1. Information on the study

Data point:	CA 5.6
Report author	Manservisi F. *et al.*
Report year	2019
Report title	The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system
Document No	Environmental Health (2019) Vol.18, 15
Guidelines followed in study	Pilot study based on OECD test guideline 443: Extended one-generation reproductive toxicity study.
Deviations from current test guideline	Yes, only one dose level for each test item and insufficient number of animals per dose level used.
GLP/Officially recognised testing facilities	No, no GLP statement delivered.
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The present pilot study examines whether exposure to glyphosate-based herbicides (GBHs) at the dose of glyphosate considered to be "safe" (the US Acceptable Daily Intake - ADI - of 1.75 mg/kg bw/day), starting from in utero life, affect the development and endocrine system across different life stages in Sprague Dawley (SD) rats. In pups, anogenital distance (AGD) at PND 4 was statistically significantly increased both in Roundup-treated males and females and in glyphosate-treated males. Age at first estrous (FE) was significantly delayed in the Roundup-exposed group and serum testosterone concentration significantly increased in Roundup-treated female offspring from the 13-week cohort compared to control animals. A statistically significant increase in plasma TSH concentration was observed in glyphosate-treated males compared with control animals as well as a statistically significant decrease in DHT and increase in BDNF in Roundup-treated males. Hormonal status imbalances were more pronounced in Roundup-treated rats after prolonged exposure.

Materials and methods

Chemicals - Glyphosate (purity of > 99.5%), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy). The representative formulated product, Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%), water (42.5%) and surfactant (16%)) was purchased from Consorzio Agrario dell'Emilia, Bologna, Italy.

Animals and experimental design - Each of the 24 virgin female SD rats (17 weeks old, 270–315 g) was mated outbred with one breeder male rat of the same age and strain. Every day, the females were examined for the presence of sperm. After evidence of mating, females were housed separately during gestation and delivery. Newborns were housed with their mothers until weaning. Weaned offspring were co-housed, by sex and treatment group, not more than 3 per cage. Cages were identified by a card indicating study protocol code, experimental and pedigree numbers, and dosage group. The cages were placed inside a single room at 22 °C ± 3 °C and at 50% ± 20% relative humidity. A light/dark cycle of 12 hours was maintained. Two groups of dams and their pups were treated with either glyphosate or MON 52276 diluted in drinking water to achieve the desired glyphosate dose of 1.75 mg/kg bw/day. The F0 female breeders received the test item from gestation day (GD) 6 to the end of lactation, while the offspring (F1) continued to be exposed after weaning for an additional 6 or 13 weeks. Glyphosate or MON 52276 solutions were freshly prepared on a daily basis taking into account body weight and water consumption. During pregnancy and lactation, embryos and offspring (F1) were all retained in the litter and received the test compounds mainly through their dams (F0). The day of birth was designated post-natal day 1 (PND 1) for pups and lactation day 1 (LD 1) for dams. After weaning, the offspring (F1) were treated via the drinking water until sacrifice. On PND 28, offspring were randomly distributed in

two cohorts: 8/sex/group of the 6-week cohort and 10/sex/group of the 13-week cohort. Altogether, 108 rats (54 males and 54 females) were enrolled in the post-weaning treatment phase.

Measurements in F0 dams and litters prior to weaning - Mean gestational length was calculated as the number of days from detection of a positive vaginal smear (GD 0) to birth of a litter. Pregnancy was confirmed by the occurrence of parturition. The body weight of the dams was recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, the body weight of the dams was recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 (last measurement before weaning). Body weight of the pups by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. Feed and water consumption of the dams was recorded twice weekly during gestation on GD 0, 3, 6, 9, 12, 15, 18, and 21, and twice weekly during lactation on LD 1, 4, 7, 10, 13, 16, 19, 21 and 25. Dead pups were removed when found and sexed when possible. Sex was determined on PND 1. The mean litter size was assessed on PND 0 (within 24 hours from delivery), 1, 4, 7, 10, 13, 16, 19, 21, and 25. Litter size included dead as well as live offspring. Dead pups were visually examined by floating the lungs in saline, to distinguish if they were stillborn (died in utero) or died shortly after birth. Live-birth index was determined on PND 0 as (number of pups born alive / total number of pups born) × 100. Survival index, calculated as (total number of live pups at designated time point / number of live pups born) × 100, was measured on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. For all the pups, anogenital distance (AGD) was measured and body weight recorded on PND 4.

Post weaning endpoints up to adulthood - After weaning, body weight was measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifice, the means of individual body weights were calculated for each group and sex. Daily water and feed consumption per cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2. Time to vaginal opening (VO) was determined by daily inspection of all female pups starting on PND 28. The body weight of each female was recorded on the day VO was observed. Time to balano-preputial separation (BPS) was determined by daily inspection of all males beginning on PND 35. The body weight of each male was recorded on the day BPS was observed. The female rats belonging to the developmental (6-week) cohort were also monitored for the time to first estrous (FE).

Estrous cycle characterization - Starting on approximately PND 95 and for the duration of 3 weeks, daily vaginal lavage was performed on female rats of the 13-week cohort. To reduce variability, vaginal cytology samples were collected by vaginal lavage at the same time of the day over the course of the experiment, in the mid-morning, between 10:00 and 13:00 h. Collection, processing and vaginal smear evaluation was performed as described previously.

Necropsy - Five days after weaning, dams were sacrificed and following tissues were collected and fixed in alcohol: mammary glands (4 sites: axillary and inguinal, right and left), adrenal glands, uterus (including cervix), ovaries, and vagina. The adrenal glands, uterus and ovaries were also weighed. For the determination of serum testosterone, blood was collected and serum prepared by centrifugation and stored at -80 °C pending analysis. All male and female pups belonging to both cohorts were sacrificed on PND 73 ± 2 for the 6-week cohort and PND 125 ± 2 for the 13-week cohort. Following tissues were collected for histopathology and fixed in alcohol: mammary gland (4 sites: axillary and inguinal, right and left), thyroid and parathyroid, adrenal glands, bladder and prostate, seminal vesicles and coagulating gland, left and right testis with epididymis (half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation), uterus (including cervix), ovaries and vagina. During necropsy, all tissues with gross lesions were removed for histopathology. Adrenal glands, bladder and prostate, seminal vesicles and coagulating gland, left testis, left epididymis, uterus (including cervix) and ovaries were weighed. In case of paired organs, both organs were preserved. Organ weight was expressed as absolute and relative organ weight. Rats were sacrificed randomly across the 4 stages of the estrous cycle. In order to determine and allow correlation with histopathology in reproductive organs and hormone analysis, the stage of estrous cycle was determined by histological appearance of the various components of the reproductive tract for F1 females of the 6-week cohort or by a vaginal smear examined on the day of necropsy for F1 females of the 13-week cohort.

Sperm analysis - Sperm analyses were performed on each male from both cohorts, at scheduled necropsies on PND 73± 2 and PND 125 ± 2. At necropsy, half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation. Spermatids resistant to homogenization and spermatozoa present in the caput/corpus and cauda epididymis were counted. The tunica albuginea was removed from the (half) testicle, and a sample of the parenchyma was weighed and homogenized in 5 mL saline-TritonX-100 at 0.05%. The samples were then diluted 10-20 times in saline, and the mature spermatids resistant to homogenization (step 17-19 spermatids) were counted using a Thoma chamber. Four fields per animal were recorded, and the numbers of spermatids per gram of testis were calculated. To calculate the daily sperm production (DSP) these values were subsequently divided by 6.1, which is the number of days step 17-19 spermatids are present in the seminiferous epithelium. Similarly, the segments of the epididymis (caput, corpus and cauda) were cut with a scissor, weighed, homogenized, diluted and counted as described for the testes. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for each segment of the epididymis calculated. The epididymal sperm transit time through the epididymal caput/corpus and cauda was calculated by dividing the number of spermatozoa present in each portion of the epididymis by the DSP of the associated testis. To assess the percentage of morphologically abnormal sperm half of left cauda epididymis of each rat was transferred to a Petri dish containing 2.5 mL (for 70 day old animals) or 3.5 mL (for 120 day old animals) of Dulbecco's PBS at 37°C, cut in 2-3 pieces and incubated for approximately 3 minutes at 37°C with gently swirling to facilitate release of sperm cells from the cauda. Dried smears of epididymal spermatozoa were stained with 1% Eosin Y for 30 minutes and evaluated at 400 x magnification. Five hundred spermatozoa per rat were evaluated and scored as morphologically normal or abnormal according to the presence or absence of head or tail defects.

Histopathology - After fixation, samples were trimmed, processed, embedded in paraffin wax, sectioned to a thickness of 4-5 μm and then processed in an alcohol-xylene series and stained with hematoxylin and eosin for microscopic evaluation. Histopathology evaluation was performed blind by at least two pathologists. At least one senior pathologist peer reviewed all lesions of oncological interest as well as any lesion of dubious interpretation. All the pathologists used the same evaluation criteria and the same classification based on international standard criteria (INHAND, NTP) described in the specific Standard Operating Procedures and long adopted at the CMCRC/RI.

Hormone analysis - Serum concentration of free (fT) and total testosterone (TT), 5α-dihydrotestosterone (DHT), 17β-estradiol (E2) and Sex Hormone Binding Globulin (SHBG) were measured in duplicates by solid phase enzyme-linked immunosorbent assays (ELISAs): "Estradiol rat ELISA" (#DEV9999), manufactured by Demeditec Diagnostics GmbH, "Rat Free Testosterone (F-TESTO) ELISA" (#CSB-E0597r), "Rat Testosterone, T ELISA" (#CSB-E05100R); "Rat dihydrotestosterone (DHT) ELISA" (#CSB-E07879r), and "Rat sex hormone-binding globulin (SHBG) ELISA" (#CSB-E12118r), manufactured by Cusabio Biotech Co. Ltd. The detection range and the Lower Limit of Detection (LLD) of each ELISA kit was 2.5-1280 pg/mL and 2.5 pg/mL for E2, 0.3-60 pg/mL and 0.15 pg/mL for fT, 0.13-25.6 ng/mL and 0.06 ng/mL for TT, 10-2000 pg/mL and 5 pg/mL for DHT, 375-6000 ng/mL and 375 ng/mL for SHBG. Each kit was used following the manufacturer's instructions and absorbance was measured at 450 nm using a 96-well plate reader. Plasma pituitary hormones were measured in duplicate using the "Rat Pituitary Magnetic Bead Panel", a Luminex® bead-based immunoassay, following manufacturers' instructions. Seven plasma pituitary hormones were measured in plasma samples from 40 pups (20 females and 20 males) randomly selected from the 6-week cohort (N = 48 total): adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF), follicle stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH), prolactin (PRL) and thyroid stimulating hormone (TSH). FSH and LH were also assessed in 40 pups (20 females and 20 males) randomly selected from the 13-week cohort (N = 60 total). BDNF and TSH results from the 6-week cohort showed marginal differences by exposure groups in male pups, it was therefore attempted to validate these results by measuring BDNF and TSH in all male pups (N = 30) from the 13-week cohort. Plasma TT was measured in duplicates in all dams (N = 24) using an ELISA kit, the "Testosterone Parameter Assay Kit", following manufacturers' instructions.

Statistical methods - Where data on a particular endpoint were collected from both sexes, analyses were conducted separately. All statistical tests were made using a significance level of $\alpha = 0.05$. For

continuous data including body weight, weight gain and organ weights, which are most often normally distributed, one-way ANOVA, followed by a Dunnett's test was used to compare treatment versus control groups. For hormone data, which are usually non-normally distributed and have high inter-individual variability, a screening for outliers was made, based on a Box and Whisker Plot procedure and considering as outliers the values that were outside the box boundaries by more than 3 times the size of the box itself. In the case of hormone ratios, the same outliers of the single evaluation were considered. Nonparametric Kruskal-Wallis' tests, using beta approximation, were used in cases where data were not normally distributed (all hormones). Counting data, not normally distributed, were also analyzed with appropriate regression models. Where the observations were grouped (such as for litter data), fixed and mixed effect models were estimated (litter as random effect) and both reported. For biological parameters related to the body weight (such as the AGD), statistical analyses were always performed including the body weight of each pup in the regression model. The incidence of pathological lesions, reported as the numbers of animals bearing lesions, were compared using a two-tail Fisher exact test. The statistical analysis was performed using Stata/IC 10.1 (for all regressions) and Statisti× 10 (for all the other tests); graphs were obtained using Microsoft Excel and Statisti× 10.

Results

No statistically significant differences were observed between the control and the glyphosate and MON 52276 groups for gestational index, mean gestational length, relative weight gain during pregnancy, relative weight gain during lactation, total number of pups delivered at PND 0, litter size, sex ratio, mean life birth index, number of dams with reported stillbirths, number of still born pups, and survival index on PND 1 and PND 21. Also no treatment related effects were observed for water or feed consumption during gestation or lactation. In pups, AGD on PND 4 was statistically significantly increased both in MON 52276 treated males and females and in glyphosate treated males. Results were still significant after running multilevel linear regression models adjusted for body weight and litter as a random effect. Post-weaning body weights as well as water and feed consumption showed no difference in both female and male offspring. In female offspring, age and body weight at vaginal opening (VO) was similar across treatment groups, however, age at FE was statistically significantly delayed in the MON 52276 exposed group. Female offspring in the control and glyphosate treated groups presented the FE within 6 days from the VO, while in the MON 52276 treated group 2/10 females presented a more than doubled interval (12 and 14 days) between VO and FE. In female pups followed up to 13-weeks (N = 30), the percent of time spent in each stage of the estrous cycle did not differ between glyphosate and MON 52276 treated animals and controls. In male offspring, exposure to glyphosate or MON 52276 did not affect BPS or sperm parameters (number of mature spermatids in the testis, daily sperm production, number and sperm transit time through caput/corpus and cauda epididymis and morphology). There were no treatment-related gross lesions in F0 and F1 reproductive and endocrine organs in either sex and there was no statistically significant effect on absolute and relative organ weight of adrenal glands, uterus and ovaries in the dams, adrenal glands, testis, epididymis, bladder/prostate and seminal vesicles/coagulating glands in male offspring (with the exception of a decrease in absolute epididymal weight in the 13-week cohort), and adrenal glands, uterus and ovaries in female offspring.

