, manually minced into 1–2 mm fragments and then placed in Hank's Balanced Salt Solution (Sigma–Aldrich) containing Liberase TL (50 µg/mL; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/mL; Sigma – Aldrich). Tissue was digested at 37°C in a CO_2

incubator for 30 min. The tissue suspension was then passed through a 40 μm cell strainer. ACK lysis buffer (invitrogen) was used to clear red blood cells.

Flow cytometric analysis

Single cell suspensions from lungs (10^6 per mL) were blocked with anti-mouse CD16/CD32 antibodies before cell-surface staining. Cells were stained with fluorescently-labeled antibodies against SiglecF, Ly-6G/C (Gr-1), in different combinations according to the experiment. Analysis was performed using a FACSCanto II cytometer and FACSDIVA software (BD Biosciences). Eosinophils were defined as being $SiglecF^+Gr\text{-}1^+$ and neutrophils as $SiglecF^-$ $Gr\text{-}1^+$.

Cytokine measurement

IL-4, IL-10, IL-13, and IFN-γ production were measured by the in vivo cytokine capture assay (IVCCA). Briefly, biotinylated cytokine-specific mAbs were injected via tail vein immediately before the last airway treatment, and blood was collected 24 h later; sera or plasma were analyzed with microtiter plates wells coated with corresponding anti-cytokine mAbs. Cytokine levels were also assessed in Bronchoalveolar lavage fluid (BALF) that was obtained 24 h after the last airway treatment. A kit for measuring in vivo IL-4 production by IVCCA, R46A2 and XMG1.2 anti-IFN-γ mAbs was purchased from Becton–Dickinson (Franklin Lakes, NJ); eBio1316H and eBio13A anti-IL-13 mAbs JES5-2A5 and JES5-16E3 anti-IL-10 mAbs ELISA Ready-SET-Go analysis kits for measurement IL-33 and IL-5 were purchased from eBioscience. Assays were performed according to the kit's manufacturer protocols.

Statistical analyses

Data were analyzed with Sigma Plot 12.0 (Systat Software, Inc., San Jose, CA). Statistically significant differences in means were determined by one-way ANOVA followed by Bonferroni multiple comparison tests. Kruskal–Wallis tests were conducted if the data did not have a normal distribution. All the data are presented as mean ± SD for each group. Probability values of <0.05 were considered significant.

Results

Exposure of air samples collected during glyphosate spray on farms stimulates airway inflammation

Wild type C57BL/6 (WT) and TLR4–/– mice were intranasally exposed to 'Real Env.' samples (PBS suspended farm air samples) daily for 7 days. 'Real Env.' exposure was found to substantially increase the cell count in both the lungs and BAL fluid of WT and TLR4–/– mice. Additionally, the increase in pulmonary infiltrate in lungs was found to be higher in TLR4–/– than in WT mice (Fig. 1A and B). Similarly, an increase in eosinophil and neutrophil levels in 'Real Env.' treated mice (Fig. 1C–F) were observed. This inflammation was also confirmed by histological examinations (Fig. 1G) and elevated IgG1 and IgG2a levels. Additional experiments were conducted using reagent grade glyphosate of different doses. Administration of reagent grade glyphosate to the airway of mice produced substantial pulmonary inflammation whether the daily dose given was 100 ng, 1 μg or 100 μg for 7 days.

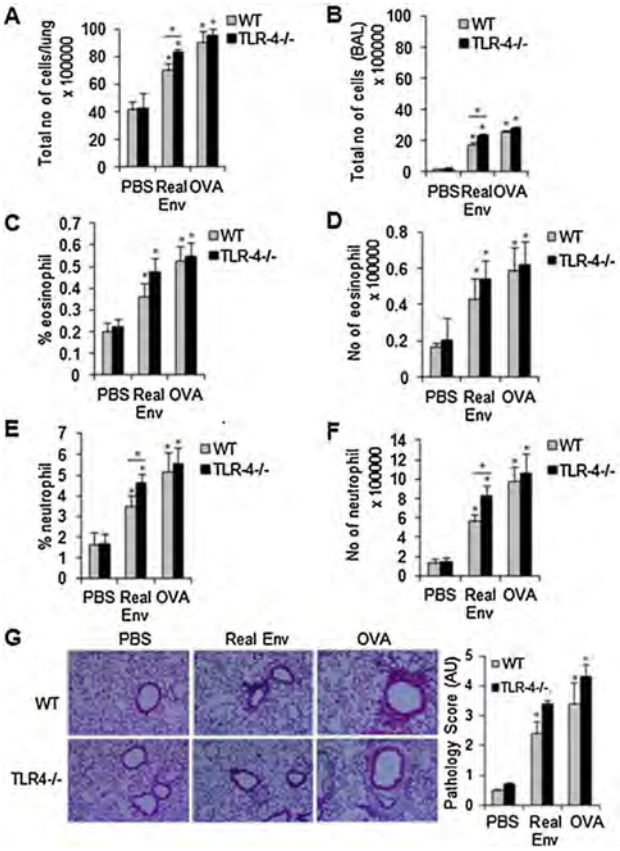


Figure 1: Increase in total number of cells, eosinophils, and neutrophils in lung and BAL fluids upon airway exposure to farm air samples ('Real Env.') and OVA for seven consecutive days (mean ± SD; *n* = 8). Increase in total number of cells in (A) lung and (B) BAL fluids. Increase in percentage (C) and total number (D) of eosinophils and neutrophils (E, F) per lung upon exposure to farm air samples ('Real Env.'). (G) Representative lung sections (H&E staining) and its pathology score from mice treated with PBS, farm air samples ('Real Env.') and OVA intranasally for 7 consecutive days (mean ± SD; *n* = 8); magnification 200x. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4–/– mice group.

In the BALF and lung digests, a significant increase in the total cell count when treated with glyphosate at 1 µg or 100 µg (Fig. 2A and D) was found. Eosinophils (Fig. 2B and C), neutrophils, (Fig. 2E and F), and IgG1 and IgG2a levels) were also increased in glyphosate-treated mice compared to controls. However, No significant changes in the total cell count, eosinophils and neutrophils, IgG1 and IgG2a at glyphosate dose of 100 ng were found. Inflammation was confirmed by histological examination (Fig. 2M). Mice treated with both reagent grade glyphosate and OVA demonstrated significantly higher cell count (Fig. 2G and J), eosinophils (Fig. 2H and K), neutrophils (Fig. 2I and L), IgG1, and IgG2a compared to PBS treated mice.

Because pulmonary mastocytosis is typically observed in protein-allergen-induced experimental asthma, the pulmonary mast cell burden in the mice were assessed.

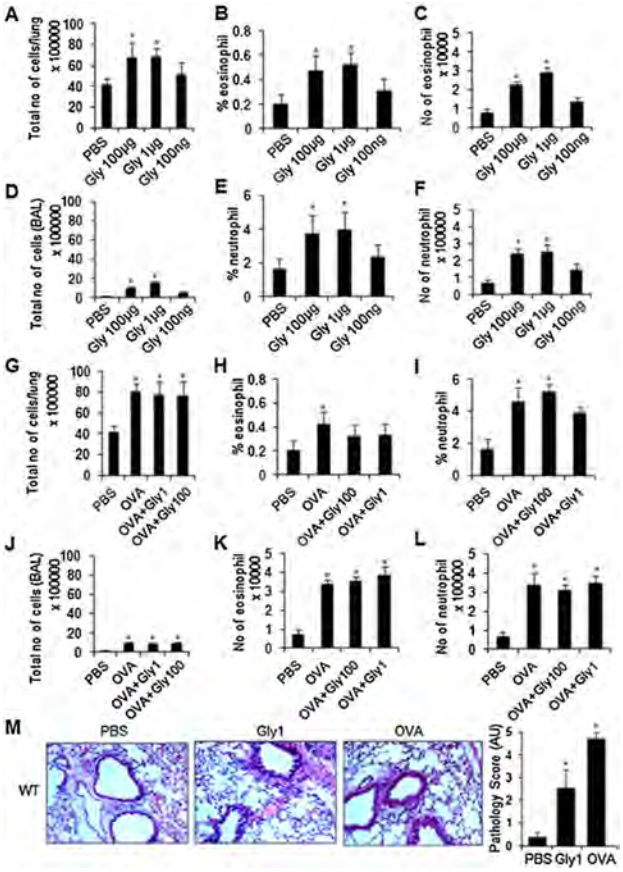


Figure 2: Increase in total number of cells, eosinophils, and neutrophils in lung and BAL fluids of WT mice upon airway exposure to glyphosate and combinations of glyphosate and OVA for seven consecutive days (mean ± SD; $n = 8$). Increase in total number of cells in (A) lungs and (D) BAL fluids upon exposure to different doses of glyphosate (100 ng, 1 µg or 100 µg). Increase in percentage (B) and total number (C) of eosinophils and neutrophils (E and F) per lung upon exposure to two doses of glyphosate. Increase in total number of cells in (G) lungs and (J) BAL fluids upon exposure to combination of glyphosate (1 µg or 100 µg) with OVA (100 µg). Increase in percentage and total number of (H and K) eosinophils and (I and L) neutrophils per lung upon exposure to OVA and combination of glyphosate, respectively. (M) Representative lung sections (H&E staining) and its pathology score from WT mice treated with PBS, glyphosate (1 µg) and OVA (100 µg) intranasally for 7 consecutive days (mean ± SD; $n = 8$); magnification 200 x. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS and treated WT mice group

A significant increase in mast cell number in lungs treated with the substances isolated from the air on active farms ('Real Env.') and reagent grade glyphosate (Fig. 3A and C) was not observed. However, the MCPT-1 levels were found to be substantially higher in both groups indicating increased mast cell degranulation in the treated mice (Fig. 3B and D).

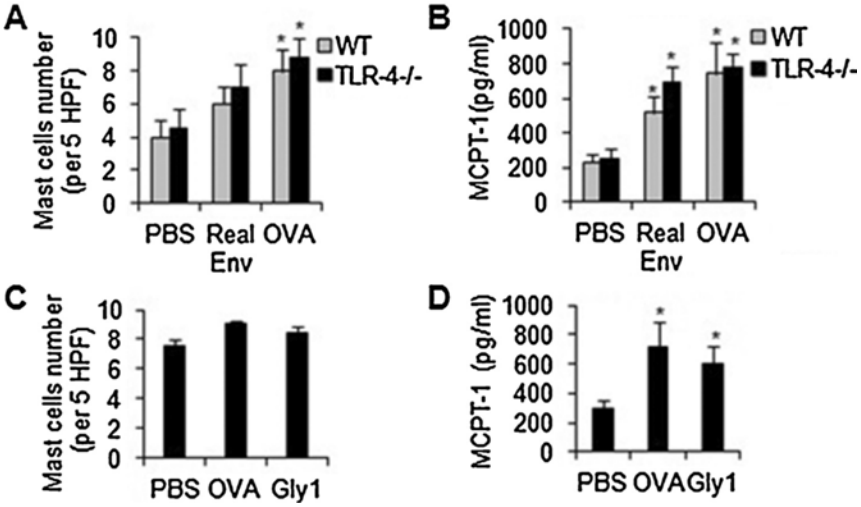


Figure 3: Farm air samples containing glyphosate as well as pure glyphosate alone induce increased mast cell degranulation but no increase in lung mast cell numbers upon airway exposure. (A) Mast cells number in CAE stained lung section and (B) serum MCPT-1 concentration in blood 4 h after last exposure of PBS, farm air samples ('Real Env.'), and ovalbumin (OVA). (C) Mast cells number in CAE stained lung section and (D) serum MCPT-1 concentration from mice treated with PBS, ovalbumin and 1 μg of glyphosate delivered to intranasally for 7 consecutive days (mean ± SD; n = 8). * Indicates statistically significant differences ($p < 0.05$) with respect to PBS and treated mice group.

Glyphosate-rich farm air samples induced airway inflammation and higher production of IL-10, IL-13, IL-5, IFN-γ and IL-4 but glyphosate alone failed to produce IL-4

To evaluate the glyphosate-induced inflammation, the systemic cytokine profile (Fig. 4A–E) of 'Real Env.' and glyphosate exposed mice were measured using IVCCA (Finkelman and Morris, 1999). Significantly higher levels of IL-5, IL-10, IL-13, and IL-4 were found upon treatment with 'Real Env.' alone in WT and TLR4–/– mice (Fig. 4A–D) approaching the levels induced by treating with OVA alone. The production of IL-5, IL-13 and IL-10 following 'Real Env.' exposures was higher in TLR4–/– than in WT mice. No significant difference in IL-4 production between TLR4–/– and WT mice were found (Fig. 4D). Production of these cytokines in mice given two different doses of glyphosate were then tested and found significantly higher levels of IL-5, IL-10, IL-13 and IFN-γ (Fig. 4F) that approached those levels induced by treating with OVA alone. Notably, there was no additional or synergistic effect when OVA was co-administered with glyphosate (Fig. 4G). Another interesting finding is that glyphosate alone was unable to induce significant levels of IL-4 while airway treatment with glyphosate with OVA did so.

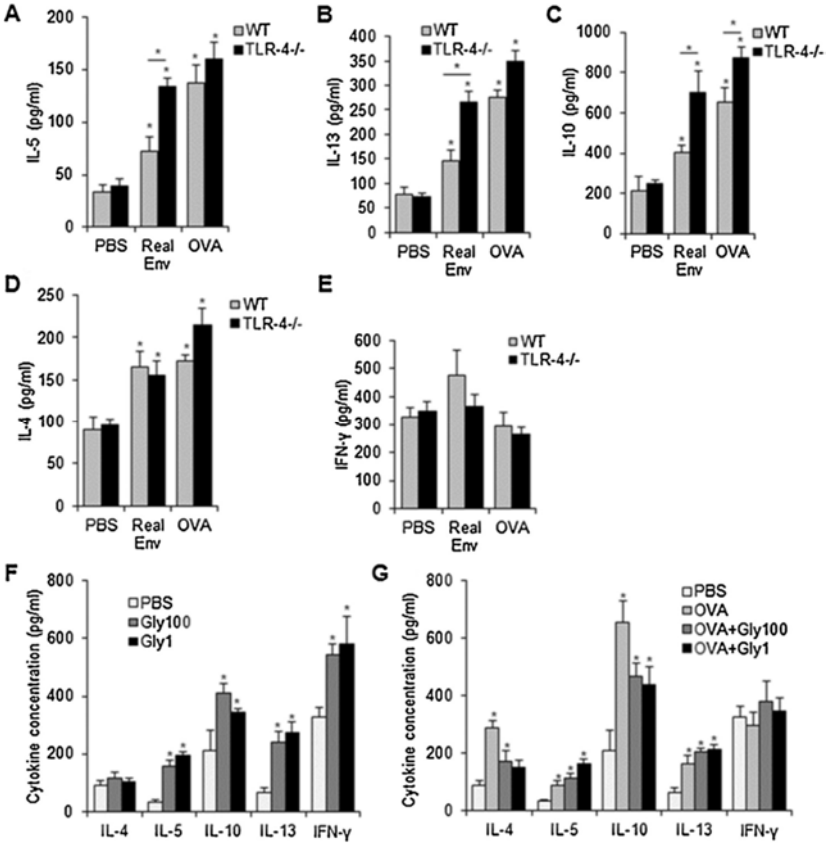


Figure 4: (A–E) Higher production of IL-5, IL-13, IL-10, IL-4 and no change in the IFN-γ levels upon exposure to farm air samples in WT and TLR4–/–mice. (F) The increased level of IL-5, IL-10, IL-13, IFN-γ and no change in the IL-4 levels upon glyphosate (1 or 100 µg) exposure to WT mice. (G) The increased level of IL-4, IL-5, IL-10, IL-13, and no change in IFN-γ levels upon combination of glyphosate (1 or 100 µg) and ovalbumin (100 µg) exposure to WT mice (mean ± SD; $n = 8$). Levels of cytokines were evaluated by IVCCA in serum of mice upon 7 consecutive days of intranasal treatment with farm air samples ('Real Env.') and glyphosate. Blood samples were collected 24 h after the last exposure. IL-5 was measured in the BAL fluids. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4–/– mice group.

IL-33 and TSLP in lungs are increased upon exposure to glyphosate-rich air samples as well as reagent grade glyphosate alone

As the cytokine profile of mice treated with 'Real Env.' and glyphosate approximated those treated with OVA, mediators known to promote type 2 pathology were examined. IL-33 and TSLP appeared to be logical choices because of their well-recognized effector functions, and due to their source – the respiratory epithelium cells which would be the first cells to encounter inhaled glyphosate. The IL-33 and TSLP content of BALF were measured directly and found an abundance of both cytokines in 'Real Env.' – treated WT and TLR4–/– mice (Fig. 5A and B). IL-33 production was observed to be significantly higher in TLR4–/– mice compared to WT mice. An abundance of both cytokines in glyphosate-treated mice were observed (Fig. 5C and D). This finding was confirmed by immunohistochemical staining of IL-33 and TSLP in lung sections of glyphosate-treated mice (Fig. 5E) and 'Real Env.' – treated WT and TLR4–/– mice, which demonstrated substantial production of both cytokines, which was limited to the respiratory epithelium after glyphosate exposure.

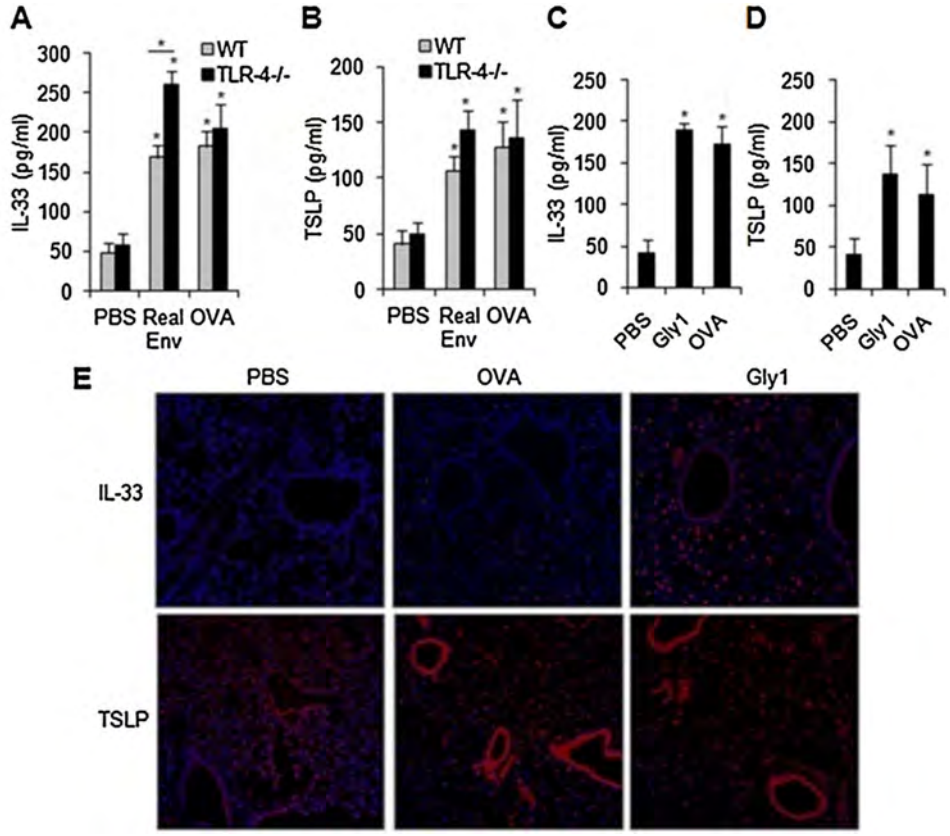


Figure 5: IL-33 and TSLP productions increased in the lung upon exposure to farm air samples and glyphosate. (A and B) ELISA based measurement of IL-33 and TSLP in BAL fluids of PBS, farm air samples and ovalbumin (100 µg) treated WT and TLR4–/– mice, respectively (mean ± SD; *n* = 8). (C and D) ELISA based measurement of IL-33 and TSLP in BAL fluids of PBS, OVA and pure glyphosate (1 µg) treated WT mice, respectively (mean ± SD; *n*=8). (E) Immunofluorescence staining of IL-33 and TSLP in the lung sections of the glyphosate treated WT mice, magnification 200x. * Indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4–/– mice group.

Glyphosate-induced pulmonary inflammation is attenuated in IL-13 –/– mice

Glyphosate as a small molecule may not be efficiently presented to conventional T cells by antigen-presenting cells. The involvement of innate pathways upon glyphosate exposure, as hypothesized, was supported by the absence of an increased production of IL-4. This absence would have been expected if type 2 innate lymphoid cells (ILC2s) were the primary source of the IL-5 and IL-13 detected. IL-33 and TSLP have been well described to induce ILC2s, which in turn causes lung pathology particularly via IL-13-dependent mechanism. To test this hypothesis, IL-13 deficient mice were exposed to glyphosate for 7 and 21 days and assessed lung inflammation. While there was no change in IL-4 levels, the inability to produce IL-13 prevented the rise in IL-5 production, but not the rise in IL-10 production, at both time points during glyphosate treatment. Deficiency in IL-13 also prevented a significant rise in IL-33 and TSLP levels at the early time point but not the latter one (Fig. 6A–D). Lack of IL-13 production was also associated with significantly less ($p < 0.05$) severe cellular infiltration noted on histology (Fig. 6E). Despite significant inflammation, no airway hyper responsiveness was found in glyphosate-treated wild type and IL-13–/– mice.