Most pituitary hormones were unaffected by exposure to glyphosate or MON 52276 in males, with the exception of a statistically significant increase in plasma TSH in the glyphosate group of the 6-week cohort and the MON 52276 group of the 13-week cohort and a statistically significant increase in BDNF in the MON 52276 group of the 6-week cohort. Apart from a statistically significant decrease in DHT in MON 52276 treated males of the 13-week cohort and a statistically significant increase in total testosterone in MON 52276 treated females of the 13-week cohort no effects were found on sex hormones. Hormone ratios were calculated as indicators of the general balance between hormones and sex steroid hormone bioavailability. The TT/SHBG ratio was statistically significantly increased in MON 52276 treated females of the 13-week cohort. The E2/SHBG ratio was statistically significantly increased in MON 52276 treated males of the 6-week cohort. The fT/TT ratio was statistically significantly decreased in glyphosate treated males of the 6-week cohort and in MON 52276 treated males of the 13-week cohort. Male and female MON 52276 treated rats of the 13-week cohort showed a marked and statistically significant decrease in DHT/TT ratio. No such differences were observed for DHT/TT ratio in males and females of the 6-week cohort and no statistically significant differences were

observed for the E2/TT ratio in males and females of the glyphosate and MON 52276 treated groups of both cohorts.

Discussion

MON 52276, when administered to rats from in utero through adulthood at a dose level corresponding to the glyphosate RfD defined by the US EPA (1.75 mg/kg bw/day), elicited subtle but potentially adverse effects on reproductive development and hormone concentrations. Overall, these effects indicate an impact on pre- and peri-pubertal sexual maturation. The effects of treatment with glyphosate were essentially limited to increased AGD and TSH concentration, and both changes were specific to males. MON 52276 seemed to affect both females and males, resulting in a statistically significant increase of AGD and sexual hormones imbalances in both cohorts. Statistically significant differences in apical endpoints (AGD and FE) together with changes in hormonal activity detected in both treatment groups should be taken into account suggesting evidence for reproductive toxicity via an endocrine disruption mechanism. A longer AGD at birth in both sexes and an increased age at FE, together with the increased TT in female offspring, are considered endpoints for androgen-mediated activity. The significant increase in AGD and the delay in the appearance of the first estrous cycle observed in MON 52276-treated female rats is consistent with increased developmental androgenization. The first ovulation is the true endpoint of a series of morphological and functional changes at different levels of the hypothalamic–pituitary–gonadal (HPG) axis, hence, it constitutes the unequivocal sign that puberty has been achieved. No difference in the achievement of vaginal opening (VO) was observed among the groups tested. In males, a prolonged, albeit of low-intensity, androgenizing effect could eventually evoke a counteracting feed-back response from the HPG axis. As apical endpoint, an increased AGD was observed in both treatment groups. Hormone profiling in males, revealed a decreased serum DHT in the MON 52276 group of the 13-week cohort, suggesting an effect on TT metabolism after a prolonged exposure. The lower conversion of TT to DHT might indicate a possible reduction in 5α-reductase enzyme activity. However, this effect was not observed in females of the 6-week cohort. It is of note that males treated with MON 52276 showed normal seminiferous tubules and sperm production which is consistent with the absence of any effect on testosterone and FSH. A statistically significant increase in TSH was observed in glyphosate treated males of the 6-week cohort and MON 52276 treated males of the 13-week cohort. Since no histological changes were observed in the thyroid gland, the increased plasma concentration of TSH can be considered as not indicative of a thyroid related effect. BDNF is a neurotrophin playing a fundamental role in survival and differentiation of selected neuronal populations during development, and in the maintenance and plasticity of neuronal networks during adulthood. A statistically significant increase in BDNF was observed in MON 52276 treated males of the 6-week cohort but not in males of the 13-week cohort. BDNF is an explorative and new endpoint for neurodevelopment and the utility of neurotrophins as potential biomarkers is not completely understood.

The present study has some limitations. First, this is a pilot study performed on a limited number of animals where only one dose was used. However, the dose was selected specifically for its relevance to human health risk assessment, as it is the chronic current RfD defined by the USEPA (1.75 mg/kg bw/day) and therefore a dose level that is expected to be “safe”. Furthermore, the number and timing of blood sample collection was limited to the final sacrifice of animals, considering that this was a pilot study and that *in vivo* blood sampling could lead to maternal and pups stress. Another source of uncertainty, which is currently difficult to assess, is the timing of blood sampling during the necropsy session (9.00 am – 3.00 pm), a circadian-dependent modulation of circulating hormones cannot be completely ruled out. Standard errors in different hormone concentrations were large, in relation to the relatively small group sizes and the physiological variability of hormone concentrations. In females, the estrous cycle status at the time of necropsy is another important source of variability when analyzing sexual hormone profiles. However, even if sacrificing animals on a specific day of the cycle might improve the ability to observe changes in the baseline hormone concentrations, the issue of sacrificing animals in the same cycling period (e.g estrous) is still controversial. The updated OECD Test Guidelines on reproductive-developmental toxicity do not require the sacrifice of females in the same stage of estrous, only the examination of the estrous cycle on the day of necropsy is recommend to allow correlation with the histopathology in reproductive organs. Finally, the adjuvant(s) present in Roundup Bioflow (corresponding to 16% of the formulation) could not be studied since the nature of the co-

formulants is a trade secret. These are supposed to be surfactants, diluents or adjuvants stabilizing glyphosate and allowing its penetration in plants. The majority of significant changes observed in hormonal status emerged in the 13-week cohort (animals sacrificed at adulthood) compared to the 6-week cohort (animals sacrificed after puberty) suggesting that more prolonged exposures were more effective in producing imbalances in the hormone concentrations. In this experiment, MON 52276 was shown to be definitely more potent than glyphosate alone.

Conclusions

The present study demonstrates that exposure to MON 52276 at a dose level equivalent to 1.75 mg glyphosate acid/kg bw/day, from the prenatal period to adulthood, induced endocrine effects and altered reproductive developmental parameters in male and female SD rats. MON 52276 exposure was associated with androgen-like effects, in particular in females, including a statistically significant increase of ano-genital distance in both males and females, a delay of first estrous and increased testosterone in females. MON 52276 exposure was also associated with altered testosterone metabolism in both males and females, where a statistically significant decrease in DHT/TT ratio was observed in the longest treated group (13-week cohort). Overall, MON 52276 elicited more pronounced effects than glyphosate, which only increased anogenital distance and TSH concentration in male rats in the peripubertal window (6-week cohort).

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this pilot study the effect of glyphosate and its reference formulation Roundup Bioflow (MON 52276) at a dose of 1.75 mg glyphosate acid eq./kg bw/day on endocrine modulation was investigated in female rats during pregnancy and lactation and in male and female rats during lactation, the peripubertal period and adulthood. The endpoints explored were body weight, water and food consumption, gestational parameters, litter parameters, landmarks of sexual development, estrous cyclicity, gross and histopathology of reproductive and endocrine tissues, sperm parameters and serum and plasma hormone levels. MON 52276 exposure was associated with statistically significant increase of ano-genital distance in males and females, a delay of first estrous and increased serum testosterone in females and altered testosterone metabolism in both males and females. MON 52276 elicited more pronounced effects than glyphosate, which only increased statistically significantly anogenital distance during the peripubertal period. The statistically significant increase in TSH levels in glyphosate and MON 52276 treated rats was not associated with histopathological changes in the thyroid and thus of minor toxicological significance. The effect of glyphosate on ano-genital distance is not corroborated by a regulatory reproductive toxicology study where rats were exposed to much higher doses of glyphosate (> 1,000 mg/kg bw/day).

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the limited number of animals used per dose level and only one dose level was tested.

Reliability criteria for *in vivo* toxicology studies

Publication: Manservisi *et al.,* 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Pilot study based on OECD test guideline 443 but with deviations.
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 > 99.5% as Pestanal™ . Source: Sigma-Aldrich, Milan, Italy.
Only glyphosate acid or one of its salts is the tested substance	N	Also representative formulated product tested. Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%), water (42.5%) and surfactant (16%)). Source: Consorzio Agrario dell'Emilia, Bologna, Italy.
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 via the drinking water.
Dose levels reported	Y	1.75 mg glyphosate acid eq./bw/day.
Number of animals used per dose level reported	Y	Dams: 8/group. Offspring: 8 M + 8F/group (6-week cohort); 10 M + 10F/group (13-week cohort).
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	Not possible, only comparison between glyphosate and MON 52276.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered as relevant for the risk assessment of glyphosate but reliable with restrictions because of the limited number of animals used per dose level and only one dose level was tested.		

1. Information on the study

Data point:	CA 5.7
Report author	Martinez A. *et al.*
Report year	2019
Report title	Effects of glyphosate and aminomethylphosphonic acid on an isogeneic model of the human blood-brain barrier.
Document No	Toxicology Letters (2019) Vol. 304, 39-49.
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 effect of acute exposure to glyphosate (GPH) on the blood-brain barrier in vitro based on induced pluripotent stem cells (iPSCs) and compared to two chemical analogs: aminomethylphosphonic acid (AMPA) and glycine (GLY), for concentrations ranging from 0.1 μM to 1000 μM was investigated.

Materials and methods

Chemicals - Glyphosate (EPA 547 1,000 μg/mL solution), aminomethylphosphonic acid (AMPA) and glycine (GLY) were purchased as analytical grade reagents from Sigma-Aldrich, St. Louis, USA. The purity of the test chemicals was not reported.

Cell culture - Induced pluripotent stem cell line IMR90-c4 iPSC (RRID: CVCL_C437) was purchased from WiCell cell repository (WiCell, Madison, WI). iPSC colonies were maintained on hPSC-grade growth factor reduced Matrigel (C-Matrigel, Corning, Corning, MA) in the presence of Essential 8 medium (E8, ThermoFisher, Waltham, MA).

iPSC differentiation - iPSCs were differentiated into brain microvascular endothelial cells (BMEC). iPSCs were seeded as single cells on T-Matrigel at a cell density of 20,000 cells/cm^2 in E8 supplemented with 10 μM Y-27632. 24 hours after seeding, cells were maintained in E8 for 5 days prior to differentiation. Cells were maintained for 6 days in unconditioned medium (UM: DMEM/F12 with 15 mM HEPES, 20% knockout serum replacement, 1% non-essential amino acids, 0.5% Glutamax and 0.1 mM β-mercaptoethanol). After 6 days, cells were incubated for 2 days in the presence of EC+/+ (EC medium supplemented with 1% platelet-poor derived serum, 20 ng/mL human recombinant basic fibroblast growth factor (bFGF) and 10 μM retinoic acid). After such maturation process, cells were dissociated by Accutase® treatment and seeded as single cells on tissue-culture plastic surface (TCPS) coated with a solution of collagen from human placenta and bovine plasma fibronectin at 80 μg/cm^2 and 20 μg/cm^2, respectively. Twenty-four hours after seeding, cells were incubated in presence of EC-/- (EC medium supplemented with 1% platelet poor derived serum (PDS)). Barrier phenotype experiments were performed 48 hours after seeding. Differentiation of iPSCs into neurons was done using an adherent 3-step differentiation method. Co-culture experiments were performed by seeding iPSC-derived BMECs at day 8 of differentiation on inserts juxtaposed over 16-days iPSC-derived neurons. BMECs were maintained in EC medium, whereas neurons were maintained in neuron maturation medium (NMM).

Glyphosate, AMPA and glycine treatment - Dilutions of glyphosate, AMPA and glycine were made immediately before the experiments and maintained in cell medium for 24 or 48 hours. In co-culture experiments, the test compounds were added in the apical chamber at a concentration of 100 μM and incubated for 6 hours. iPSC-derived neuron monocultures exposed to similar concentrations served as controls.

Cell metabolic activity - Following treatment, CellTiter Aqueous® MTS reagent ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) was added to each sample following recommendations by the manufacturer. Cells were maintained for 60 minutes at 37 °C followed by a measurement of absorbance at 490 nm using an ELISA plate reader. Absorbance obtained from the test samples were subtracted from background absorbance and normalized against controls (untreated cells).

iPSC-derived BMECs barrier function - iPSC-derived BMECs were seeded at a seeding density of 10^6 cells/cm^2 on Transwells (polyester, 0.4 μm pore size, Corning) and coated as previously described. Barrier function was assessed 48 hours after seeding of iPSC-derived BMECs monolayers. Barrier tightness was measured by assessing both the transendothelial electrical resistance (TEER) and paracellular diffusion. TEER was measured using an EVOHM STX2 chopstick electrode. For each experiment, three measurements were performed for each insert and the average resistance obtained was used for the determination of the barrier function.

Fluorescein, glyphosate and mannitol permeability assay - To assess changes in paracellular permeability, sodium fluorescein was added in the apical (top) chamber at a final concentration of 10 μM. 100 μL aliquots were sampled from the basolateral (donor) chamber every 15 minutes for up to 60 minutes. Each aliquot sample was replaced with 100 μL of cell medium. Fluorescein content in the samples was assessed using a fluorimeter ELISA plate reader. Glyphosate permeability was assessed by incubating cells in the presence of 100 μM glyphosate dissolved in EC-/- in the apical chamber. Sampling in the basolateral chamber occurred as previously described. For the determination of glyphosate the samples taken were alkalinized with 17 μL of borax solution followed by the addition of 17 μL of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) solution for the derivatization of glyphosate. Samples were allowed to incubate in the dark under gentle shaking for 2 hours. The derivatization process was terminated by adding 137 μL dichloromethane. The sample was homogenized and centrifuged at 2000 rpms for 5 minutes to separate the organic phase and analysis by spectrophotometry at 265 nm. Blank EC-/- medium was used as the blank, whereas glyphosate dissolved in EC-/- at concentrations ranging from 10 nM to 10 μM was used to establish a standard curve. For the measurement of mannitol permeability, [^{14}C] D-mannitol was added in the apical chamber with sampling in the basolateral chamber as described previously. Radioactivity was assessed by adding 100 μL sample to 5 mL liquid scintillation cocktail and counted using a Beckman-Coulter LS6500 liquid scintillation counter. The permeability across BMECs monolayers was obtained by calculating the clearance slope from both samples and blank inserts and by the calculation of the Pe value following the method of Perriere *et al*.

Immunocytochemistry - Cells were stained on tissue culture polystyrene (TCPS) plates and fixed with 4% paraformaldehyde. Cells were blocked for 1 hour at room temperature in PBS supplemented with 10% normal goat serum (PBS-G) with 0.2% Triton-X100 and were then incubated overnight in the presence of claudin-5, occludin, GLUT1 or βIII tubulin. Cells were washed with PBS containing 1% bovine serum album, and incubated in the presence of Alexa Fluor®-488 conjugated secondary antibodies for 1 hour at room temperature. Thereafter the cells were counterstained with DAPI and observed on a Leica inverted epifluorescence microscope. Micrograph pictures were acquired using Leica Acquisition Suite X and processed using ImageJ. Semi-quantitative analysis was done by measuring the average fluorescence intensity of each micrograph picture using the built-in measure tool in ImageJ. Average fluorescence values from negative controls were subtracted from the fluorescence values obtained in the test samples.

Flow cytometry - iPSC-derived BMECs at Day 10 of differentiation were treated with 100 μM glyphosate, AMPA or glycine for 24 hours. Cells were harvested by enzymatic dissociation using Accutase® and fixed with 4% paraformaldehyde. Cells were blocked in PBS-G supplemented with 0.2% Triton-X100 dissolved in PBS for 30 minutes, following by an overnight incubation at 4 °C in primary antibody solution (GLUT-1, SPM498 dissolved in PBS-G). Cells were then washed with PBS containing 1% BSA and incubated in the presence of Alexa Fluor® 555-conjugated antibody. As isotype control, cells were exposed to mouse IgG as primary antibody and analyzed using a BD FACSVerse®, with a fluorescence photomultiplier tube (PMT) adjusted to IgG isotype control. Fluorescence intensity for

each sample was obtained from a count of 10,000 cellular events. Median fluorescence intensity (MFI, geometric mean) was determined for each sample and corrected against IgG isotype.

Glucose and doxorubicin uptake assay - Glucose uptake assays were performed by incubating cells grown on TCPS in presence of cell medium supplemented with [^{14}C] D-glucose. Cells were incubated for 60 minutes at 37 °C. Afterwards the cells were washed with ice-cold PBS and homogenized with PBS + 0.2% Triton-X100 for 10 minutes. Radioactivity was assessed by adding 100 µL sample to 5 mL liquid scintillation cocktail and counted using a Beckman-Coulter LS6500 liquid scintillation counter. The doxorubicin uptake assay was performed by pre-incubating iPSC-derived BMECs in the presence of glyphosate, AMPA or glycine at 100 µM for 2 hours. Doxorubicin was added to obtain 5 µM as a final concentration and allowed to incubate for 1 hour. Cells were homogenized as previously described and total fluorescence assessed by fluorimetry. Total protein content obtained from cell homogenates was determined using a BCA protein assay.

Statistical analysis - Cells were randomly assigned treatment conditions prior each experiment. Data are represented as mean ± S.D. from three or more independent experiments. One-way analysis of the variance (ANOVA) coupled with Dunnett (or Kruskal-Wallis) tests analysis were performed using Prism 7.0 built-in package (GraphPad Software). A p-value < 0.05 was considered as statistically significant.

Results

BMEC cell viability – For the assessment of the effect of glyphosate on the viability of BMECs cell monolayers a range of concentrations was used partially overlapping the levels found in patients reported as asymptomatic, minor and moderate (17, 241, and 428 µM, respectively). Treatment with glyphosate, AMPA or glycine for 24 hours at concentrations ranging from 10 to 1000 µM resulted in no changes in cell metabolic activity. This indicates that glyphosate and AMPA unlikely have toxicity towards the blood-brain barrier.

Fluorescein permeability in BMECs monolayers - Changes in the barrier function in BMECs monolayers were measured using TEER and fluorescein permeability. In addition to the previous concentrations used, 2 concentrations (0.1 and 1 µM) were included to reflect average plasma concentrations reported in occupational exposure. No changes in TEER were noted for any of the concentrations tested of glyphosate, AMPA or glycine. However, a biphasic response was noticed in fluorescein permeability. At 0.1 µM, a slight but not statistically significant decrease in fluorescein permeability was found for glyphosate, AMPA and glycine followed by a statistically significant increase for both glyphosate and AMPA at 1 and 10 µM but not at 100 and 1,000 µM. To confirm the increase in the paracellular profile of glyphosate, changes in paracellular permeability were investigated using [^{14}C]-mannitol, an alternative paracellular flux marker at 1 and 10 µM. A modest but statistically significant increase in mannitol permeability was observed at 10 µM glyphosate. No significant increase was noted following AMPA or glycine treatment.

Tight junction complexes integrity - To better understand the effect of glyphosate and AMPA on the barrier function, changes in tight junction complexes were investigated, in particular changes in claudin-5 and occludin, by immunocytochemistry. No changes in claudin-5 immunolocalization were observed. However, a dose-dependent decrease in claudin-5 relative expression was noted in all groups as quantified by fluorescence intensity. Glyphosate decreased claudin-5 fluorescence intensity at 100 and 1000 µM, but treatment with AMPA already decreased significantly claudin-5 fluorescence intensity at 10 µM. No changes in occludin localization occurred following treatment although a significant decrease in occludin protein levels was noted in all treatment groups with the exception of 10 µM glyphosate. Unlike claudin-5, this effect appeared to be dose-independent.

Diffusion across the BBB - The ability of glyphosate to cross the blood brain barrier (BBB) was investigated following a single exposure at 100 µM in the apical chamber for 2 hours and measurement of the amount of glyphosate present in the basolateral chamber. After 2 hours of diffusion, the amount of glyphosate capable of crossing BMEC monolayers was about 1.67 ± 0.31% of the applied dose. It

was found that the permeability of glyphosate was significantly greater than fluorescein ($18.67 \pm 3.55 \times 10^{-6}$ cm/min versus 10.59×10^{-6} cm/min) or mannitol ($13.10 \pm 2.03 \times 10^{-6}$ cm/min). Also the effect of glyphosate and AMPA on drug efflux transporters was investigated using doxorubicin as a drug efflux substrate. With the exception of AMPA that showed a 2-fold increase over control, exposure to 100 μM glyphosate or glycine for 24 hours showed no differences compared to controls.

Modulation of glucose uptake in BMECs - As previous studies reported changes in glucose levels in certain vertebrates following exposure to glyphosate and AMPA changes in GLUT1 (the main glucose transporter at the BBB) localization and expression in BMECs following treatment by immunocytochemistry were studied. Following exposure at 100 μM glyphosate, but not at 1,000 μM, an apparent increase in GLUT1 immunoreactivity was noted. Similar results were obtained with AMPA although less pronounced. A flow cytometry analysis was performed where changes in mean fluorescence indexes were compared following exposure at 100 μM for 24 hours. Exposure to glyphosate yielded an increase in GLUT1 expression levels compared to control. Although AMPA showed no differences in GLUT1 expression, glycine exposure resulted in a significant decrease compared to control.

Barrier function of neurons co-cultured with BMECs - As glyphosate showed the ability to cross the BBB and produced changes in GLUT1 expression and glucose uptake in BMECs monolayers, the effects of glyphosate on neurovascular coupling using a BMEC/neurons co-culture model was investigated. First, the ability of such co-cultures to yield barrier function was assessed by measuring differences in TEER between BMECs monocultures and BMECs co-cultured with iPSC-derived neurons. A 3-fold increase in TEER in BMECs co-cultured with neurons compared to BMECs maintained in monocultures was observed. These co-cultures were then exposed to 100 μM glyphosate, AMPA or glycine for 24 hours. TEER measurements indicated that there was no statistically significant difference in barrier tightness when compared to controls. A mild increase in fluorescein permeability was noted with glyphosate when compared to control, but not with AMPA or glycine.

Neurovascular coupling - The effect of glyphosate on neurovascular coupling was investigated by measuring changes in neural cell metabolic activity using MTS following exposure to 100 μM for 6 hours. A significant decrease in cell metabolic activity was observed in co-cultured neurons when compared to monocultures. When co-cultured neurons were exposed to glyphosate and AMPA the metabolic activity was statistically significantly increased when compared to controls. Glyphosate and AMPA produced no statistically significant changes in metabolic activity of mono-cultured neurons. When the effect of glyphosate, AMPA and glycine was investigated in iPSC-derived neuron colonies by immunocytochemistry against βIII-tubulin, no changes were observed both in monocultures and co-cultures, suggesting that changes in cell metabolic activity is unlikely due to cell death.

Neuron progenitor cells (NPC) - The effect of glyphosate on differentiating and differentiated neurons was investigated by exposing cells to a concentration considered representative of the amount crossing the BBB i.e. 0.1–1 μM. First, the effect of an exposure of 24 hours on the cellular metabolic activity of undifferentiated NPCs was investigated using the MTS assay. A significant decrease in cell metabolic activity was seen at concentrations of glyphosate and glycine of 1 μM although immunofluorescence analysis of these NPCs showed no major alterations in the relative cell density and nestin (a cellular marker of neural stem cells/progenitor cells) immunoreactivity. Then, differentiating NPCs were treated continuously for 16 days (by replacing cell medium every 48 hours) in the presence of 0.1 μM glyphosate, AMPA or glycine. The concentration tested is representative of plasma concentrations reported in occupational workers and is 20 times higher than values reported in non-occupational population. No significant changes in cell metabolic activity were observed between the different groups compared to controls. After 16 days of exposure to glyphosate or AMPA no changes in gross morphology of iPSC-derived neuron colonies were evident.

Neurites density - The exposure of iPSC-derived neurons seeded at low density (50,000 cells/cm^2) to glyphosate or AMPA for 24 hours at concentrations ranging from 1 μM to 1000 μM decreased statistically significantly cell metabolic activity at 10 μM and beyond. AMPA showed similar results, albeit not statistically significant. Treatment with glycine showed only small effects on cell metabolic

activity. Changes in cell density and neurites formation by immunocytochemistry were also investigated and, with the exception of glycine, no depletion in neurites was observed with glyphosate and AMPA. Upon quantification of cell nuclei and neurites per surface area a progressive decrease in neuron density was noted for both glyphosate and AMPA, with a significant decrease at 1000 µM. However, no differences in neurite density were noted with glyphosate and AMPA, with the exception of glycine at 100 and 1000 µM.

Discussion

The toxicity of acute glyphosate poisoning on blood-brain barrier integrity was investigated by assessing its activity on the different cell types of the neurovascular unit. Cells were exposed to glyphosate and AMPA at concentrations ranging from 10 to 1000 µM. This is the concentration range of glyphosate plasma values in patients with self-inflicted poisoning. An increase in fluorescein permeability was noted for glyphosate and AMPA at 1 µM and 10 µM. A similar outcome was noted for mannitol in cells exposed to 1 µM glyphosate. This suggests a possible detrimental effect of glyphosate and AMPA on blood-brain barrier function. Although no major changes in tight junction complexes localization were observed, a decrease was found in both claudin-5 and occludin protein levels after exposure to glyphosate, AMPA and glycine, suggesting that glyphosate and AMPA may interfere with tight junction complexes integrity. Yet, the interference of such compounds on tight junction proteins remains unclear. Although not statistically significant, a 50% increase in paracellular permeability was noted with glycine at 100 and 1000 µM. The data from this study suggest that high levels of glycine may increase the permeability of the BBB and disrupt tight junction complexes. In addition to changes in barrier function, glyphosate permeability in BMEC monolayers was assessed. It is estimated that about 1% of the applied dose (100 µM) diffused across BMECs monolayers. However, the permeability for glyphosate was significantly higher than that for fluorescein despite its high hydrophilicity (xLogP = -4.63). This indicates that glyphosate crosses the BBB via carrier-mediated diffusion. Amongst the different cell types, neurons displayed the most important changes in metabolic activity following exposure to glyphosate and AMPA. Significant changes in neuronal cell metabolic activity were observed following exposure to glyphosate, AMPA or glycine whereas such changes we not observed in BMECs. Such decrease in cell metabolic activity was unlikely to be considered as neurotoxicity since these effects didn't translate in changes in neuronal cell density and neurites formation. The change in cell metabolic activity observed may be due to changes in glucose metabolism, as changes in glucose uptake in BMECs, as well as some changes in GLUT1 expression levels were noted.