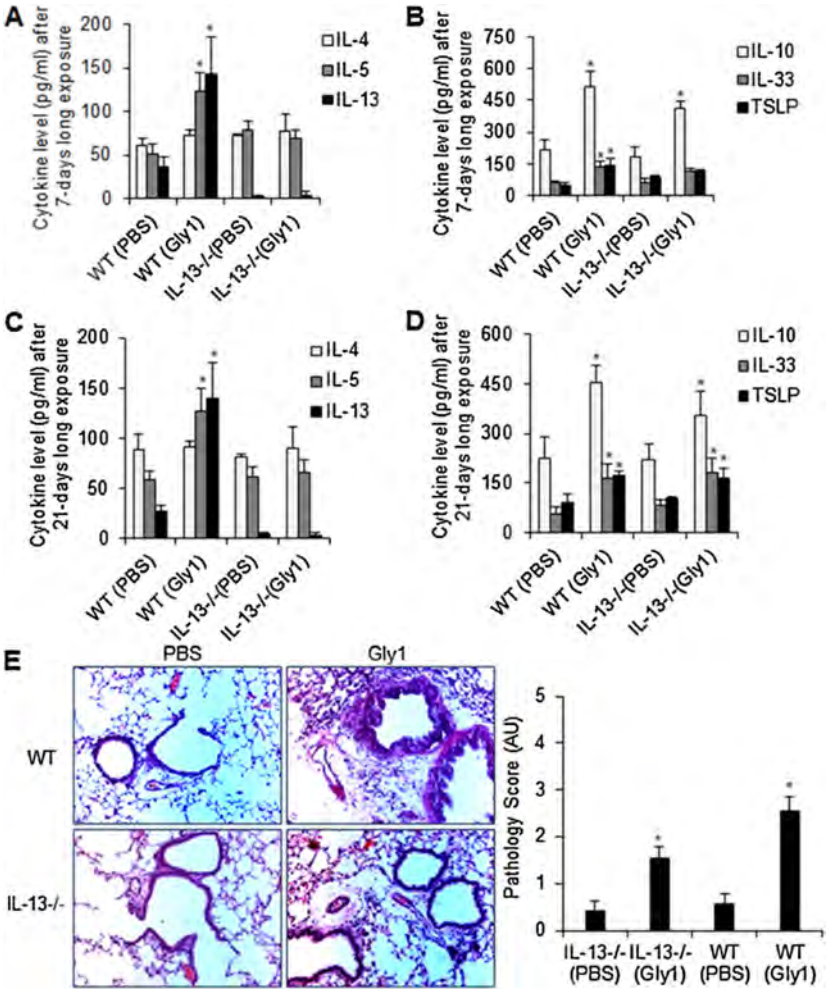


Figure 6: IL-13-deficient mice demonstrated diminished inflammatory response upon glyphosate exposure. (A, C) Diminished production of IL-5 but no change in IL-4 level, and (B, D) diminished production of TSLP, IL-33, IL-10 levels, between IL-13-deficient mice and WT mice upon glyphosate exposure (1 µg) for 7 or 21 days, respectively (mean ± SD; *n*=8). (E) Representative lung sections (H&E staining) from mice treated with PBS and glyphosate (1 µg) intranasally 3 times a week for 21 days; magnification 200 x (left panel). Arbitrary scores were based on inflammatory cells infiltration in lungs parenchyma, peribronchial, and perivascular regions. Analysis was performed in a double-blinded manner (right panel). * Indicates statistically significant differences (p <0.05) with respect to PBS treated control group.

Conclusion

The results demonstrate the capacity of glyphosate-rich air samples from farms as well as pure glyphosate to induce type 2 airway inflammation, over both short and longer time courses. Furthermore, glyphosate induced inflammation was found to be associated with induction of IL-33 and TSLP. This work also highlights the production of IL-13 as well as modulation of innate immune system by glyphosate, which may play an important role in exacerbation of airway inflammation by this low molecular weight chemical.

3. Assessment and conclusion

Assessment and conclusion by applicant:
This study evaluated nose-only exposure to glyphosate and collected farm samples containing glyphosate and evaluated the immune response in the lungs. This is not a guideline study nor an endpoint used in risk assessment. This study in not usable for risk assessment in terms of hazard assessment. In terms of exposure, the study determined that average amount of glyphosate per filter

from environmental samples after spray application to fields was 17.33 µg, which correspond to average airborne concentration of 22.59 ng/m^3. The method for the collection and analysis of the air samples was not validated and the assumptions and calculations used in the determination of the average airborne concentration were not provided, therefore the results cannot be verified. While the study itself is acceptable, it is unreliable in terms of usable endpoints for risk assessment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the method for the collection and analysis of the air samples was not validated.

Reliability criteria for *in vivo* toxicology studies

Publication: Kumar *et al*., 2014.	**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 is reagent grade. Source: Sigma - Aldrich, St. Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Sampling of aerosols from field spraying also contain co-formulants of the GBH applied.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	Female mice of wild type and IL-13-/- BALB/c background.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Intranasal application of air samples taken during glyphosate field application (24 hours) and glyphosate.
Dose levels reported	Y	Field air sample and 100 ng, 1 µg or 100 µg of glyphosate delivered intranasally.
Number of animals used per dose level reported	Y	
Method of analysis described for analysis test media	Y	Glyphosate measured in air sample with ELISA kit.
Positive control	Y	Ovalbumin.
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	Y	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 method for the collection and analysis of the air samples was not validated.		

1. Information on the study

Data point:	CA 5.4
Report author	Kwiatkowska M. *et al.*
Report year	2017
Report title	DNA damage and methylation induced by glyphosate in human peripheral blood mononuclear cells (in vitro study)
Document No	Food and Chemical Toxicology (2017) Vol. 105, 93-98
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

The purpose of this study was to assess DNA damage (determination of single and double strand-breaks by the comet assay) as well as to evaluate DNA methylation (global DNA methylation and methylation of p16 (CDKN2A) and p53 (TP53) promoter regions) in human peripheral blood mononuclear cells (PBMCs) exposed to glyphosate. PBMCs were incubated with the compound studied at concentrations ranging from 0.1 to 10 mM for 24 h. The study has shown that glyphosate induced DNA lesions, which were effectively repaired. However, PBMCs were unable to repair completely DNA damage induced by glyphosate. A decrease in global DNA methylation level at 0.25 mM of glyphosate was also observed. Glyphosate at 0.25 mM and 0.5 mM increased p53 promoter methylation, while it did not induce statistically significant changes in methylation of p16 promoter. To sum up, it was shown for the first time that glyphosate (at high concentrations from 0.5 to 10 mM) may induce DNA damage in leucocytes such as PBMCs and cause DNA methylation in human cells.

Materials and methods

Chemicals – Glyphosate (95% purity) commercially obtained from Sigma-Aldrich, USA.

Isolation of PBMCs - PBMCs were isolated from leucocyte-buffy coat from blood collected from 9 healthy volunteers (aged 18-55 years) with no symptoms of infectious disease. For each parameter, 3 leucocyte-buffy coats were taken from 3 blood donors. After dilution with PBS (1:4) PBMCs were isolated from the buffy coat by centrifugation using lymphocyte separation medium (LSM). The PBMCs were collected, suspended in erythrocyte lysis buffer and incubated. Afterwards PBS was added and the cells centrifuged. The supernatant was decanted and the cells were washed twice with RPMI with L-glutamine and 10% fetal bovine serum (FBS). The cells were resuspended in RPMI medium with L-glutamine, 10% FBS and penicillin-streptomycin (0.5%) and counted in the hemocytometer. The final PBMCs density used in the experiments after addition of glyphosate was $1x10^6$ cells/mL. After incubation, PBMCs were diluted to a density of 5×10^4 cells/mL for the comet assay and condensed to a density of 5×10^6 cells/mL for the conduct of epigenetic methods. The viability of the cells was over 94%.

Treatment of PBMCs - Glyphosate was dissolved in PBS and the final concentrations of glyphosate in the comet assay were in the range of 0.25 to 10 mM, while epigenetic changes were assessed after exposure to glyphosate at 0.25 and 0.5 mM. For the determination of DNA damage the cells were incubated with glyphosate for 24 hours and repair of the DNA lesions was assessed after a recovery period of 120 minutes following incubation. In the comet assay, 0.25 mM was chosen as the lowest concentration of glyphosate which produced any statistically significant changes in DNA damage. Epigenetic changes were assessed at 0.25 mM which did not induce DNA damage and at 0.5 mM which induced statistically significant DNA damage. For the assessment of cell viability PBMCs were incubated for 24 hours with different concentrations of glyphosate. Afterwards, the cells were

centrifuged and the test solution discarded. The cells were then resuspended in RPMI medium and incubated for an additional 2 hours. Cell viability was 90.0%, 90.2%, 90.4%, 88.5%, and 87.8% for control, 0.5, 5, 7.5 and 10 mM of glyphosate, respectively. Each DNA damage experiment included hydrogen peroxide as the positive control.

Alkaline comet assay - The comet assay was performed under alkaline conditions with some modifications. A freshly prepared cell suspension in 0.75% low melting point agarose dissolved in PBS was layered onto microscope slides pre-coated with 0.5% normal melting point agarose. The cells were lysed for 1 hour at 4 ^{o}C in an alkaline buffer and DNA was allowed to unwind for 20 minutes in an alkaline solution. Electrophoretic separation was performed in an alkaline solution at 4 ^{o}C for 20 minutes at an electric field strength of 0.73 V/cm (28 mA). Then, the slides were washed in water and stained with 2 mg/mL 4',6-diamidino-2-phenylindole (DAPI) and covered with cover slips. The comets were observed at 200 x magnification in an Eclipse fluorescence microscope. Fifty images (comets) were randomly selected from each sample and the mean value of DNA in the comet tail was taken as an index of DNA damage (expressed in percent). For one blood donor, 3 parallel tests with aliquots of the sample of the cells were performed for a total number of 150 comets. A total number of 450 comets from 3 blood donors was recorded to calculate mean ± SEM.

DNA repair - The control samples and the PBMCs treated with glyphosate at 0.5, 5, 7.5 and 10 mM were washed and resuspended in fresh RPMI 1640 medium with L-glutamine preheated to 37 ^{0}C. Aliquots of the suspension were taken immediately after incubation at time "zero" and 120 minutes later. The samples were placed in an ice bath to stop DNA repair. The repair was assessed as a decrease in the extent of DNA damage measured after 120 minutes of post-incubation using the alkaline version of the comet assay.

Methylation of p16 (CDKN2A) and p53 (TP53) promoter regions - Chemical modification of 500 ng of genomic DNA was performed with the Cells-to-CpG™ Bisulfite Conversion Kit. For methylation analysis, a quantitative methylation-specific real-time PCR assay (qMSP) was conducted in 3 independent experiments including 3 blood donors with FastStart SYBR Green Master. All samples were amplified in triplicate. To determine the methylation status of a particular gene expressed as the methylation index (MI) in percentage, the Ct values of the methylated gene of interest were compared with the Ct values of the unmethylated gene of interest.

Global DNA methylation - Global DNA methylation was determined by means of DNA quantification using 5-methyl cytosine (5-mC) monoclonal antibodies in an ELISA-like reaction using the Methylflash Methylated DNA Quantification Kit. DNA (100 ng) isolated from whole blood PBMCs was used for analysis. Each sample was analyzed in duplicate and the determination was repeated whenever there was a failure in detection. The calculation of the amount of 5-mC was done with the use of a standard curve. Methylation levels were calculated relative to the methylated control DNA and expressed as a percentage of methylated DNA.

Statistical analysis - The mean value was calculated for three independent experiments (3 blood donors), whereas for each individual an experimental point was a mean value of at least 2 (methylation analysis) or 3 replications. Statistical analysis was conducted using the Mann-Whitney test, the Student's t-test and one-way analysis of variance (ANOVA) with a post hoc multiple comparisons procedure. The differences were considered to be statistically significant when $p<0.05$.Data analysis was performed using STATISTICA software.

Results

Analysis of DNA strand-breaks and DNA repair - DNA damage (single and double strand-breaks and alkali-labile sites formation) was statistically significantly increased from a glyphosate concentration of 0.5 mM on. At 10 mM the statistically significant increase in DNA damage exceeded 13 times the control value. After 120 minutes of recovery significant repair was observed of the DNA lesions induced by glyphosate (5.61% vs 2.93%, 10.51% vs 5.07%, , 14.91% vs 7.76%, and 27.49% vs 11.05% at 0.5 mM, 5 mM, 7.5 mM, and 10 mM of glyphosate, respectively.

DNA methylation status - As compared to control cells, the percentage of the global DNA methylation level was statistically significantly decreased by glyphosate at 0.25 mM, but not at 0.5 mM. On the contrary, p53 promoter methylation was statistically significantly increased at both concentrations of glyphosate. Methylation of the p16 gene promotor was increased after treatment with glyphosate but this change was not statistically significant.

Discussion and Conclusions

This study revealed that glyphosate decreased global DNA methylation in PBMCs statistically significantly at a concentration of 0.25 mM. Surprisingly, the effect of glyphosate at 0.5 mM on global DNA methylation was not statistically significant although the mean value from three independent experiments was still decreased as compared to control. Along with the decreased global DNA methylation, a statistically significant increase in methylation of the p53 promoter region was observed at 0.25 and 0.5 mM. Altered p53 promoter hypermethylation is an epigenetic pattern frequently observed in human cancers. Thus, the results of this study suggest that glyphosate at high concentrations (≥ 0.25 mM) may cause a down-regulation of p53 gene expression and activate proto-oncogenes or retrotransposable sequences which may induce genomic alterations by insertion and/or homologous recombination. This study showed for the first time that glyphosate may induce DNA damage in human leucocytes and cause epigenetic alterations in animal cells.

3. Assessment and conclusion

Assessment and conclusion by applicant:
It was the objective of this study to investigate the effect of high glyphosate concentrations on DNA integrity and DNA methylation in PBMCs *in vitro*. It was demonstrated that glyphosate increased statistically significantly DNA damage (single and double strand-breaks and alkali-labile sites formation) from 0.5 mM up to 10 mM. Repair of the DNA lesions was significant at all concentrations tested after 120 minutes of recovery. The percentage of the global DNA methylation level was statistically significantly decreased by glyphosate at 0.25 mM but not at 0.5 mM. On the contrary, p53 promoter region methylation was statistically significantly increased as compared to control cells at 0.25 and 0.5 mM.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the lowest concentration at which DNA damage was observed (0.5 mM) is higher than the blood concentrations in rats (0.3 mM) obtained after dosing at the limit dose of 2000 mg/kg bw where no MN effects were seen.

Reliability criteria for *in vitro* toxicology studies

Publication: Kwiatkowska *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: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	Salt not mentioned
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		
Test concentrations in physiologically acceptable range (< 1 mM)	Y/N	0.25 to 10 mM
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	For DNA damage. The concentration at which DNA damage was observed is higher than the blood concentrations in rats obtained after dosing at the limit dose of 2000 mg/kg bw for the detection of MN in vivo. The results obtained are not corroborated by regulatory in vivo genotoxicity studies.
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 lowest concentration at which DNA damage was observed (0.5 mM) is higher than the blood concentrations in rats (0.3 mM) obtained after dosing at the limit dose of 2000 mg/kg bw where no MN effects were seen.		

1. Information on the study

Data point:	CA 5.8.1
Report author	Kwiatkowska M. *et al.*
Report year	2020
Report title	Evaluation of apoptotic potential of glyphosate metabolites and impurities in human peripheral blood mononuclear cells (*in vitro* study)
Document No	Food and chemical toxicology (2020) Vol. 135, pp. 110888
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 (Research conducted in an academic laboratory)
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The study aimed to assess the effect of glyphosate, its metabolites: aminomethylphosphonic acid (AMPA), methylphosphonic acid and its impurities: PMIDA, N-methylglyphosate, hydroxymethylphosphonic acid and bis (phosphonomethyl)amine on apoptosis induction in human peripheral blood mononuclear cells (PBMCs). PBMCs were exposed to the compounds studied at concentrations ranging from 0.01 to 5 mM for 4 h. It was observed an increase in reactive oxygen species (including hydroxyl radical) and cytosolic calcium ions levels as well as reduction of transmembrane mitochondrial potential (ΔΨm) in PBMCs exposed to the compounds examined. All substances studied changed PBMCs membrane permeability, activated caspase-8, -9, -3 and caused chromatin condensation, which showed that they were capable of inducing apoptosis both via extrinsic and particularly intrinsic pathway. Generally the study demonstrated that there were no differences between apoptotic changes induced by glyphosate, its metabolites or impurities, and observed changes were provoked by high concentrations of investigated compounds. Since clear changes were only seen at high concentrations, a low apoptotioc potential of these compounds was concluded.

Materials and methods

Chemicals; The investigated compounds i.e., aminomethylphosphonic acid (AMPA) (purity 98%), methylphosphonic acid (purity 98%), N-(phosphonomethyl)iminodiacetic acid (PMIDA) (purity 98%), N-methylglyphosate, hydroxymethylphosphonic acid (purity 98%) and bis-(phosphonomethyl)amine (purity 97%) were synthetized by the Institute of Industrial Organic Chemistry, Warsaw, Poland. Glyphosate [N-(phosphonomethyl)glycine] (purity 95%) in acid form was bought from Sigma-Aldrich, USA. The investigated compounds were dissolved in phosphate-buffered saline (pH 7.4). Other chemicals were purchased from POCh (Poland) and Roth (Germany) and were of analytical grade. Different concentrations of glyphosate have been selected in this study:

• 0.01 mM (low concentration) that is quite similar to the concentration determined in blood of humans who were not directly exposed to this herbicide,

• 0.05 mM to 0.5 mM (moderate to high concentration) that corresponds to the concentration that may penetrate into human blood as a result of glyphosate formulation poisoning,

• 5 mM to 10 mM (very high concentration) that corresponds to the concentration detected in humans after acute poisoning with glyphosate formulation.

Cells isolation; PBMCs were isolated from leucocyte-buffy coat obtained from blood collected in Blood Bank in Lodz, Poland. Blood was taken from healthy, non-smoking volunteers who showed no signs of infection disease symptoms at the time the blood samples were collected. The study was approved by the Bioethics Committee of the University of Lodz No. KBBN-UŁ/I/3/2013. The final PBMCs density

used in the experiments (after addition of glyphosate, its metabolites or impurities) was $2x10^6$ per 1 mL. The viability of the cells was over 95% as determined by flow cytometry.

Quantitative determination of apoptosis (YO-PRO-1/PI staining); Apoptosis is characterized by changes in cell membrane permeability. Apoptotic cells that have altered membrane permeability (membrane integrity is maintained) are permeable for a marker YOPRO-1 (carbocyanine nucleic acid stain), which exhibits green fluorescence, whereas they are not permeable for the dye propidium iodide (PI) that shows red fluorescence. The cells were treated with glyphosate, its metabolites and impurities in the final concentrations ranging from 0.01 to 10 mM and incubated for 4 h at 37 °C in total darkness. Apoptosis was induced with 10 μM of camptothecin. After the incubation, the samples were centrifuged at 300 g for 5 min at 4 °C, the supernatant was removed, and the cells were supplemented with RPMI with L-glutamine and 10% FBS. Then, the mixture of YO-PRO-1 and PI (0.1 μM each) was added to the samples, which were incubated for 20 min on ice in total darkness. The samples were analysed by flow cytometry (LSR II, Becton Dickinson) with excitation maximum at 488 nm to visualize the YO-PRO-1 green fluorescence (520/30 bandpass filter) and PI red fluorescence (610/20 bandpass filter). FMC gate on PBMCs has been established for data acquisition and the data were recorded for a total of 10,000 events per sample.

Determination of biochemical and morphological hallmarks of apoptosis

Cytosolic calcium ion level; Calcium ion accumulated in mitochondria and endoplasmatic reticulum is an important secondary messenger in controlling apoptotic cell death. The level of cytosolic calcium ions was analysed by flow cytometry (LSR II, Becton Dickinson) using a fluorescent probe Fluo-3/AM according to manufacturer's protocol. Fluo-3/AM passes through membrane of living cells, in which it is cleavaged by intracellular esterases to Fluo-3. Inside the cell, Fluo-3 exhibits green fluorescence after complexation with calcium ions. PBMCs treated with glyphosate, its metabolites or impurities in the final concentrations ranging from 0.01 to 10 mM were incubated for 4 h at 37 °C in total darkness. Next, the cells were centrifuged at 300 g for 5 min at 4 °C, suspended in Fluo-3/AM (1 μM) solution and incubated at 37 °C for 20 min in total darkness. Then, Hanks' Balanced Salt Solution (HBSS), composed of inorganic salts and supplemented with glucose (with 1% of BSA) was added to the cells suspension, and PBMCs were incubated for 40 min at 37 °C in total darkness. The cells were centrifuged at 300 g for 5 min at 4 °C and washed twice with HEPES buffer. After centrifugation, PBMCs were suspended in HEPES buffer and incubated for 10 min at 37 °C in total darkness. The samples were analysed using flow cytometer (LSR II, Becton Dickinson) with excitation at 490/500 nm to visualize the Fluo-3 fluorescence. FMC gate on PBMCs has been established for data acquisition and the data was recorded for a total of 10,000 events per sample.