Conclusion

The data from this study demonstrate the relative safety of glyphosate and AMPA with regard to the blood-brain barrier after acute exposure with minimal effects observed at concentrations significantly higher than baseline exposure levels, occupational and non-occupational alike. The presence of an active uptake and diffusion of glyphosate across the blood-brain barrier suggests the need of extensive brain-centered studies to evaluate the pharmacokinetics and pharmacodynamics of glyphosate on the central nervous system during acute exposure and in individuals exposed to high amounts of such pesticides.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effect of glyphosate, AMPA and glycine was investigated on he integrity of the blood-brain barrier *in vitro* using an induced pluripotent stem cell line differentiated into brain microvascular endothelial cells (BMEC) and neurons. The endpoints investigated were BMEC cell viability, fluorescein permeability in BMEC cell monolayers, tight junction complexes integrity, diffusion across the blood-brain barrier, modulation of glucose uptake in BMECs, barrier function of neurons co-cultured with BMECs, neurovascular coupling, differentiation of neuron progenitor cells and neurites density. The results of this study indicate that glyphosate or AMPA unlikely present toxicity towards the blood-brain barrier, glyphosate and AMPA at 1 and 10 µM may increase the barrier permeability in BMECs monolayers, glyphosate may increase paracellular permeability in BMECs

monolayers to fluorescein via partial disruption of tight junction complexes integrity, exposure to high levels of glycine or AMPA (100 µM) may impair glucose uptake and metabolism in BMEC monolayers via an alteration in GLUT1 expression and/or activity, exposure to high concentrations of glyphosate (100 µM) may impair neurovascular coupling, chronic exposure to low levels (0.1 µM) of glyphosate or AMPA failed to show any signs of neurotoxicity and that low concentrations (< 10 µM) of glyphosate and AMPA may not have detrimental effects on iPSC-derived neurons.

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

Reliability criteria for *in vitro* toxicology studies

Publication: Martinez *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 glyphosate and AMPA not reported. Source: Sigma-Aldrich, St. Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also glycine and AMPA tested.
AMPA is the tested substance	Y	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Concentration range *in vitro* from 0.1 to 1000 µM for some tests.
Cytotoxicity tests reported	Y	
Biochemical methods described	Y	Some could be better documented.
Analytical method described	Y	The method for the analysis of glyphosate.
Positive and negative controls	N	No positive controls were used.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	For some tests
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 sufficiently characterized and no positive controls were used in any of the assays conducted.

1. Information on the study

Data point:	CA 5.7
Report author	Martínez M. et al.
Report year	2018
Report title	Neurotransmitter changes in rat brain regions following glyphosate exposure
Document No	Environmental Research 161 (2018) 212 - 219
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The effects of glyphosate oral exposure (35, 75, 150 and 800 mg/kg bw, 6 days) on brain region monoamine levels of male Wistar rats were examined. Glyphosate-treated rats (35, 75, 150 and 800 mg/kg bw, 6 days), had no visible injury, i.e., no clinical signs of dysfunction were observed. After last dose of glyphosate, serotonin (5-HT), dopamine (DA) and norepinephrine (NE) and its metabolites levels were determined in the brain regions striatum, hippocampus, prefrontal, cortex, hypothalamus and midbrain, by HPLC. Glyphosate caused statistically significant changes in the 5-HT and its metabolite 5-hydroxy-3-indolacetic acid (5-HIAA), DA and its metabolites 3,4-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and NE and its metabolite 3metoxy-4-hydroxyphenylethyleneglycol (MHPG) levels in a brain regional- and dose-related manner. Moreover, glyphosate, dose-dependent, evoked a statistically significant increase in 5-HT turnover in striatum and hypothalamus and in DA turnover in prefrontal cortex and hippocampus, and a statistically significant decrease in NE turnover in prefrontal cortex and hypothalamus. The present findings indicate that glyphosate significantly altered central nervous system (CNS) monoaminergic neurotransmitters in a brain regional- and dose-related manner, effects that may contribute to the overall spectrum of neurotoxicity caused by this herbicide.

Materials and methods

Chemicals; Glyphosate [N-(phosphonomethyl) glycine], molecular formula $C_3H_8NO_5P$ CAS RN 107-83-6, purity ≥ 98%, serotonin (5-HT) and its metabolite [5-hydroxy-3-indolacetic acid (5-HIAA)], dopamine (DA) and its metabolites [3,4-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] and norepinephrine (NE) and its metabolite [3-metoxy-4-hydroxyphenylethyleneglycol (MHPG)] were purchased from Sigma-Aldrich, St Louis, MO, 63103 USA. All other chemicals were of the highest quality grade and obtained from commercial sources.

Animals and experimental design; All experiments using live animals were undertaken in accordance with the ethics requirements and authorized (protocol number 086) by the official ethical committee of our university. Male Wistar rats of 60 days old each weighing 200–210 g (Charles River Inc., Margate, Kent, UK) were used. The animals were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2 °C and 50 ± 10% relative humidity) with a 12 h light/dark cycle (light from 08.00 to 20.00 h). Food (A04 rodent diet, Scientific Animal Food & Engineering, SAFE, Augy, France) and water were available *ad libitum*. Thirty male rats were assigned randomly to five groups of 6 animals each, a control group and four glyphosate treated groups. Animal treated groups received glyphosate orally at the dose of 35, 75, 150 and 800 mg/kg bw [equivalent to 1/160, 1/75, 1/37 and 1/7 of the acute oral rat LD50 ≈ 5.6 g/kg bw] for 6 consecutive days. The doses were chosen taking into account the LD50 oral value as well as the NOAEL (no observed adverse effect level) described in the literature. The glyphosate treated group rats were deprived of food for 6 h before the oral administration of glyphosate, but were allowed water ad libitum. Glyphosate was

dissolved in water and was administered orally by gavage in a maximum volume of 2 mL/rat. Control animals received the vehicle (water) on the same schedules. The animal body weights were measured during the study and food and water consumption of each animal was also assessed. The animals received the treatment at the same time each day, specifically between 10.0h and 11.0h a.m. Three hours after the last dose, the animals were sacrificed by decapitation. The brain was removed quickly and hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex tissues rapidly dissected out at 4 °C. Tissues were rapidly weighed and stored at -80 °C until analysis.

Determination of monoamine levels; The five brain regions analyzed in the present study were hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex. Following sample collections, 300–800 µL of 0.4 M $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ was added to the tissues, and the mixture was homogenized (1 min) by sonication (Labsonic U-Braun). The homogenates were centrifuged (RC5C, Sorvall Instruments) at 12,000g for 20 min at 4 °C and aliquots of supernatants were taken for analysis of 5-HT and its metabolite 5-HIAA, DA and its metabolites DOPAC and HVA and NE, using a high performance liquid chromatography (HPLC) technique with electrochemical detection. Also, aliquots of supernatants were taken for analysis of the norepinephrine metabolite MHPG by HPLC with fluorimetric detection. An acid-catalyzed procedure was used to hydrolyze MHPG-sulphate in homogenates of brain region tissues. Volumes of 200–300 µL of the supernatants (in 0.4 M $HClO_4$) were treated for 3 min at 100 °C in a water bath. The samples were then cooled and 30 - 45 µL of 2 M NaOH were added (final pH: ca. 1.5) and aliquots were injected into a reverse phase HPLC system. For the analysis of catecholamines NE, DA, DOPAC and HVA, the mobile phase consisted of 0.1 M $Na_2HPO_4 \cdot 2H_2O$, 0.1 M citric acid (pH 3.5), 1.6 mM octane sulphonic acid, 0.9 mM EDTA and 10% (v/v) methanol. For the analysis of the indolalkylamines 5-HT and 5-HIAA, the mobile phase consisted of 0.1 M $Na_2HPO_4 \cdot 2H_2O$, 0.1 M citric acid (pH 3.5) and 10% (v/v) methanol. Elution was performed at a flow rate of 1 mL/min and the working electrode potential was set at 0.8 V for catecholamines and 0.7 V for indolalkylamines. The HPLC system consisted of a Shimadzu liquid chromatograph, model LC-9A equipped with a 5 µm particle size C18-Nucleosil reversed phase column (4 mm i.d. × 125 mm) proceeded by a C18 precolumn, an electrochemical detector (Shimadzu, model L-ECD-6A), a sample injector (20 µL valve) and an integrator (Shimadzu, model C-R6A Chromatopac). For the analysis of the norepinephrine metabolite (MHPG), the mobile phase consisted of 0.06 M $Na_2HPO_4 \cdot 2H_2O$, 0.03 M citric acid and 6% (v/v) methanol. Elution was performed at a flow rate of 1.5 mL/min. The HPLC system consisted of a Shimadzu liquid chromatograph, model LC-10AS, a 25 µm particle size Tracer Extrasil ODS reversed phase column (4 mm i.d. × 125 mm), a fluorescence detector (Shimadzu, model RF-551), a sample injector (20 µL valve) and an integrator (Shimadzu, model C-R6A Chromatopac). Excitation and emission wavelengths of the detector were 275 and 315 nm, respectively. Peak areas from the sample chromatograms were used to quantify the analytes by external standard technique using solutions of catecholamines (NE, DA, DOPAC y HVA), indolalkylamines (5-HT and 5-HIAA) and norepinephrine metabolite (MPHG) reference standards (Sigma Chemical Co., St Louis, MO, USA). For tissue specimens as determined by use of a linear least squares regression procedure, a linear relationship existed in the calibration curve of catecholamines (NE, DA, DOPAC, HVA), indolalkylamines (5-HT, 5-HIAA) and norepinephrine metabolite (MPHG) over the range of 0.002–100 µg/g, which always yielded a correlation coefficient exceeding 0.9998. Overall mean recovery of catecholamines (NE, DA, DOPAC and HVA), indolalkylamines (5-HT and 5-HIAA) and norepinephrine metabolite (MPHG) from tissues was 100% for every analyte. Within- and between-day variation was < 4%. Quantification limit (LOQ) was 2 ng/g for NE, DA, DOPAC, 5-HT and 5-HIAA and 10 ng/g for HVA and MPHG in the different tissue matrices. NE, DA and 5-HT turnover were calculated as ratios of metabolites to neurotransmitter.

Data analysis; Statistical analysis of data was performed using GraphPad Prism 6 for Windows. Results are presented as mean ± S.D. of 6 animals per group. Results significantly different from controls are also presented as percentage change over control. One-way ANOVA was carried out to determine significant dose-dependent effect of glyphosate on 5-HT, DA, NE and metabolite levels and the corresponding turnover values in the brain regions studied, followed by Tukey's post hoc test. Statistical significance was set at $P < 0.05$. ANOVA's F values are presented in the Tables. F distribution was calculated with numerator degrees (DFn) and denominator degrees of freedom (DFd).

Results

The glyphosate-treated rats at oral doses of 35, 75, 150 and 800 mg/kg bw/day for 6 days had no visible injury. These doses were selected based on preliminary experiments where the doses and route of administration did not show any adverse effects, abnormal clinical signs as well as changes in body weight, food and water consumption in the animals (Table 1).

Table 1
Effect of glyphosate on body weight gain and food and water consumption in male rats.

Parameter	Animal groups				
	Control	Glyphosate (35 mg/kg bw, 6 days)	Glyphosate (75 mg/kg bw, 6 days)	Glyphosate (150 mg/kg bw, 6 days)	Glyphosate (800 mg/kg bw, 6 days)
Body weight gain (g)	29.17 ± 2.64	27.67 ± 4.23	29.33 ± 3.08	30.00 ± 4.00	27.33 ± 4.50
Food consumption (g)	96.33 ± 7.45	98.00 ± 7.27	95.66 ± 7.39	93.67 ± 9.24	95.50 ± 9.05
Water consumption (mL)	133.83 ± 20.92	131.50 ± 20.12	129.5 ± 21.50	140.50 ± 24.77	151.00 ± 9.19

Results are presented as means ± SD for six rats.
Results are not significantly different from control group.
Continuous probability distribution (F) for all parameters were lower than 1.140 (DFn = 4, DFd = 25).

All the rat groups exposed to glyphosate by oral route did not show statistically difference on weight of tissues (brain regions) or the ratio weight tissue/body weight (%) compared to control group (data not shown). Glyphosate at a dose of 35 mg/kg bw did not affect the 5-HT, DA, NE and metabolite levels in the brain regions studied. In this study, 35 mg/kg bw might be identified as the NOAEL based on neurotransmitter changes in CNS. Glyphosate at doses of 75, 150 and 800 mg/kg bw produced in a dose-dependent manner a significant decrease of 5-HT content respect to control in striatum. Moreover, glyphosate at doses of 150 and 800 mg/kg bw produced a significant decrease of 5-HT content respect to control in hippocampus and prefrontal cortex and only at a dose of 800 mg/kg bw in hypothalamus and midbrain. In addition, the highest dose (800 mg/kg bw) of glyphosate resulted in a significant decrease in the 5-HIAA levels in hippocampus compared to control group. Also, glyphosate at doses of 150 and 800 mg/kg bw significantly increased the turnover (5-HIAA/5-HT) in striatum and hypothalamus compared to control groups (Table 2).

Table 2
Effect of glyphosate on 5-HT and 5-HIAA levels and turnover (5-HIAA/5-HT) in brain regions of male rats.