Mitochondrial transmembrane potential ($\Delta\Psi m$); Mitochondrial dysfunction leading to collapse of transmembrane mitochondrial potential has been shown to participate in the induction of apoptosis. Transmembrane mitochondrial potential was shown as red fluorescence intensity of MitoTracker Red CMXRos (excitation/emission maxima – 579/599 nm). This probe is cell permeable and contains mildly thiol-reactive chloromethyl moiety for mitochondrial labeling. Nigericin and valinomycin (1 μM) were used to increase and decrease $\Delta\Psi m$, respectively. PBMCs were exposed to glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. Then, the samples were centrifuged at 300 g for 5 min at 4 °C, the supernatant was decanted, and the cells were suspended in PBS. The cells were stained with MitoTracker CMXRos in the final concentration of 1 μM for 15 min at 37 °C in total darkness, and then analysed in 96-well plates using a microplate reader (Cary Eclipse, Varian).

Caspase-8, -9 and -3 activity; Caspases are critical enzymes of apoptosis. The caspases are able to break down peptide bonds via cysteine residues in various substrates, and therefore they catalyze the apoptotic cell death irreversibly in mammals including human beings. Analysis of caspase-8 and -3 was executed according to the manufacturer's protocols. The assays were based on the hydrolysis of the peptide substrates such as acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETDAMC) by caspase-8 and acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, which resulted in the release of the fluorescent 7-amino-4-methylcoumarin (AMC). The excitation and emission wavelengths of AMC were 360 nm and 460 nm, respectively. Caspase-9 colorimetric assay was based on hydrolysis of the substrate acetyl-Leu-Glu-His-Asp-p-nitroaniline (Ac-LEHD-pNA),

which led to the release of p-nitroaniline (pNA) that absorbance was determined at 405 nm. Camptothecin (10 μM) was used to induce apoptosis. Detection of caspase-3 and -8 activities was executed using fluorescent microplate reader (Fluoroskan Ascent FL, Labsystem) and determination of caspase-9 activity was performed using absorbance microplate reader (BioTek ELx808, Bio-Tek).

Hoechst 33342/PI staining; Chromatin condensation paralleled by DNA fragmentation is one of the most important criteria, which are used to identify apoptotic cells. Morphological changes of chromatin in PBMCs were assessed by double staining with Hoechst 33342 and PI. The cells were exposed to glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. After incubation, PBMCs were centrifuged at 200 g for 3 min at 4 °C. The supernatant was removed, and the cells were suspended in PBS (0.5 ml). Then the mixture of 1 μl of Hoechst 33342 and 1 μl of PI (1 mg/ml each) was added. After 1 min incubation at 37 °C in total darkness, the cells were analysed by fluorescence microscope (Olympus IX70, Japan) at 400x magnification. PMBCs were classified on the basis of their morphological staining characteristics: viable (blue fluorescence), early apoptotic (intensive bright blue fluorescence), late apoptotic (blue-violet fluorescence) and necrotic (red fluorescence).

Determination of reactive oxygen species level

Oxidation of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; ROS play a central role in regulation of the main pathways of apoptosis mediated by mitochondria, death receptors and the endoplasmic reticulum (ER). The rate of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) oxidation was assessed by flow cytometry. 6-CarboxyH2DCF-DA is a compound widely used for the detection of intracellular oxidants production. The probe diffuses across cellular membrane where it is hydrolyzed by intracellular esterases to 6-carboxy-2′,7'dichlorodihydrofluorescein (6-carboxy-H2DCF) that after oxidation, yields highly fluorescent 6-carboxy-2′,7′-dichlorofluorescein (DCF). The cells were treated with glyphosate, its metabolites or impurities for 4 h at 37 °C in total darkness. Next, fluorescent marker was added to PBMCs, which were stained for 15 min at 37 °C in the dark. The final concentration of the fluorescent probe was 10 μM. Positive control consisted of hydrogen peroxide (2 mM). FMC gate on PBMCs has been established for data acquisition, and fluorescence was measured with the excitation and emission maxima of 488 and 530 nm, respectively. The data was recorded for a total 10,000 events per sample.

Oxidation of hydroxyphenyl fluorosceine; Highly reactive oxygen species (mainly hydroxyl radical) were assessed using flow cytometer (Becton Dickinson, LSR II) and 3-(p-hydroxyphenyl)-fluorescein (HPF). HPF is nonfluorescent until it reacts with hydroxyl radical. As a result of oxidation, the probe exhibits bright green fluorescence (excitation/emission maxima – 490/515 nm). Formation of hydroxyl radical was provoked by the addition of the mixture of ferrous perchlorate(II) (0.1 mM) and hydrogen peroxide (1 mM) to PBMCs suspension. Finally, the cells were treated with HPF in the final concentration of 2 μM and incubated for 15 min at 37 °C in total darkness. The data was recorded for a total of 10,000 events per sample.

Statistical analysis; The statistical analysis was performed with STATISTICA 8 data analysis software (2000 StatSoft, Inc., Tulsa, OK, USA). In this study, one-way analysis of variance (ANOVA) (p and F added to the description of the results) with post hoc multiple comparisons procedure (Tukey test) was used to assess statistical differences in case of normal distribution (significance marked on the charts). The difference was considered to be significant for $P < 0.05$. The individual analysis was performed on blood from 4 to 5 donors, while each experiment was repeated trice.

Results

Apoptotic changes; After 4 h incubation, all compounds studied caused an increase in the number of apoptotic cells. Apoptotic changes were observed in PBMCs treated with 0.5 mM and 5 mM of glyphosate, PMIDA and hydroxymethylphosphonic acid. Two glyphosate metabolites: AMPA and methylphosphonic acid as well as two glyphosate impurities: N-methylglyphosate and bis-(phosphonomethyl)amine caused apoptotic changes only at their highest concentration of 5 mM (Fig. 1).

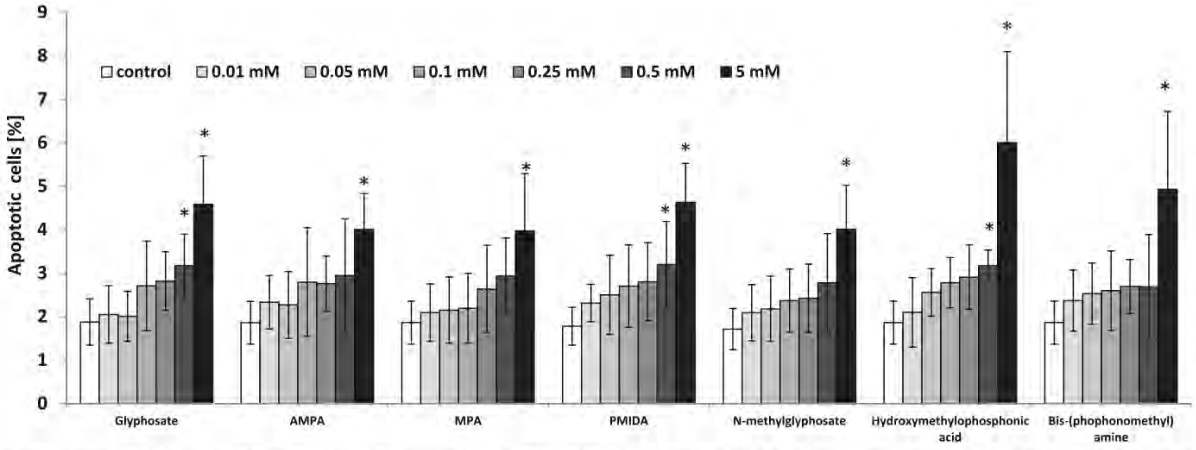


Fig. 1. Changes in the number of apoptotic PBMCs (expressed in per cent) after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to the control ($P < 0.05$).

Morphological changes of chromatin; Cell staining with Hoechst 33342 and PI allowed for the observation of early apoptotic cells (blue color), late apoptotic cells (blue-violet color) and necrotic cells (red color) in the population of PBMCs exposed to the compounds examined for 4 h. Selected photographs showing the presence of individual types of apoptotic and necrotic PMBCs are presented below (Photo 1).

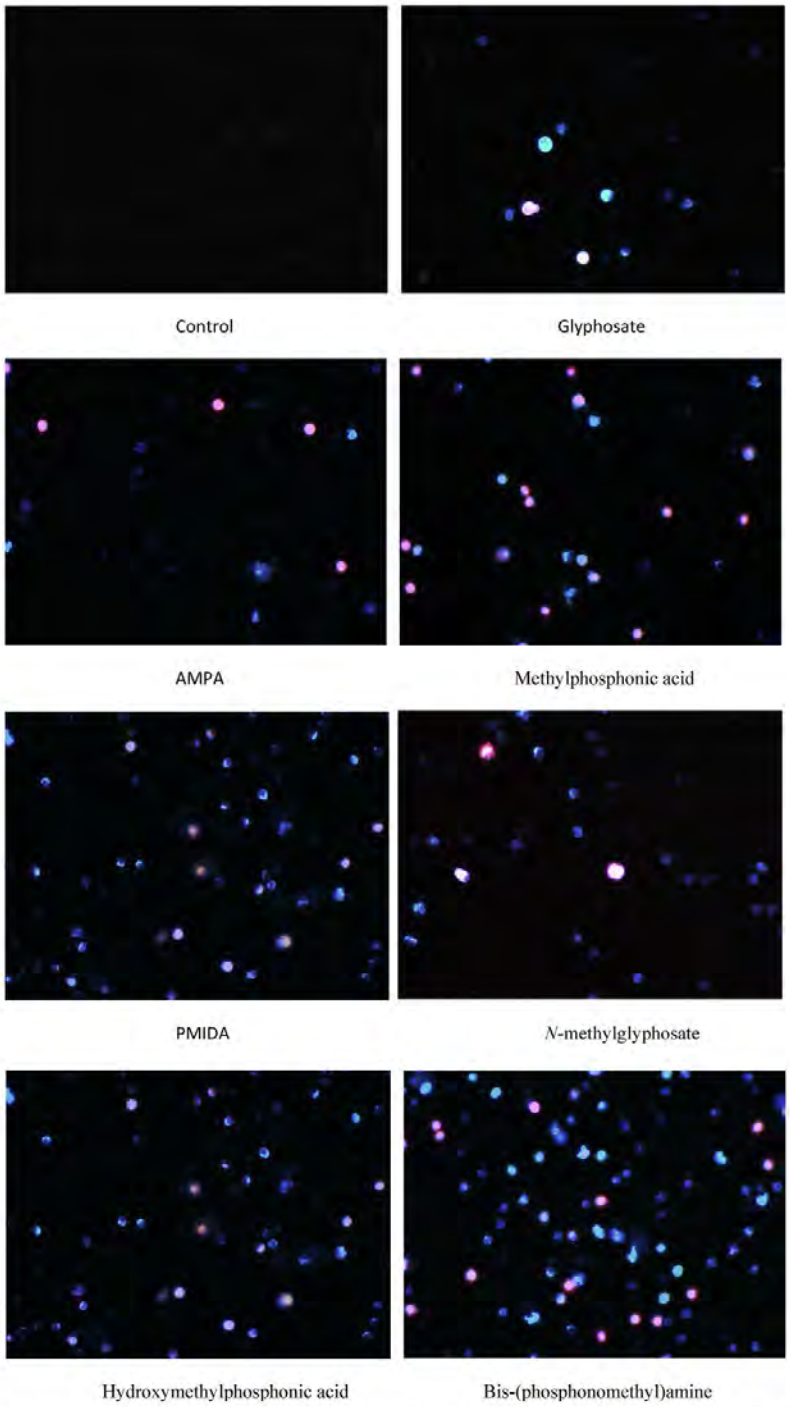


Photo 1. The representative photomicrographs of Hoechst 33324/PI-stained PBMCs pretreated with PBS (control) and with 5 mM of glyphosate, AMPA, methylphosphonic acid, PMIDA, *N*-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine. Viable cells (blue fluorescence), early apoptotic cells (intensive bright blue fluorescence), late apoptotic cells (blue-violet fluorescence) and necrotic cells (red fluorescence).

Cytosolic calcium ion level; A statistically significant increase in cytosolic calcium ion level was observed after incubation of PBMCs with glyphosate and other compounds studied (except for PMIDA). Statistically significant changes were observed for the highest concentration (5 mM) of glyphosate, AMPA and N-methylglyphosate. Glyphosate metabolite - methylphosphonic acid and glyphosate impurity - bis(phosphonomethyl)amine from the concentration of 0.5 mM caused an increase in cytosolic calcium ion level, while hydroxymethylphosphonic acid induced changes in the parameter studied from the concentration of 0.25 mM (Fig. 2).

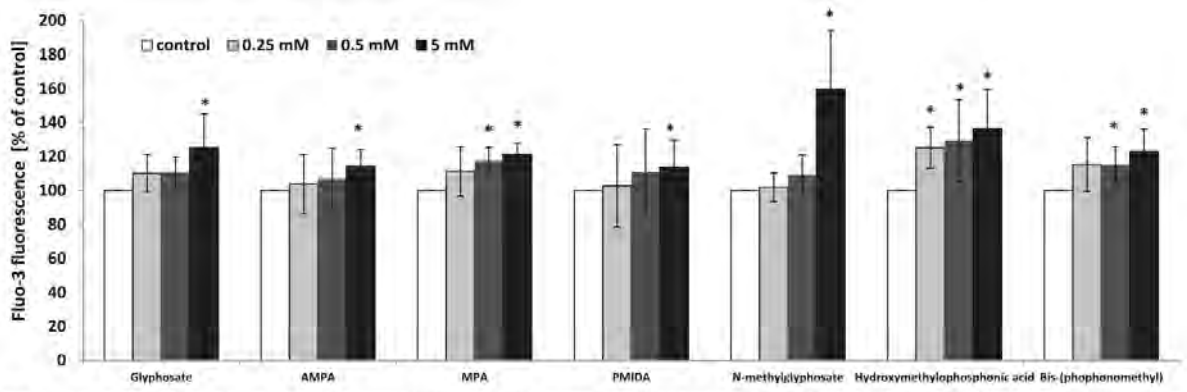


Fig. 2. Changes in cytosolic calcium ion level in control PBMCs and PBMCs incubated with glyphosate, its metabolites and impurities in the concentrations ranging from 0.25 to 5 mM for 4 h (*) Statistically significant changes compared to control ($P < 0.05$).

Transmembrane mitochondrial potential (ΔΨm); Most of the compounds studied caused a decrease in transmembrane mitochondrial potential, while hydroxymethylphosphonic acid (at 0.25 mM) caused statistically significant increase in the parameter examined. Glyphosate and one of its production impurities: bis(phosphonomethyl)amine (from the concentration of 0.05 mM) decreased ΔΨm. Other glyphosate metabolites: AMPA and methylphosphonic acid, and two glyphosate impurities: PMIDA and N-methylglyphosate decreased the parameter studied from the concentration of 0.1 mM (Fig. 3).

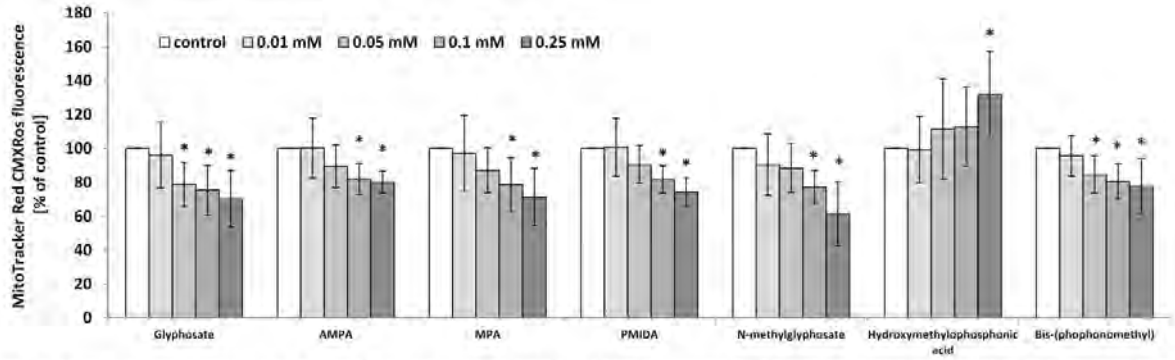


Fig. 3. Changes in transmembrane mitochondrial potential in control PBMCs and PBMCs incubated for 4 h with glyphosate, its metabolites and impurities in the concentrations ranging from 0.01 to 0.25 mM (*) Statistically significant changes compared to control ($P < 0.05$).

Caspase-8 and -9 activity; Glyphosate, AMPA, PMIDA and bis-(phosphonomethyl)amine increased in caspase-8 activity. The changes were observed in PBMCs treated with 0.5 mM of glyphosate and AMPA and with the highest concentration (5 mM) of PMIDA and bis-(phosphonomethyl)amine. Other compounds studied such as methylphosphonic acid, N-methylglyphosate and hydroxymethylphosphonic acid did not cause statistically significant changes in caspase-8 activity (Fig. 4). Glyphosate, its metabolites and impurities caused a substantial increase in caspase-9 activity. Changes in the enzyme activity were observed for the highest concentration (5 mM) of glyphosate, its two metabolites: AMPA and methylphosphonic acid and all glyphosate impurities: PMIDA, N-methylglyphosate, hydroxymethylphosphonic acid and bis(phosphonomethyl)amine (Fig. 5).

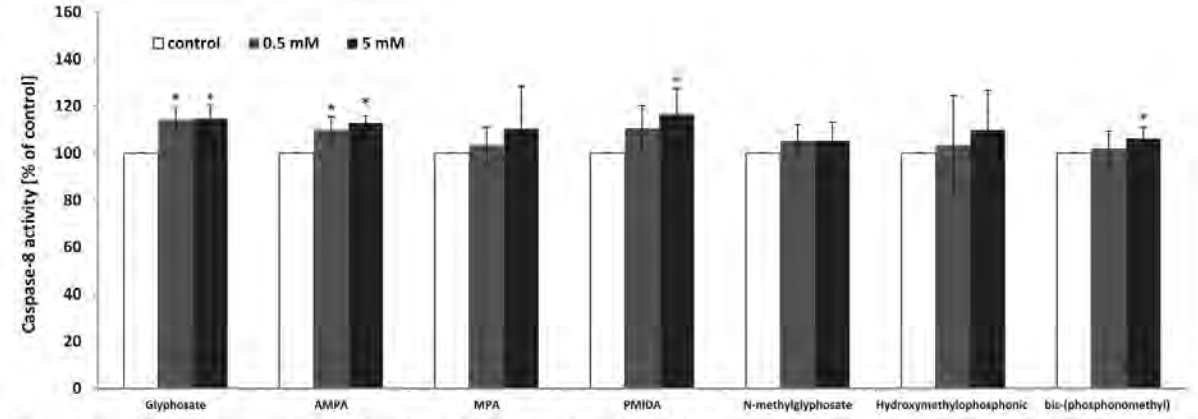


Fig. 4. Changes in caspase-8 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

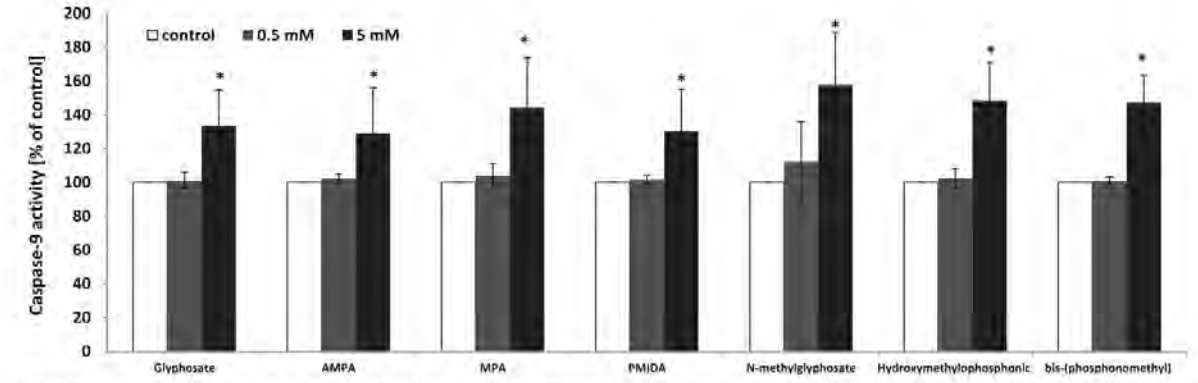


Fig. 5. Changes in caspase-9 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

Caspase-3 activity; An increase in caspase-3 activity was noted in PBMCs treated with 0.25 mM of glyphosate and PMIDA and with 0.5 mM of AMPA, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine. Other compounds tested: N-methylglyphosate and methylphosphonic acid only at the highest concentration of 5 mM caused an increase in caspase-3 activity (Fig. 6).