Parameter	Animal groups: oral dose of glyphosate during 6 consecutive days	Brain regions				
		Striatum	Hippocampus	Prefrontal cortex	Hypothalamus	Midbrain
5-HT (ng/g)	Control	696.28 ± 14.11	321.60 ± 12.99	710.24 ± 39.86	1698.90 ± 15.31	1551.79 ± 70.09
	35 mg/kg bw	650.05 ± 38.04	319.00 ± 8.17	675.01 ± 49.65	1642.00 ± 74.65	1541.00 ± 62.59
	75 mg/kg bw	557.50 ± 112.42** (−20)[a]	315.16 ± 6.98	603.64 ± 133.99	1575.00 ± 147.39	1515.42 ± 51.99
	150 mg/kg bw	445.57 ± 85.33*** (−36)[a]	293.97 ± 6.23* (−9)[a]	507.20 ± 42.11*** (−29)[a]	1360.75 ± 452.82	1495.03 ± 64.02
	800 mg/kg bw	355.11 ± 16.48*** (−49)[a]	291.06 ± 27.67* (−9)[a]	482.80 ± 69.26*** (−32)[a]	1103.07 ± 111.29*** (−35)[a]	1210.46 ± 134.13*** (−22)[a]
		27.45[b]	5.793[b]	10.51[b]	7.330[b]	18.18[b]
5-HIAA (ng/g)	Control	365.50 ± 75.88	390.31 ± 99.55	299.85 ± 48.05	743.32 ± 78.12	865.34 ± 123.9
	35 mg/kg bw	366.30 ± 55.29	383.04 ± 6.35	297.00 ± 48.98	755.00 ± 33.45	846.00 ± 95.25
	75 mg/kg bw	366.49 ± 68.09	351.54 ± 8.99	295.10 ± 64.32	736.09 ± 55.73	820.32 ± 120.66
	150 mg/kg bw	368.70 ± 31.54	307.93 ± 91.06	270.69 ± 58.58	733.13 ± 116.03	805.00 ± 66.93
	800 mg/kg bw	352.41 ± 60.91	269.81 ± 55.41* (−31)[a]	259.39 ± 37.20	726.33 ± 40.54	708.82 ± 151.74
		0.06677[c]	3.669[c]	0.7277[c]	0.175[c]	1.661[c]
5-HIAA/5-HT	Control	0.53 ± 0.12	1.22 ± 0.30	0.42 ± 0.06	0.45 ± 0.04	0.56 ± 0.09
	35 mg/kg bw	0.56 ± 0.06	1.20 ± 0.04	0.45 ± 0.11	0.47 ± 0.20	0.55 ± 0.08
	75 mg/kg bw	0.66 ± 0.08	1.12 ± 0.04	0.5 ± 0.15	0.52 ± 0.19	0.54 ± 0.09
	150 mg/kg bw	0.86 ± 0.26*** (63)[a]	1.06 ± 0.35	0.54 ± 0.14	0.59 ± 0.18* (30)[a]	0.54 ± 0.04
	800 mg/kg bw	0.99 ± 0.14*** (88)[a]	0.93 ± 0.21	0.54 ± 0.09	0.69 ± 0.15*** (53)[a]	0.59 ± 0.13
		20.8[c]	1.591[c]	1.355[c]	13.31[c]	0.3139[c]

** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.01$.
*** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.001$.
* Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.05$.
[a] Percentage change over control in parenthesis.
[b] F (continuous probability distribution; DFn = 4, DFd = 25).

With respect to DA, DOPAC and HVA levels and turnover in brain regions (Table 3), glyphosate at doses of 75, 150 y 800 mg/kg bw produced, in a dose-dependent manner, a statistically significant decrease of DA levels in prefrontal cortex and midbrain compared to control groups. Likewise, glyphosate at the highest dose (800 mg/kg bw) decreased significantly the DA levels in hypothalamus, striatum and hippocampus compared to control group. Moreover, glyphosate at doses of 150 and 800 mg/kg bw decreased significantly the DOPAC metabolite levels in hypothalamus and glyphosate at highest dose only produced a significant decrease of DOPAC levels in hippocampus respect to control group. In addition, the HVA levels significantly decreased after doses of glyphosate 75, 150 and 800 mg/kg bw in hypothalamus, after doses of glyphosate 150 and 800 mg/kg bw in midbrain; and after dose of 800 mg/kg bw in prefrontal cortex respect to control groups. Glyphosate (75, 150 and 800 mg/kg bw) produced a significant increase of the turnover (DOPAC+HVA/DA) in prefrontal cortex respect to control groups. Glyphosate at highest dose only produced a significant increase of the turnover (DOPAC+HVA/DA) in hippocampus (Table 3).

Table 3
Effect of glyphosate on DA, DOPAC and HVA levels and turnover (DOPAC+HVA/DA) in brain regions of male rats.

Parameter	Animal groups: oral dose of glyphosate during 6 consecutive days	Brain regions				
		Striatum	Hippocampus	Prefrontal cortex	Hypothalamus	Midbrain
DA (ng/g)	Control	6406.35 ± 502.83	248.48 ± 59.36	209.00 ± 45.43	600.20 ± 35.36	776.95 ± 34.09
	35 mg/kg bw	6332.00 ± 296.00	231.00 ± 34.60	186.00 ± 21.33	581.00 ± 51.76	732.00 ± 20.86
	75 mg/kg bw	5614.83 ± 1040.66	213.55 ± 29.46	109.14 ± 16.61*** (−48)[a]	555.48 ± 102.85	676.48 ± 28.33*** (−13)[a]
	150 mg/kg bw	5563.85 ± 223.28	177.84 ± 39.32	97.28 ± 10.78*** (−53)[a]	528.52 ± 39.21	666.07 ± 29.05*** (−14)[a]
	800 mg/kg bw	5009.86 ± 468.29** (−22)[a]	73.07 ± 67.49*** (−71)[a]	34.99 ± 33.65*** (−83)[a]	480.02 ± 23.93* (−20)[a]	642.72 ± 22.98*** (−17)[a]
		6.044[b]	12.49[b]	36.86[b]	4.001[b]	23.71[b]
DOPAC (ng/g)	Control	691.04 ± 64.66	9.51 ± 1.77	21.43 ± 2.68	93.22 ± 21.18	48.75 ± 13.78
	35 mg/kg bw	689.00 ± 74.87	9.44 ± 1.29	21.00 ± 2.23	85.00 ± 18.34	47.00 ± 8.38
	75 mg/kg bw	688.17 ± 221.53	9.00 ± 1.22	19.19 ± 1.23	68.19 ± 21.13	45.78 ± 9.33
	150 mg/kg bw	689.62 ± 37.04	7.66 ± 1.26	18.46 ± 1.01	57.55 ± 6.11** (−38)[a]	44.81 ± 4.09
	800 mg/kg bw	683.38 ± 72.08	6.66 ± 1.95* (−30)[a]	20.04 ± 0.84	57.26 ± 4.70** (−39)[a]	43.78 ± 1.93
		0.00389[b]	4.005[b]	2.958[b]	6.156[b]	0.3051[b]
HVA (ng/g)	Control	907.87 ± 258.95	18.29 ± 1.95	50.75 ± 8.78	68.91 ± 14.12	71.46 ± 3.82
	35 mg/kg bw	906.00 ± 132.32	18.00 ± 3.48	50.60 ± 4.97	64.00 ± 8.34	68.00 ± 5.32
	75 mg/kg bw	901.87 ± 260.40	17.45 ± 4.11	50.33 ± 6.29	44.34 ± 17.40** (−36)[a]	65.10 ± 4.41
	150 mg/kg bw	846.17 ± 48.38	16.17 ± 1.26	46.38 ± 4.27	34.77 ± 5.77*** (−50)[a]	52.91 ± 6.04*** (−26)[a]
	800 mg/kg bw	839.75 ± 32.86	16.40 ± 2.59	30.84 ± 11.55** (−39)[a]	36.82 ± 6.41*** (−47)[a]	38.09 ± 11.10*** (−47)[a]
		0.226[b]	0.6519[b]	7.480[b]	11.50[b]	25.26[b]
(DOPAC+HVA)/DA	Control	0.25 ± 0.05	0.12 ± 0.02	0.36 ± 0.09	0.28 ± 0.25	0.15 ± 0.02
	35 mg/kg bw	0.25 ± 0.04	0.12 ± 0.01	0.39 ± 0.02	0.27 ± 0.06	0.16 ± 0.02
	75 mg/kg bw	0.3 ± 0.11	0.13 ± 0.03	0.64 ± 0.07** (78)[a]	0.22 ± 0.07	0.16 ± 0.02
	150 mg/kg bw	0.28 ± 0.02	0.14 ± 0.03	0.67 ± 0.07** (86)[a]	0.18 ± 0.02	0.15 ± 0.01
	800 mg/kg bw	0.31 ± 0.04	0.98 ± 0.91*** (150)[a]	2.88 ± 2.62*** (153)[a]	0.20 ± 0.02	0.13 ± 0.02
		1.269[b]	5.254[b]	39.28[b]	2.009[b]	2.647[b]

*** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at P < 0.001.
** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at P < 0.01.
* Results are presented are means ± SD from six animals in each group and are significantly different from the control value at P < 0.05.
[a] Percentage change over control in parenthesis.
[b] F (continuous probability distribution; DFn = 4, DFd = 25).

In relation to NE and MHPG levels and turnover, glyphosate at doses of 75, 150 and 800 mg/kg bw produced a significant decrease of the NE levels in striatum and midbrain compared to control. Moreover, glyphosate at doses of 150 and 800 mg/kg bw produced a significant decrease of NE levels in hippocampus and only at a dose of 800 mg/kg bw in prefrontal cortex respect to control groups. Moreover, MHPG levels significantly decreased after doses of glyphosate 150 and 800 mg/kg bw in hippocampus and after dose of glyphosate 800 mg/kg bw in striatum, prefrontal cortex, hypothalamus and midbrain. Glyphosate at dose of 800 mg/kg bw produced a significant decrease of the turnover (MHPG/NE) in prefrontal cortex and hypothalamus compared to control group (Table 4).

Table 4
Effect of glyphosate on NE and MHPG levels and turnover (MHPG/NE) in brain regions of male rats.

Parameter	Animal groups: oral dose of glyphosate during 6 consecutive days	Brain regions				
		Striatum	Hippocampus	Prefrontal cortex	Hypothalamus	Midbrain
NE (ng/g)	Control	210.64 ± 79.72	190.74 ± 13.01	145.81 ± 3.08	1006.77 ± 195.00	452.04 ± 76.01
	35 mg/kg bw	202.00 ± 37.93	183.00 ± 11.02	145.00 ± 9.34	1005.00 ± 74.85	422.00 ± 39.44
	75 mg/kg bw	121.07 ± 42.09* (−43)[a]	159.24 ± 20.21	138.54 ± 11.18	1000.88 ± 76.64	357.03 ± 55.11* (−21)[a]
	150 mg/kg bw	118.13 ± 24.65* (−44)[a]	150.02 ± 30.98* (−21)[a]	137.29 ± 6.11	901.07 ± 72.69	358.61 ± 31.77* (−21)[a]
	800 mg/kg bw	102.74 ± 27.10** (−51)[a]	137.34 ± 15.13** (−28)[a]	127.64 ± 12.35* (−12)[a]	878.22 ± 71.54	358.92 ± 17.12* (−21)[a]
		7.194[b]	7.981[b]	3.908[b]	2.007[b]	5.083[b]
MHPG (ng/g)	Control	161.88 ± 21.25	67.07 ± 8.13	109.60 ± 28.56	50.29 ± 8.27	71.69 ± 15.56
	35 mg/kg bw	159.00 ± 35.00	61.00 ± 5.85	100.00 ± 12.67	46.00 ± 11.27	70.00 ± 9.32
	75 mg/kg bw	155.46 ± 36.00	54.90 ± 6.67	84.67 ± 12.91	43.40 ± 7.21	69.11 ± 8.54
	150 mg/kg bw	132.94 ± 31.34	51.22 ± 9.28* (−24)[a]	84.46 ± 12.41	43.03 ± 21.58	67.72 ± 10.57
	800 mg/kg bw	108.95 ± 10* (−33)[a]	50.47 ± 9.85* (−25)[a]	71.11 ± 10.32** (−35)[a]	20.22 ± 9.25** (−60)[a]	29.01 ± 4.66*** (−60)[a]
		3.748[b]	4.451[b]	4.810[b]	5.198[b]	18.61[b]
MHPG / NE	Control	0.85 ± 0.29	0.35 ± 0.06	0.77 ± 0.18	0.05 ± 0.01	0.16 ± 0.03
	35 mg/kg bw	0.84 ± 0.33	0.34 ± 0.05	0.69 ± 0.09	0.05 ± 0.02	0.17 ± 0.02
	75 mg/kg bw	1.42 ± 0.59	0.35 ± 0.08	0.61 ± 0.08	0.04 ± 0.01	0.20 ± 0.04
	150 mg/kg bw	1.14 ± 0.23	0.35 ± 0.08	0.62 ± 0.10	0.05 ± 0.03	0.19 ± 0.03
	800 mg/kg bw	1.10 ± 0.19	0.37 ± 0.07	0.56 ± 0.05* (−23)[a]	0.02 ± 0.01** (−55)[a]	0.18 ± 0.01
		2.733[b]	0.1513[b]	2.792[b]	5.423[b]	1.923[b]

* Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.05$.
** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.01$.
*** Results are presented are means ± SD from six animals in each group and are significantly different from the control value at $P < 0.001$.
[a] Percentage change over control in parenthesis.
[b] F (continuous probability distribution; DFn = 4, DFd = 25).