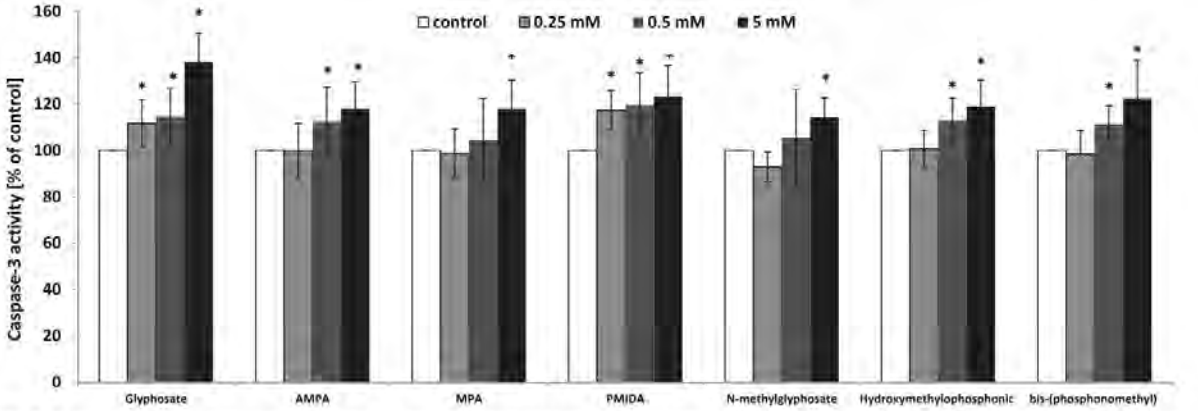


Fig. 6. Changes in caspase-3 activity in PBMCs after 4 h incubation with glyphosate, its metabolites and impurities. (*) Statistically significant changes compared to control ($P < 0.05$).

ROS level; It was observed that glyphosate, its metabolites and impurities induced a statistically significant increase in H2DCFDA oxidation in PBMCs. Changes in ROS level were observed after 4 h of exposure of PBMCs to 0.25 mM of glyphosate, methylphosphonic acid, PMIDA, N-methylglyphosate and hydroxymethylphosphonic acid. Other compounds studied like AMPA and bis-(phosphonomethyl)amine from the concentration of 0.5 mM caused an increase in the H2DCFDA oxidation (Fig. 7).

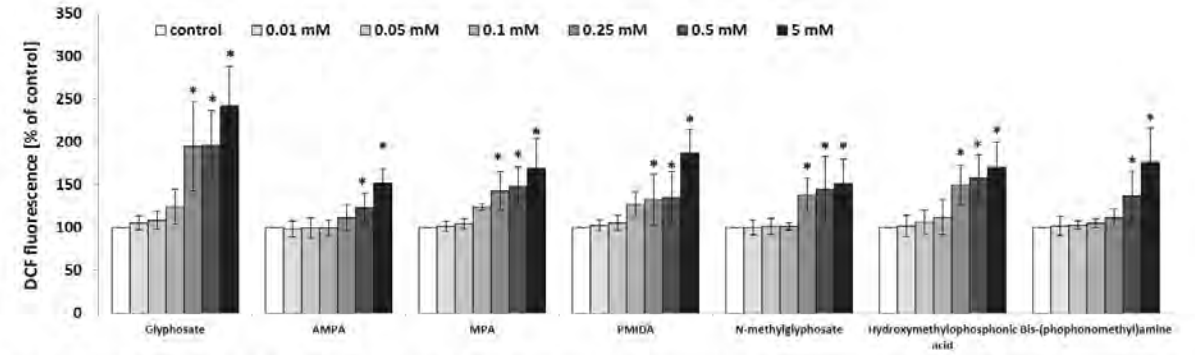


Fig. 7. Changes in total ROS level in PMBCs incubated with glyphosate, its metabolites and impurities for 4 h (*) Statistically significant changes compared to the control ($P < 0.05$).

Hydroxyl radical level; Glyphosate, its metabolites and impurities increased highly reactive oxygen species level, including hydroxyl radicals in PMBCs. Statistically significant changes were observed for most of the compounds studied from the concentration of 5 mM. An increase in HPF fluorescence was noted in cells treated for 4 h with the highest concentration (5 mM) of glyphosate, its impurities: N-methylglyphosate, hydroxymethylphosphonic acid and bis-(phosphonomethyl)amine and metabolites: AMPA and methylphosphonic acid. The strongest changes in HPF oxidation were caused by PMIDA, which changed this parameter from the concentration of 0.5 mM (Fig. 8).

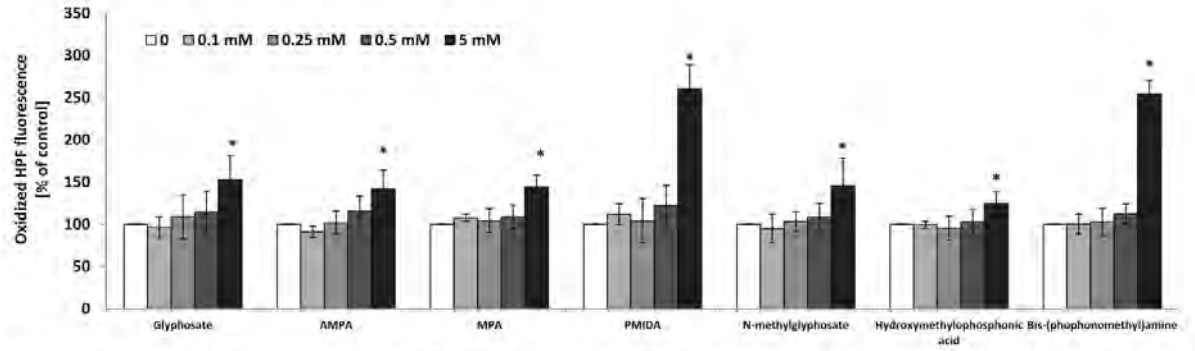


Fig. 8. The level of highly reactive oxygen species (mainly hydroxyl radical) in human PMBCs incubated with glyphosate, its metabolites and impurities for 4 h (*) Statistically significant changes compared to the control ($P < 0.05$).

Discussion

Apoptotic potential of glyphosate has been evaluated in various cell types; however, the effect of glyphosate, its metabolites (including AMPA and methylphosphonic acid) and its impurities on apoptotic changes in human leucocytes has not been assessed. This study has described apoptotic potential of glyphosate, its metabolites and impurities in human PBMCs. Besides a quantitative determination of apoptotic cells (staining with YO-PRO-1/PI fluorescent probes) the analysis concerned evaluation of the mechanism of action of these substances by measurement of a variety of parameters involved in the programmed cell death. The activities of caspases, of both initiator caspase-8 and -9, as well as executor caspase-3 were determined. Alterations in cytosolic calcium ion and ROS levels were also analysed. Moreover, changes in transmembrane mitochondrial potential and chromatin condensation were assessed. PBMCs were exposed to tested compounds for 4 h, the time necessary to observe apoptotic changes. Flow cytometry analysis has demonstrated a statistically significant increase in the number of apoptotic cells exposed to all compounds studied. Apoptotic changes induced by glyphosate, PMIDA and hydroxymethylphosphonic acid were observed from the concentration of 0.5 mM, while those induced by AMPA, methylphosphonic acid, N-methylglyphosate and bis-(phosphonomethyl)amine from the concentration of 5 mM. A slight increase in the number of apoptotic cells (5.76%) treated with glyphosate was observed, compared to the control (1.22%).

It was observed that methylphosphonic acid at lower concentration (0.25 mM) (in comparison to other analysed compounds) caused a statistically significant increase of cytosolic calcium ion level in PBMCs. Other compounds such as methylphosphonic acid and bis(phosphonomethyl)amine induced apoptosis from the concentration of 0.5 mM, while glyphosate, AMPA and N-methylglyphosate from 5 mM. It was also observed that aminomethylphosphonic acid did not cause any statistically significant increase in this parameter.

Our study demonstrated that all compounds analysed caused reduction of transmembrane mitochondrial potential. Glyphosate and its impurity - bis(phosphonomethyl)amine, from the concentration of 0.05 mM, caused reduction of $\Delta\Psi m$, while other compounds analysed induced the same changes from the concentration of 0.1 mM (except for hydroxymethylphosphonic acid, that caused reduction of the discussed parameter at the concentration of 0.25 mM). An increased ROS production was observed in PBMCs exposed with all analysed compounds. The obtained results are consistent with data published, which observed ROS formation in human PBMCs treated with glyphosate, AMPA and particularly glyphosate preparation - Roundup 360 PLUS. It has been shown that the increase in ROS level, and hydroxyl radical in particular may contribute to DNA damage, and thus apoptotic cell death. For that reason, the formation of highly reactive oxygen species (including hydroxyl radical) was assessed in PBMCs treated with glyphosate, its metabolites and impurities. It has been noted that glyphosate, its impurities: N-methylglyphosate, hydroxymethylphosphonic acid and bis(phosphonomethyl)amine as well as its metabolites: AMPA and methylphosphonic acid at the concentration of 5 mM caused an increase in hydroxyl radical level. The highest increase in this parameter was noted in PBMCs exposed to PMIDA (from 0.5 mM Similarly, our study showed that AMPA at lower concentration did not induce hydroxyl radical formation, and the increase in discussing parameter was noted only at its highest level of 5 mM. The proteolytic activity of caspases involves cleavage of their substrates at aspartate residues. In this study we have observed that glyphosate, its metabolites and impurities caused chromatin condensation in human PBMCs. Based on our own research, and also on studies of other authors it may be suggested that glyphosate, its metabolites and impurities induce apoptosis both via the intrinsic pathway (evidenced by the observed increase in total ROS and hydroxyl radical levels, a decrease in transmembrane mitochondrial potential and an increase in caspase-9 activity), and - to a lesser extent via the extrinsic pathway (evidenced by changes in caspase-8 activity) (except for methylphosphonic acid, Nmethylglyphosate and hydroxymethylphosphonic acid).

Conclusion

Toxicity of pure glyphosate versus its metabolites and impurities: The results obtained in this study do not indicate an essential role of metabolites and production impurities of glyphosate in toxic (proapoptotic) action of glyphosate and possibly glyphosate-based preparations. Research presented in this paper indicated that both AMPA and MPA exerted a smaller effect on ROS and hydroxyl radical formation than glyphosate. For other analysed parameters, no significant differences have been demonstrated between glyphosate and its metabolites activities. Studies on toxicity of glyphosate metabolite - MPA - are even more limited, while our results clearly demonstrated a relatively low toxicity of this compound. The study also revealed that some of glyphosate impurities were characterized by a slightly stronger proapoptotic potential than the parent compound. They constitute, however, a minor impurities of glyphosate and should not significantly increase toxicity of N-(phosphonomethyl)glycine-based products (contrary to surfactants).