Discussion

Pesticides are widely used in agricultural and other settings, resulting in continuing human exposure. The nervous system represents a prime target for both the acute and chronic effects of pesticides. Among them are the organochlorines, pyrethroids, organophosphates, neonicotinoids, herbicides and some novel agents. Acute symptoms can include headache, nausea, dizziness, and sensory paresthesia. Toxicity often involves neuronal hyper-excitability, and disorders of cognition. Toxicological in vitro and in vivo studies have demonstrated specific neurodegenerative effects from exposure to certain pesticides, and human case reports have suggested a causal relationship between certain pesticide exposures and Parkinson's disease (PD) typically associated with degeneration of the dopaminergic neurons. Evidence is also now accumulating that organophosphate pesticides target serotonin and

noradrenergic systems contributing to adverse outcomes related to emotional and social behaviours. Ingestion of the herbicide glyphosate may cause significant toxicity including nausea, vomiting, diarrhea, oral and abdominal pain, renal and hepatic impairment, and pulmonary oedema. Impaired consciousness and seizures have also been reported as sequelae but there are limited data of glyphosate on central nervous system (CNS) toxicity. This study was designed to investigate the effects of glyphosate on CNS monoaminergic neurotransmitter contents (5-HT, DA and NE) in male Wistar rats in order to generate more data on the glyphosate neurotoxicity. The current study showed that exposure to glyphosate, in a region and dose-dependent manner, was accompanied by a significant decrease in the 5-HT, DA and NE contents in the brain regions studied (striatum, hippocampus, prefrontal cortex, hypothalamus, hypothalamus and midbrain), which indicated that glyphosate transfer across the blood-brain barrier, enters the brain, probably accumulates in significant quantity, and exerts neurotoxicity altering the serotonergic, dopaminergic and noradrenergic systems. Researchers have reported similar changes of these brain neurotransmitters after exposure to the insecticide pyrethroid cyfluthrin. It should be noted that the rats treated with glyphosate at dose of 35 mg/kg bw per day did not exhibit any effects on the 5-HT, DA and NE contents in the brain regions studied. In our study, this NOAEL observed (35 mg/kg bw per day) was lowest to that identified on the maternal and developmental toxicity studies (NOAEL of 50 mg/kg bw per day) and used to establish the ADI. For current regulatory evaluation of risks associated with glyphosate exposure, a NOAEL of 35 mg/kg bw per day could be used instead of a NOAEL of 50 mg/kg bw per day. In this regard, taking into account that glyphosate probably accumulates in the CNS and considering that in the present study the glyphosate exposure was only during only 6 days, further research with a longer period of exposure should be necessary to corroborate the proposed NOAEL of 35 mg/kg bw per day. In the present study, quantitative analysis of 5-HT, DA and NE contents showed that the loss of these neurotransmitters was mainly observed in the striatum [5-HT and NE contents decreased significantly (-49% and -51%, respectively) after the highest dose of 800 mg glyphosate/kg bw] and in prefrontal cortex and hippocampus [DA contents decreased significantly (-83% and -71%, respectively) after the highest dose of 800 mg glyphosate/kg bw]. Previously, Researchers also showed in rats that glyphosate decreased DA but not 5-HT levels in striatum as well as reduced the locomotor activity suggesting that the decrease in striatal DA levels could also explain a behavioral hypoactivity. Moreover, in our study, glyphosate after the highest dose of 800 mg/kg bw produced a significant increase of the (5-HIAA/5-HT) turnover in striatum (88%) and of the (DOPAC+HVA/DA) turnover in prefrontal (153%) and hippocampus (150%), but a significant decrease of the (NE/MHPG) turnover in prefrontal cortex (-23%) and hypothalamus (-55%), critical brain regions that regulate cognitive functions. The cooperation of the hippocampus and the prefrontal cortex is vital in spatial working memory performance and decision making. Disconnection or damage to either of the two brain regions induces impaired cognitive behaviors. Because cognitive functions are quite complex, more details are required for the complete understanding of glyphosate-induced neurotoxicity. It would be of interest to investigate the developmental changes of the hippocampus and prefrontal cortex in prenatal glyphosate.

Conclusion

In conclusion, the results demonstrate that glyphosate leads to loss of 5-HT, DA and NE levels in the CNS. The neurochemical effects observed in the present study are an important public health concern. Although we have not data on humans, glyphosate could exert its neurotoxicity, notably on monoamine systems, by inducing DNA damage, neuronal inflammation and oxidative stress mechanisms. Further investigation is needed to involve the glyphosate herbicide with neurodegenerative diseases. Nevertheless, our results support the hazard of glyphosate herbicide and show the importance of minimizing its herbicide use.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Although the study concludes “loss of 5-HT, DA and NE levels in the CNS”, no historical controls are available to assess and compare the changes in the treatment-groups to ascertain if the effects are within background or if they are biologically relevant.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because there was historical control to determine if changes in the levels of neurotransmitters were within historical controls. No positive control included either. Also no analytical verification of dose levels.

Reliability criteria for *in vivo* toxicology studies

Publication: Martínez M. *et al.*, 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Non-guideline
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 purity of ≥98%. Source: Sigma-Aldrich. No information on storage,
Only glyphosate acid or one of its salts is the tested substance	Y	Yes
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	Wistar rat (male)
Test conditions clearly and completely described	Y	Yes
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	35, 75, 150, 800 mg/kg bw/day for 6 days
Positive control	N	-
Number of animals used per dose level reported	Y	6/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	N	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	No historical control data for comparison, no analytical verification of dose or stability.
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because there was historical control to determine if changes in the levels of neurotransmitters were within historical controls. No positive control was included. Also no analytical verification of dose levels.		

1. Information on the study

Data point:	CA 5.9 Exposure study
Report author	McGuire M. K. *et al.*
Report year	2016
Report title	Glyphosate and aminomethylphosphonic acid are not detectable in human milk
Document No	The American journal of clinical nutrition, (2016) Vol. 103, No. 5, pp. 1285-90.
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

It was sought to determine whether glyphosate and its metabolite aminomethylphosphonic acid (AMPA) could be detected in milk and urine produced by lactating women and, if so, to quantify typical consumption by breastfed infants. Milk (n = 41) and urine (n = 40) samples from healthy lactating women living in and around Moscow, Idaho and Pullman, Washington was collected. Milk and urine samples were analyzed for glyphosate and AMPA with the use of highly sensitive liquid chromatography–tandem mass spectrometry methods validated for and optimized to each sample matrix. The milk assay, which was sensitive down to 1 μg/L for both analytes, detected neither glyphosate nor AMPA in any milk sample. Mean ± SD glyphosate and AMPA concentrations in urine were 0.28 ± 0.38 and 0.30 ± 0.33 mg/L, respectively. Because of the complex nature of milk matrixes, these samples required more dilution before analysis than did urine, thus decreasing the sensitivity of the assay in milk compared with urine. No difference was found in urine glyphosate and AMPA concentrations between subjects consuming organic compared with conventionally grown foods or between women living on or near a farm/ranch and those living in an urban or suburban non-farming area.

Materials and Methods

Human subjects – A total of 41 healthy lactating women living in and around Pullman, Washington, and Moscow, Idaho, were included in the study. To be eligible for participation, women had to be 1–3 months postpartum, breastfeeding and/or pumping milk 5 times/day and more, and aged 18 years and older. All but 1 subject also completed a 5-question survey documenting potential glyphosate exposure from the environment and diet.

Milk and urine collection and preservation - After cleaning the breast, approx. 30 mL milk was collected with an hospital-grade electric breast pump into a single-use sterile collection container. A midstream urine sample was also collected into a single-use sterile collection container. The sample containers were immediately placed in ice, separated into aliquots and frozen at -20^{0}C pending analysis. One subject failed to provide a urine sample.

Glyphosate and AMPA analyses - Milk and urine samples were analyzed for glyphosate and AMPA using liquid chromatography-tandem mass spectrometry in the multiple reaction monitoring mode. Two precursor product ion transitions for each analyte and a stable isotope labelled internal standard for each analyte were used to ensure the selectivity of the analytical method. Although 2 quantitative precursor-product ion transitions were monitored, the results were reported using the most sensitive transition for each analyte. The assay was validated separately for milk and urine. Limits of detection (LOD) and quantification (LOQ) for glyphosate in milk were 1.0 and 10.0 μg/L, respectively. The LOD and LOQ for glyphosate in urine were 0.02 and 0.10 μg/L, respectively. The LOD and LOQ for AMPA in milk

were 1.0 and 10.0 µg/L, respectively and those for AMPA in urine were 0.03 and 0.10 µg/L, respectively. Glyphosate and AMPA concentrations in milk were independently confirmed by another laboratory using the same liquid chromatography-tandem mass spectrometry method with minor modifications. Because of differences in instrumentation, the LODs with the more sensitive quantitative ion transitions were 6.0 and 9.0 µg/L for glyphosate and AMPA in human milk, respectively. The LOQ for human milk was 25.0 µg/L for both analytes. Duplicate aliquots from each milk sample were sent to each laboratory separately.

Statistical analyses - For glyphosate and AMPA concentrations in urine a generalized linear mixed model was used assuming a Poisson distribution with a logarithmic link function. For concentrations less than the respective LOD values, one-half LOD (0.01 and 0.015 µg/L for glyphosate and AMPA, respectively) nominal values were used for assessment. For concentrations between the LOD and LOQ, one-half LOQ (0.05 µg/L for both glyphosate and AMPA) nominal values were used for assessment. All values are presented as means ± SDs.

Results

Description of study population and glyphosate exposure - Women were aged 29 ± 5 years, 67 ± 17 days postpartum, and had a BMI (kg/m^2) of 26.8 ± 8.6. 75% of them lived in an urban or suburban non-farming region of the Palouse, and 58% of them reported that they made no effort to eat foods characterized as organic, although they sometimes included them in their diets for convenience. 15% reported ever having personally mixed or used any type of weed killer. All but one of the women having reported ever doing so had mixed or used a weed killer containing glyphosate.

Glyphosate and AMPA concentrations in milk - Regardless of where the samples were analyzed, none of the milk samples contained detectable amounts of either glyphosate or AMPA.

Glyphosate and AMPA concentrations in urine - Glyphosate was detectable in nearly all (n =37) of the urine samples and was quantifiable in 29 of them. Glyphosate values ranged from below the LOD (<0.02 µg/L) to 1.93 µg/L, with a mean concentration of 0.28 ± 0.38 µg/L. AMPA was also detectable in nearly all (n = 38) of the urine samples and quantifiable in 29 of them. Urine AMPA values ranged from below the LOD (<0.03 mg/L) to 1.33 µg/L, with a mean concentration of 0.30 ± 0.33 µg/L. There were no statistically significant effects of consuming organic compared with conventional foods or living on/near a farm compared with living in an urban/suburban region on concentrations of glyphosate in urine, respectively. Neither were there statistically significant effects of consuming organic compared with conventional foods or living on/near a farm compared with living in an urban/suburban region on concentrations of AMPA in urine. Adjusting for potential covariates (age, time postpartum, BMI, parity) did not alter these conclusions. When raw, untransformed values were used in the analysis, there was a statistically significant positive correlation between urinary glyphosate and AMPA concentrations.

Discussion and Conclusion

The results provide evidence that the concentrations of glyphosate and AMPA in milk produced by healthy women are below the detection limits of available validated analytical assays. In urine, glyphosate and AMPA were detectable in many samples, but the concentrations were very low (<0.02 to 1.93 and <0.03 to 1.33 µg/L, respectively) and well below values reported in other healthy adult populations. The RfD for glyphosate is 1.75 mg/kg bw/day. US EPA considers AMPA to be of similar or lesser toxicity than glyphosate and determined that it should be exempt from regulation regardless of concentrations observed in food or feed. Thus, a woman with a typical weight for the study participants, 75 kg, could consume as much as 131.25 mg glyphosate/day with no expected negative effects. Taking an oral bioavailablity of 20% into account and assuming that all glyphosate absorbed is excreted within 24 hours and all absorbed glyphosate is excreted in urine, the urinary output would be 26,250 µg/day. In the current study, the highest reported glyphosate concentration in urine was 1.93 µg/L. As such, even allowing for a relatively high urine output (3 L/day), the highest glyphosate excretion in our study would be 5.79 µg/day, a value which is more than 4,500 times lower. Applying similar parameters and logic

to a 5-kg infant with a mean milk intake of 0.7 L/day and a milk glyphosate concentration of 1 µg/L (the LOD value), then the maximum daily consumption of glyphosate would be 0.7 µg/day which is more than 12,000 times lower than the RfD. It is important to emphasize that the larger international study from which these samples originate was not designed to detect small differences in urine glyphosate and AMPA concentrations based on dietary choices, location of residence (e.g., urban compared with rural), or occupational glyphosate exposure. Detecting small-effect sizes at statistically significant concentrations and adequate statistical power would require 4–5 times as many observations than used in this study.

3. Assessment and conclusion

Assessment and conclusion by applicant:
In this study breast milk and urine samples from lactating women were analyzed for glyphosate and AMPA. The results provide evidence that the concentrations of glyphosate and AMPA in milk produced by healthy women are below the detection limits of available validated analytical assays. In urine, glyphosate and AMPA were detectable in many samples, but the concentrations were very low and well below the values reported in other healthy adult populations.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with all the reliability criteria of an exposure study.

Reliability criteria for exposure studies

Publication: McGuire *et al*., 2016	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.		
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Reference material (glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	Exposure mainly via food
Study		
Study design clearly described	Y	Monitoring of glyphosate in urine and breast milk of lactating women
Population investigated sufficiently described	Y	
Exposure circumstances sufficiently described	Y	
Sampling scheme sufficiently documented	Y	
Analytical method described in detail	Y	
Validation of analytical method reported	Y	
Monitoring results reported	Y	
Overall assessment		
Reliable	Y	
Reliable with restrictions		

Publication: McGuire *et al*., 2016	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with all the reliability criteria of an exposure study.		

1. Information on the study

Data point:	CA 5.3
Report author	Mesnage R. *et al*
Report year	2018
Report title	Comparison of transcriptome responses to glyphosate, isoxaflutole, quizalofop-p-ethyl and mesotrione in the HepaRG cell line
Document No	Toxicology Reports (2018) Vol. 5, 819–826
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

Together with 3 other herbicide active ingredients (quizalofop-p-ethyl, isoxaflutole and mesotrione) the effect of glyphosate on the transcriptome and metabolome profile of differentiated HepaRG cells was investigated.

Materials and methods

Chemicals - Glyphosate (purity ≥ 96%) was purchased from Sigma-Aldrich, Gillingham, Dorset, UK.

HepaRG cell culture - Differentiated HepaRG™ cells (HPR 116) were purchased from Biopredic International (Rennes, France). Cells were thawed, suspended and plated in general purpose medium (Williams' E medium + GlutaMAX™) containing the ADD670 supplement. Cells were kept in general purpose medium until day 8, when the culture becomes well organized and includes well-delineated trabeculae and many canaliculi-like structures. At this time, the culture is composed of primitive biliary epithelial cells and mature hepatocytes with basal metabolic activities similar to freshly isolated primary cells. From day 8 to day 14, cells were switched to the test medium composed of Williams' E medium + GlutaMAX™ supplemented with 2% fetal bovine serum and 1% DMSO, as well as different concentrations of glyphosate or solvent as a control. Glyphosate was tested at 0.06 μM (concentration representative of low environmental exposure), 6 μM, and 600 μM.