Obtained results clearly indicate low proapoptotic properties of all analysed compounds. Initial clear apoptotic effects are associated with their highest analysed concentrations, which correspond to the concentrations to which a human organism could be exposed only as a result of acute or subacute poisoning with glyphosate.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The study describes in-vitro investigations of glyphosate, its metabolites (AMPA and methylphosphonic acid) and impurities (PMIDA, N-methylglyphosate, hydroxymethylphosphonic acid, and bis-(phosphonomethyl)amine) on six intermediate endpoints of apoptosis (membrane permeability, cytosolic calcium concentration, mitochondrial transmembrane potential, caspase activity, chromatin condensation, and ROS quantitation by two methods) in human peripheral blood mononuclear cells. The reason for selection of this model is not stated but is possibly as potential target tissue for Non-Hodgkins Lymphoma. The methodologies used are frequently reported in literature but are not a standardized or validated method by GLP standards; there is no OECD guideline. Positive control results are not presented and it is unclear if positive controls were used for all assays; wording is sufficiently poor that it may be inferred that some positive controls were used, e.g. nigericin and valinomycin in the studies of mitochondrial transmembrane potential, campothecin in the caspase assays, although these may alternatively be reagents for the assay. It is unclear if assays were conducted in duplicate or triplicate (the stated term was "trice" which may be either twice or thrice), which may then also influence statistical evaluation. However, the methodology appears basically sound.

Apoptotic or pre-apoptotic activity was seen generally consistently across the assays. While glyphosate, its metabolites, and impurities were seen to increase apoptotic endpoints in these assays (0.5 mM and higher), clear effects occurred only at high concentrations.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no proper cytotoxicity tests were performed, no positive controls were used and the concentration range at which most of the effects were observed is beyond the acceptable physiological range (> 1 mM). The concentration range at which the glyphosate impurities were tested is the same as that for glyphosate which is not a realistic approach for risk assessment of impurities.

Reliability criteria for *in vitro* toxicology studies

Publication: Kwiatkowska *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 (95%). Source: Sigma-Aldrich, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also glyphosate impurities were tested.
AMPA is the tested substance	Y	
Study		
Test system clearly and completely described	Y	PBMCs
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Partly	0.01, 0.05, 0.25, 0.5, 5, 10 mM
Cytotoxicity tests reported	?	

Positive and negative controls	N	No positive controls.
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 no proper cytotoxicity tests were performed, no positive controls were used and the concentration range at which most of the effects were observed is beyond the acceptable physiological range (> 1 mM). The concentration range at which the glyphosate impurities were tested is the same as that for glyphosate which is not a realistic approach for risk assessment of impurities.		

1. Information on the study

Data point:	CA 5.4
Report author	Mañas F. *et al.*
Report year	2013
Report title	Oxidative stress and comet assay in tissues of mice administered glyphosate and AMPA in drinking water for 14 days
Document No	Journal of Basic & Applied Genetics (2013) Vol. 24(2), Article 7 - research
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

In this study the levels of thiobarbituric acid reactive substances (TBARs); quantified superoxide dismutase (SOD) and catalase (CAT) activity in liver, kidney, lung and heart were determined, and the comet assay in blood and liver of mice administered glyphosate (40 or 400 mg/kg/day) or AMPA (100 mg/kg/day) in drinking water for 14 days was performed. Exposure to glyphosate 400 mg/kg induced a statistically significant ($p < 0.05$) decrease of SOD activity in heart and an increase in CAT activity in kidney. In the comet assay there were statistically significant differences in all the treatments and tissues studied in comparison to control animals ($p \leq 0.01$). The major results of this study were that mice administered glyphosate or AMPA in drinking water for 14 days induced a significant increase in DNA damage in liver and blood but minor effects on oxidative stress parameters. DNA effects on liver and blood indicate that these compounds could be of concern in terms of their potential to damage the genetic material, and that oxidative stress does not seem to be the mechanism causing that effect.

Materials and Methods

Chemicals - Analytical grade glyphosate (96% purity), and analytical grade AMPA, (99% purity) were obtained from Sigma-Aldrich, Argentina.

Animals - Twenty-four Balb C mice of approximately 45 days of age were used in this study. Four groups of six animals each (a control group, 2 groups treated with glyphosate and a group treated with AMPA) were used for the determination of oxidative stress (TBARs, SOD and CAT) and DNA damage in the comet assay. During 7 days prior to the beginning of the study, the volume of water ingested per day was measured in every 2 animals per cage to determine the concentration of glyphosate and AMPA in the drinking water to be supplied to each group. Mice received approximately 40 or 400 mg/kg bw/day of glyphosate and 100 mg/kg bw/day of AMPA. The control group received drinking water without test compound. After 14 days of exposure, before sacrifice, peripheral blood was drawn from the tail vein to perform the comet assay. At necropsy, heart, lungs, liver, and kidneys were removed for the determination of TBARs, SOD and CAT and frozen at -80 ºC pending analysis. A part of the liver was homogenized in phosphate buffer (pH 7.4) immediately after excision for the conduct of the comet assay. All determinations were performed in triplicate.

Single cell gel electrophoresis assay in mouse blood and liver - The protocol followed the general guidelines proposed by Singh et al. (Exp. Cell Res., 175, 184-191, 1988) with minor modifications. The slides were fixed in absolute ethanol, stained with ethidium bromide and scored using fluorescence microscopy. Images of 100 “nucleoids” counted for each animal were captured with a camera attached to the fluorescence microscope and linked to the Comet Score 1.5 software. Tail moment (TM),

percentage of DNA in tail (% of DNA) and tail length (TL) were used to estimate DNA damage (in arbitrary units).

TBARs, SOD and CAT determinations - Tissue homogenates (10%) were prepared in chilled 0.05 M potassium phosphate buffer at pH 7.4. TBARs concentrations, expressed as nmol of malondialdehyde MDA/g of tissue, were measured spectrophotometrically at 532 nm in liver and kidney homogenates. TBARs concentrations were determined using a standard curve at different concentrations of MDA versus optical density, individually prepared for each tissue. Superoxide dismutase (SOD) activity was assessed spectrophotometrically in the supernatant of liver homogenates. One unit of enzymatic activity has been defined as the amount of enzyme capable of causing 50% inhibition of auto-oxidation of epinephrine. Catalase (CAT) activity was measured at 240 nm by the decomposition of the H_2O_2.

Statistical analysis
Statistical analysis was performed using Prism software (PRISM, 1997). The Kolmogorov-Smirnov test was performed to verify if the results follow a normal distribution. ANOVA followed by the Dunnett's test, or the Kruskal-Wallis test followed by the Dunn's test were performed for data with and without normal distribution, respectively.

Results

The volume of water ingested per animal/day did not show any statistically significant differences among the experimental groups throughout treatment. No statistically significant differences were found in liver, kidney, lung and heart for all oxidative stress parameters measured with the exception of a decrease in SOD in the heart and an increase in CAT in the kidney at a daily glyphosate dose of 400 mg/kg bw. A non-statistically significant increase in CAT was observed in the lung. There were no statistically significant changes in the concentrations of MDA/g of tissue at 40 and 400 mg/kg bw/day of glyphosate and at 100 mg/kg bw/day of AMPA. Tail intensity, tail length and tail moment were statistically significantly elevated in blood and liver of all dosed groups with the exception of tail intensity in liver at 40 mg/kg bw/day glyphosate.

Discussion and Conclusion

The presence of glyphosate or AMPA in drinking water did not affect water consumption. Statistically significant differences in oxidative stress (decrease in SOD activity in the heart and increase in CAT activity in the kidney) were only observed in mice treated with glyphosate at 400 mg/kg bw for 14 days. The increase in CAT activity in the lungs was not statistically significant. A non-statistically significant decrease in SOD activity was observed in all tissues of animals treated with 100 mg/kg bw AMPA but no effect of AMPA was found on CAT activity. Liver was the only tissue where no effects were recorded, neither in TBA reactive substances nor in SOD and CAT activity. In the comet assay of blood statistically significant differences from control were seen for glyphosate and AMPA in tail intensity, tail length and tail moment. Tail moment values in the comet assay were similar for both glyphosate and AMPA treatments. In this study, genotoxic changes were observed with glyphosate at 40 and 400 mg/kg bw/day and AMPA at 100 mg/kg bw/day. However, statistically significant changes in the levels of SOD and CAT especially in heart and kidney were only seen with glyphosate at 400 mg/kg bw/day. This suggests that the genotoxic effects of glyphosate and AMPA are much more important than the indicators of oxidative stress.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to investigate the effect of glyphosate and AMPA on indicators of oxidative stress and DNA integrity in mice after oral exposure for 14 days via the drinking water. The results of this study indicate that no statistically significant differences have been found in liver, kidney, lung and heart for all oxidative stress parameters measured with the exception of a decrease in SOD activity in the heart and an increase in CAT activity in the kidney at a daily glyphosate dose

of 400 mg/kg bw. There was an increase in CAT activity in the lung but this was not statistically significant and didn't show a dose-effect relationship. A statistically significant increase in DNA damage parameters was observed for glyphosate and AMPA with the exception of tail intensity in the liver for glyphosate at 40 mg/kg bw/day. No clear dose-effect relationship was evident for DNA damage parameters in blood after treatment with glyphosate. A dose-effect relationship was present for tail length and tail moment in the liver.

This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the increased DNA damage seen (only 2 dose levels tested for glyphosate with too few animals) didn't show a dose-effect relationship in blood and occurred at dose levels (40 and 400 mg/kg bw/day) that are much lower than the 2000 mg/kg bw used in regulatory *in vivo* MN tests in the mouse with negative results.

Reliability criteria for *in vivo* toxicology studies

Publication: Manas et al., 2013	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	2 dose groups for glyphosate, one dose group for AMPA
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	Very old determination methods applied
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 96%. Source: Sigma-Aldrich, Argentina.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	Y	Purity of 99%. Source: Sigma-Aldrich, Argentina.
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Exposure via drinking water
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	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported		Only 2 dose levels used. DNA damage (comet) at 40 and 400 mg/kg bw/day (no dose-effect relationship) not

		confirmed by regulatory genotoxicity (MN) studies up to 2000 mg/kg bw/day
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the increased DNA damage seen (only 2 dose levels tested for glyphosate with too few animals) didn't show a dose-effect relationship in blood and occurred at dose levels (40 and 400 mg/kg bw/day) that are much lower than the 2000 mg/kg bw used in regulatory in vivo MN tests in the mouse with negative results.		