Library generation and RNA-sequencing - A 100 ng aliquot of total RNA from each sample was used to prepare total RNA libraries using the KAPA Stranded RNA-Seq Kit with RiboErase, and samples were randomised before preparation. Polymerase chain reaction (PCR) was performed for 14 cycles for final library amplification. Resulting libraries were quantified using the Qubit 2.0 spectrophotometer and average fragment size assessed using the Agilent 2200 Tapestation. The transcriptome of HepaRG cells exposed to glyphosate was sequenced employing this strategy, except that the libraries were prepared as previously described. A total of 3 separate sequencing pools were created using equimolar quantities of each sample with compatible indexes: 2 with 17 samples each, and one with 16 samples. Paired-end reads of 75bp were generated for each library using the Illumina NextSeq®500 in con-junction with the NextSeq®500 v2 High-output 150-cycle kit.

Mass spectrometry-based metabolomics - Approximately 5,000,000 HepaRG cells per sample were harvested from the 6 well-plates to obtain a sufficient quantity of material to perform the metabolomics experiment. Cells were detached using 0.05% trypsin EDTA, and centrifuged to eliminate trypsin residues. Finally, cell pellets were frozen at -80 °C pending analysis. Metabolomics analysis of the frozen cell pellets was conducted by Metabolon Inc. The sample extracts were stored overnight under nitrogen before preparation for analysis. The resulting extract was analysed on four independent instrument platforms: two different separate RP/UPLC-MS/MS with positive ion mode electrospray

ionization (ESI), a RP/UPLC-MS/MS with negative ion mode ESI, as well as by hydrophilic-interaction chromatography(HILIC)/UPLC-MS/MS with negative ion mode ESI as previously described. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Biochemical identifications are based on 3 criteria: retention index within a narrow retention time/index (RI) window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The use of all three data points can be utilized to distinguish and differentiate biochemicals. Peaks were quantified using the area-under-the-curve method.

Statistics - Metabolome data analysis was performed using in-house services of Metabolon Inc. Biochemical data were normalized with respect to protein concentration as determined by the Bradford assay. The Welch's two-sample t-test was used to test whether control and treatment group means are different from two independent populations. This version of the two-sample t-test allows for unequal variances. FDR methods and estimated q-values were used to account for the highest number of false positive results caused by the high number of statistical tests. The RNA-seq data analysis was performed using the new version of the Tuxedo protocol with HISAT2, StringTie and Ballgown. A standard linear model-based comparison of transcript abundance was performed without adjusting for other covariates to identify differentially expressed transcripts for each group. Although 3 concentrations were tested for glyphosate, no multigroup comparisons were used because dose spacing was too large to allow reliable conclusions to be drawn from these methods. Instead, pairwise comparisons were used.

Results

Control, untreated cell cultures presented no visible signs of aging after a 6-day exposure. Transcriptome profiles of HepaRG cells were then determined using the Illumina-based RNA sequencing platform. The highest concentration of glyphosate tested caused significant changes in transcriptome profiles. Alterations in gene expression caused by the 2 lowest concentrations (0.06 and 6 μM) failed to pass the statistical threshold that took into account the high number of tests performed. A total of 7 transcripts had their levels altered ($p < 0.05$) with glyphosate at 600 μM. The number of genes disturbed by the exposure to glyphosate was insufficient to use a functional annotation tool for pathway enrichment analysis. It is not clear if glyphosate lacks hepatic toxic effects at these concentrations or if this experimental design lacks sensitivity to detect hepatic effects of weak toxicants. To further explore changes in liver metabolism caused by glyphosate in greater detail, a global metabolome profiling of HepaRG cells exposed to three concentrations of glyphosate was explored. The Metabolon HD4 platform detected 802 named biochemicals in the HepaRG samples. Overall, glyphosate did not cause significant alterations in metabolome composition. However, exposure did cause a significant decrease in long chain fatty acids (LCFAs) and polyunsaturated fatty acids (PUFAs). HepaRG cells exposed to the lowest concentration of glyphosate tested (0.06 μM) showed the most dramatic effects in the levels of these fatty acids as either significant or trends towards significant lower levels. At the higher glyphosate concentrations of 6 μM and 600 μM, lower lipid levels were also observed but these did not reach statistical significance.

Discussion and conclusion

An in-depth investigation was conducted of transcriptome profile alterations in HepaRG human liver cells caused by exposure to pesticide active ingredients. Glyphosate was found to be only weakly toxic inducing little change in transcriptome profiles. Interestingly, a follow-up metabolomics analysis of HepaRG cells treated with the lowest (0.06 μM) concentration of glyphosate revealed a significant decrease in the levels of LCFAs and PUFAs. Although these findings from an *in vitro* tissue culture model system cannot be readily translated to effects *in vivo*, they are nevertheless indicative of differences in toxicity potency between pesticide ingredients. The exact nature of this low dose effect of glyphosate cannot be determined from this single experiment, but it is possible that at higher concentrations, more overtly toxic mechanisms are masking the effects on lipids. Another possibility is that a saturation effect is occurring once the low dose is exceeded bearing in mind that glyphosate levels found in the HepaRG cells during the metabolomics analysis increased by 3.7-and 336.35-fold at the

intermediate and highest concentrations tested compared to the negative controls. Glyphosate was the least toxic of the compounds tested in this study.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Together with 3 other herbicide active ingredients (quizalofop-p-ethyl, isoxaflutole and mesotrione) the effect of glyphosate on the transcriptome and metabolome profile of differentiated HepaRG cells was investigated at 0.06, 6 and 600 µM. Glyphosate was found to be only weakly toxic inducing little change in transcriptome profiles when compared with the other herbicides tested. A follow-up metabolomics analysis of HepaRG cells exposed to glyphosate at 0.06 µM revealed a significant decrease in the levels of long chain fatty acids (LCFAs) and polyunsaturated fatty acids (PUFAs). At the higher glyphosate concentrations of 6 and 600 µM, lower lipid levels were also observed but these did not reach statistical significance. It is not clear, however, how these findings from an *in vitro* tissue culture model can be translated to effects *in vivo*.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because no positive control was used and no cytoxicity tests were performed to optimise the concentration range to be explored.

Reliability criteria for *in vitro* toxicology studies

Publication: Mesnage *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	Glyphosate (purity ≥ 96%). Source: Sigma-Aldrich, Gillingham, Dorset, UK.
Only glyphosate acid or one of its salts is the tested substance	N	Also three other pesticide active ingredients were tested (quizalofop-p-ethyl, isoxaflutole and mesotrione).
AMPA is the tested substance	N	
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	0.06, 6 and 600 µM.
Cytotoxicity tests reported	N	
Transcriptomics and metabolomics methods described	Y	
Positive and negative controls	N	No positive controls included.
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 no positive control was used and no cytoxicity tests were performed to optimise the concentration range to be explored.		

1. Information on the study

Data point:	5.8.2/016
Report author	Mesnage , R. *et al.*
Report year	2018
Report title	Ignoring Adjuvant Toxicity Falsifies the Safety Profile of Commercial Pesticides
Document No	doi.org/10.3389/fpubh.2017.00361
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

Currently, the health risk assessment of pesticides in the European Union and in the United States focuses almost exclusively on the stated active principle. Nonetheless, adjuvants can also be toxic in their own right with numerous negative health effects having been reported in humans and on the environment. Despite the known toxicity of adjuvants, they are regulated differently from active principles, with their toxic effects being generally ignored. Adjuvants are not subject to an acceptable daily intake, and they are not included in the health risk assessment of dietary exposures to pesticide residues. Here, this gap in risk assessment by reference to glyphosate, the most used pesticide active ingredient is illustrated. The case of neonicotinoid insecticides, which are strongly suspected to be involved in bee and bumblebee colony collapse disorder is also investigated. Authors of studies sometimes use the name of the active principle (for example glyphosate) when they are testing a commercial formulation containing multiple (active principle plus adjuvant) ingredients. This results in confusion in the scientific literature and within regulatory circles and leads to a misrepresentation of the safety profile of commercial pesticides. Urgent action is needed to lift the veil on the presence of adjuvants in food and human bodily fluids, as well as in the environment (such as in air, water, and soil) and to characterize their toxicological properties. This must be accompanied by regulatory precautionary measures to protect the environment and general human population from some toxic adjuvants that are currently missing from risk assessments.

Materials and methods

Not applicable because this is a review article.

In this review, a focus was done on the co-formulants included in the commercial formulations of glyphosate-based herbicides, in particular the polyethoxylated tallow amines (POEAs), which is considered as a representative good model system because they are the most used pesticides worldwide.

Results

A total of 750 different formulations of glyphosate-based herbicides are marketed worldwide. Indeed, each name of a given formulation represents a different mixture of active principle and co-formulants (Table 1). As a result of the variability in co-formulants, and since most of them are not compulsorily declared, the effects of pesticides are complex and combinatorial. The literature is quite heterogeneous because these co-formulants vary between commercial pesticide formulations and thus have different and/or additive side effects between themselves and with glyphosate. In fact, this causes confusion in the scientific community, with authors not always declaring the formulation that they tested. Authors even sometimes confuse commercial formulations with the active ingredient; they state that “glyphosate” was used when in reality they employed a formulation in their studies.

The problem of reproducibility and consistency in the results of toxicological studies could be partly due to the fact that comparisons are performed between different formulations. Some studies even compare formulations and glyphosate alone treating the two as equivalent and therefore ignoring the effects of adjuvants in the former. This adds yet further confusion and questions over the reliability of the data obtained. For example, a recent study investigating effects of a Roundup formulation on damselfly larvae concluded that "the toxicity of Roundup cannot be fully attributed to the surfactant POEA" and that "Roundup® […] contains POEA as surfactant". This is not accurate as not all Roundup formulations contains a POEA surfactant and that the manufacturer company Monsanto has moved away from the use of POEA-based surfactants in their latest generation of Roundup formulations. The authors of this study do not indicate which commercial formulation of Roundup they have tested on damselfly larvae, and thus it is unknown if POEA was present. Therefore, although they present interesting results on the toxicity of Roundup on an environmental toxicity indicator organism, at the same time they bring confusion to the field by concluding that their study "confirms the toxicity of the surfactant POEA".

Séralini and colleagues have conducted the most extensive study on the composition and toxicity of the different ingredients that constitute glyphosate-based herbicides. They have compared the toxicity of different brands of glyphosate-based herbicides in tissue culture cell assays and showed that several commercial formulations were up to 1,000 times more toxic than glyphosate alone, the regulated active ingredient. In addition, adjuvant mixtures generally contain several ingredients, and these can sometimes be mixtures themselves. For example, POEAs are mixtures of diethoxylates of tallow amines with different toxicological properties, which are characterized by their oxide/tallow amine ratio. The toxicity of POEA increases as the tallow amine chain is shortened. The most commonly used POEA is POE tallow amine (POE-15), which was used in the first formulations of glyphosate commercialized under the trade name "Roundup." By using cell culture model systems, Séralini and colleagues demonstrated that the toxicity of glyphosate-based formulations was proportional to their concentration of POE-15 or other ethoxylated surfactants. The formulations that did not contain ethoxylated surfactants were approximately 100 times less toxic to human cells. It was quite a surprising finding to see that the toxicity of two formulations of the same active ingredient could differ by a factor of 100. Thus, the consumer could buy one or another glyphosate-based herbicide formulation without being aware of this difference in toxicity.

The study by Séralini and colleagues based on tissue culture cell lines clearly has its limitations, including exacerbating the observed differential toxicity profiles of the formulations tested. However, the findings of greater toxicity of commercial formulations over glyphosate alone *in vitro* have been replicated *in vivo* in laboratory animals, other animal model systems such as sea urchins, microorganisms (bacteria, microalgae, protozoa), and crustaceans. A more recent study on two life stages of the Pacific oyster shows that POEA-based adjuvants can be very toxic to embryonal and larval development (EC50, 262 µg/L). Metamorphosis tests revealed that although EC_{50} values exceeded 100,000 µg/L for glyphosate and its metabolite ami-nomethylphosphonic acid, they were as low as ~6,000 µg/L for some commercial formulations. Studies have also revealed that some ethoxylated adjuvants can be endocrine disruptors at lower non-toxic concentrations. Recently, it has been reported that POEA-based adjuvants promote triglyceride accumulation in 3T3-L1 adipocytes at concentrations from 0.1 to 10 µM. This is in contrast to glyphosate alone, which did not promote lipid accumulation in this same adipocyte cell line (Mesnage and Antoniou, unpublished results). Another study has shown that ethoxylated adjuvants can inhibit aromatase activity disrupting estrogen–androgen balance.

Table 1. Overview of adjuvants used in pesticide formulations.

Adjuvant type	Example
Penetration agents	Petroleum or mineral oils, vegetable oils, organosilicon
Oder masking agent	1-octanal
Dyes	fd&c blue no. 1, fd&c red no. 40
Preservatives	Hexamethylenetetramine, potassium benzoate, sorbic acid
Stabilizer	Diisopropanolamine, hydroxyethylidene diphosphonic acid, silver nitrate
Diluents	Aluminum hydroxide
Surfactants	Anionic: alkylbenzene sulfonates, sodium laureth sulfate, soap Cationic: dioctadecyldimethylammonium chloride Amphoteric: cocamidopropyl betaine, cocamidopropyl hydroxysultaine Non-ionic: alkoxylated alcohol, ethoxylated alcohol, nonylphenol ethoxylate, tallow amine ethoxylate, alkyl polyoxyethylene ether
Emulsifers	Alkanoic and alkenoic acids, monoesters and diesters of α-hydro-ω-hydroxypoly (oxyethylene), glyceryl monostearate, sodium metasilicate
Propellant	1,1-Difluoroethane, butane, propane
Solvents	*N*-methyl-2-pyrrolidone, polychloromethanes, chlorinated volatile organic compounds, xylene, isopropanol
Antifoaming agent	silicones (e.g., dimethylpolysiloxane), fatty acids
Carriers	Biochar, cyanobacteria, clay minerals, siliceous minerals, zinc-layered hydroxide, polymeric materials such as chitosan, lignin, and poly(ethylene) glycol

This non-exhaustive list presents compounds grouped by category that are classically used as adjuvants in commercial pesticide formulations. Some of these molecules can have dual roles. For instance, surfactants (wetters) are also used as plant penetration agents.

A comparison of the effects of a glyphosate-based herbicide and glyphosate at an equivalent concentration of 25 mg/kg/day on the composition and metabolism of the gut microbiome in Sprague-Dawley rats found that the commercial formulation but not glyphosate alone affected the numbers of observed species in both the cecum and the colon. Although glyphosate has been patented as an antiparasitic agent and suggested to be a bacterial antibiotic (US patent number: US7771736 B2), it is likely that the effects of glyphosate-based herbicides on the gut microbiome could be due to the damaging properties of surfactants present in the adjuvant mixture on the integrity of the intestinal epithelium. In support of this possibility is the observation that emulsifiers have been shown to alter gut microbiome composition in mice by sweeping the lining of the gut, which consequently gave rise to colitis and metabolic syndrome.

More recently, it was shown that the chronic (2 years) administration of a glyphosate-based herbicide (Roundup) induced liver toxic effects in rats at an environmental concentration and daily intake of active ingredient was declared safe by regulatory agencies worldwide. However, further research is required to elucidate whether the glyphosate, the adjuvants, or the combination of the two is at the basis of the observed kidney and especially liver toxicity seen in these animals. It is difficult to attribute the toxicity of a commercial formulation to a given ingredient if they are not tested in parallel in an experiment. Glyphosate-based herbicides can not only contain POEA but also contain multiple adjuvants having intrinsic toxicological properties. These formulations can also include methylchloroisothiazolinone having allergenic properties, light aromatic petroleum distillates having liver toxic effects, or sodium o-phenylphenate considered as possibly carcinogenic to humans.

This and other work has led the European Commission to recommend a ban on the use of POEA-type adjuvants in glyphosate-based herbicide products. Although this can be seen as a positive step forward for public health, this does not exclude the use of POEA in other non-glyphosate-based products. For example, in France, 126 formulations of glyphosate were removed from the market in July 2016, but other POEA-containing pesticides can still be bought. In addition, French farmers can still source POEA as a separate adjuvant mixture (product name Regain, authorization 9300433) to mix with a glyphosate formulation in the spray tank (https://ephy.anses.fr/adjuvant/regain). POEA is also still authorized as a co-formulant in pesticide formulations containing other ingredients such as 2,4-D. Thus, farmers and the general public can still readily be exposed to POEA despite it being banned in glyphosate-based herbicides.

Furthermore, the finding that POEA is widely found in fields in the United States where glyphosate-based herbicides are applied raises concerns that this and other classes of pesticide adjuvants may be entering the food and feed chain undetected, with as yet unknown health consequences.

Discussion and conclusion

The study of the effects of chemical mixtures on health indicators is frequently aired as a priority for the field of toxicology in the twenty-first century. However, within this framework, ignoring the toxicity of the combination of each active ingredient with its adjuvants could lead to misrepresentations of the safety profile of commercial pesticides. Therefore, it is recommended the following actions to protect the public from toxicity that may arise from ingestion of adjuvants:

1. Biomonitoring of different human population groups to identify the true body burden of adjuvant classes of chemicals.
2. Surveying of food products to accurately identify sources of exposure.
3. Long-term laboratory animal toxicity studies comparing commercial formulations with their active principle to measure adverse outcomes stemming from the adjuvants.
4. The gaps in knowledge and consequent uncertainties in risk assessment concerning the toxicity of chemical mixtures, including adjuvants, need to be acknowledged by regulators. Thus, an additional safety factor needs to be added when calculating MRL and ADI values.
5. All ingredients used in the manufacture of commercial formulations of pesticides should be subjected to the same risk assessment. The classification as inert or active has no scientific basis.

Given the all-pervasive nature of adjuvants in products used in both an agricultural and urban/domestic environment, potential toxicity arising from exposure to these chemical mixtures can be greater than from any pesticide active principle. Although considering that all chemicals have intrinsic toxicological properties and that hazardous chemical properties do not necessarily translate into a risk for human health, it is scientifically not sound to argue that adjuvants are so safe that they can be ignored. The implementation of the above recommendations will allow major progress to be made in protecting the environment and general human population from these toxicants. Current practices in risk assessment and regulation fall far short of providing such protection.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This review article is presenting information on unverified effects of a small subset of co-formulants in some Glyphosate-based formulations, with an emphasis on POEA. This co-formulant is a surfactant banned in the EU glyphosate-based products.

Literature review section reaffirms low toxicity of the active ingredient compared with formulated products. No new data presented.

1. Information on the study

Data point:	CA 5.8.3
Report author	Mesnage R. *et al.*
Report year	2017
Report title	Evaluation of estrogen receptor alpha activation by glyphosate-based herbicide constituents
Document No	Food and Chemical Toxicology (2017) Vol. 108, 30-42
Guidelines followed in study	None
Deviations from current test guideline	NA
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable without restrictions

2. Full summary of the study according to OECD format

In this study, the estrogenic potential of glyphosate, commercial GBHs and polyethoxylated tallowamine adjuvants present as co-formulants in GBHs was evaluated. Glyphosate (≥10,000 μg/L or 59 μM) promoted proliferation of estrogen-dependent MCF-7 human breast cancer cells. Glyphosate also increased the expression of an estrogen response element-luciferase reporter gene (ERE-luc) in T47DKBluc cells, which was blocked by the estrogen antagonist ICI 182,780. Commercial GBH formulations or their adjuvants alone did not exhibit estrogenic effects in either assay. Transcriptomics analysis of MCF-7 cells treated with glyphosate revealed changes in gene expression reflective of hormone-induced cell proliferation but did not overlap with an ERα gene expression biomarker. Calculation of glyphosate binding energy to ERα predicts a weak and unstable interaction (-4.10 kcal mol-1) compared to estradiol (-25.79 kcal mol-1), which suggests that activation of this receptor by glyphosate is via a ligan- independent mechanism. Induction of ERE-luc expression by the PKA signaling activator IBMX shows that ERE-luc is responsive to ligand-independent activation, suggesting a possible mechanism of glyphosate-mediated activation.

Materials and Methods

Chemicals - Glyphosate used was the PESTANAL® analytical standard (≥98.0%) obtained from Sigma-Aldrich (UK). The batch of glyphosate (≥98.0%) purchased from AccuStandard (New Haven, CT, USA) was tested exclusively in the ERE transcription luciferase reporter gene assay. Glyphosate-based formulations available on the market were Glyphogan (France, 39-43% iso-propylamine salt of glyphosate, 13-18% of POEA), Roundup Grand Travaux Plus (France, 450 g/L of glyphosate, 90 g/L of ethoxylated etheralkylamine), Roundup Original DI (Brazil, 445 g/L of glyphosate diammonium salt, 751 g/L of other ingredients) and Roundup Probio (UK, 441 g/L of the potassium salt of glyphosate, and other ingredients). POEA was purchased from ChemService (West Chester, PA, USA). The agricultural spray adjuvant was Gen-amin T200 (France, 60-80% of POE-15).

Cell culture - MCF-7, MDA-MB-231 and T47D cell lines were obtained from Prof Joy Burchell (Research Oncology Department, King's College London). T47D-KBluc cells were purchased from the American Type Culture Collection (ATCC, Teddington, UK) and harbour a stably integrated copy of a luciferase reporter gene under control of a promoter containing ERE. All cells were grown in a maintenance medium. Stock solutions of glyphosate, glyphosate-based herbicide formulations, POEA and Genamin T200 surfactant formulation were prepared in serum-free medium and adjusted to pH 7.2. Stock solutions of with estradiol, 3-isobutyl-1-methylxanthine (IBMX) and BPA were prepared by dissolution in ethanol. The solutions for testing were prepared by dilution of the stock solutions in test medium taking care that the solvent concentrations were always kept below 0.5% for the cell assays and below 0.0005% for transcriptome profiling. Cells were released from the flask substrate with trypsin and counted with a hemocytometer prior to seeding. A 24-hour recovery period was allowed for cell adherence in DMEM maintenance medium before cultures were subjected to the desired tests.

E-screen assay - The E-screen allows the determination of estrogenic effects by measuring ER-mediated cell proliferation in hormone responsive cells (MCF-7, MDA-MB-231, T47D). Cells were seeded into 48-well plates at a density of 20,000 cells per well in 250 µL maintenance medium. Following a 24-hour incubation to allow cell attachment, the medium was changed to the medium containing the test compounds. The test medium was refreshed after 3 days. Following another 3-day period of incubation, an MTT assay was performed. The MTT test allows the measurement of cytotoxic effects since the activity of mitochondrial dehydrogenase enzymes indirectly reflects cellular mitochondrial respiration. Cells were incubated with 250 µL of MTT solution for 2 hours. The test was terminated by lysing the cells with dimethyl sulfoxide (DMSO) and optical density was measured at 570 nm using the SPECTROstar Nano plate reader. The proliferative effect was expressed as a percentage of the control cell culture receiving no treatment.

ERE-luciferase reporter gene assay - The ERE-mediated transcription of a luciferase reporter gene was determined in T47D-KBluc cells using the Steady-Glo® luciferase assay system following the manufacturer's instructions. T47D-KBluc cells were seeded in 96-well plates at a density of 20,000 cells per well in 50 µL of maintenance medium and allowed to attach overnight. Prior to adding the test substance an initial 24-hour incubation was performed in the absence of test substance to improve residual estrogen clearance and assay sensitivity. After incubation, Steady-Glo® luciferase reagent was added. The plates were left to stand for 10 minutes in the dark at room temperature to allow cell lysis. Bioluminescence was measured using the Orion II microplate luminometer. ER-mediated gene activation was confirmed by ascertaining if the observed effects were subject to inhibition by addition of the estrogen antagonist ICI 182,780.

Microarray gene expression profiling - MCF-7 cells were seeded into 96-well plates with maintenance medium at a density of 20,000 cells per well. After steroid deprivation in hormone free medium, the cells were treated with test substance in triplicate in three independent experiments. RNA extraction was performed using the Agencourt RNAdvance Cell V2 kit according to the manufacturer's instructions. The samples were checked for RNA quality and quantified. Subsequently, technical replicates of samples, were pooled appropriately such that the final input amount of each biological replicate was 3 ng. Transcriptome gene expression profiles were determined using the Affymetrix Human Transcriptome 2.0 Array. Data were imported and normalized together in Omics Explorer 3.0 using the Robust Multi-array Average (RMA) sketch algorithm. These microarray data were submitted to Gene Expression Omnibus (NCBI) and are accessible through accession number GSE86472.

RNA-sequencing gene expression profiling – Gene expression profiling was performed by applying Illumina sequencing by synthesis technology. The RNA-seq data were submitted to Gene Expression Omnibus (NCBI) and are accessible through accession number GSE8770.

Statistical analysis - The ERE transcription luciferase reporter gene assay and the E-Screen assay were performed 4 and 6 times in triplicate, respectively. The concentrations required to elicit a 50% response (AC_{50}) were determined using a nonlinear regression fit. For the transcriptome analysis, pair-wise comparisons were performed using a t-test controlling for batch effects in Omics Explorer 3.0. Affymetrix microarray and RNA-seq gene expression profiling were performed 3 times in triplicate. Data used for the functional analysis were selected at cut off p-values of <0.05 with fold change >1.2 to evaluate the ER activation signature. Gene and disease ontology were analyzed using the Thomson Reuters MetaCore Analytical Suite recognizing network objects (proteins, protein complexes or groups, peptides, RNA species, compounds among others). The p-values are determined by hypergeometric calculation and adjusted using the method of Benjamini and Hochberg.

Results

E-screen assay - The AC_{50} of 17β-estradiol and BPA in MCF-7 human breast cancer cells was 0.0013 µg/L and 46 µg/L, respectively. Glyphosate induced cell proliferation starting between 1,000 and 10,000 µg/L and peaking around 1,000,000 µg/L. Similar but less pronounced results were observed with the T47D cell line with cells retaining a response to glyphosate but not to Roundup Probio. No proliferative

effects were observed in the ER-negative, hormone-independent MDA-MB-231 cell line, suggesting that the proliferative effects were mediated via the ER. Roundup Probio, tested in MCF-7 cells induced a non-statistically significant trend of cell proliferation .

ERE-luciferase reporter gene assay – Glyphosate stimulated ERE-mediated transcription of the luciferase reporter gene starting at a concentration of 1,000 µg/L. Roundup Probio did not show ER activity at a glyphosate equivalent concentration, which induced a cell proliferative effect when glyphosate is tested alone. This may be explained by the potentially higher toxicity of the glyphosate-based formulation, which could have resulted in cell death at the higher concentrations tested. Two other commercial formulations, Roundup Original DI and Roundup Grand Travaux Plus, also gave negative results. To evaluate whether the luciferase reporter gene stimulatory effects observed with glyphosate were mediated through the ER, experiments were performed with the addition of the potent ER antagonist ICI 182,780. This antagonist was effective at suppressing ER activation induced by 0.001 and 0.01 µg/L but not at 0.1 µg/L of 17β-estradiol. The addition of ICI 182,780 effectively blocked the stimulatory effects of glyphosate at 2,000-20,000 µg/L, confirming its agonist-like mode of action.

Microarray gene expression profiling - MCF-7 cells treated for 48 hours with glyphosate, Roundup Probio, POEA, or bisphenol A, and 17β-estradiol were subjected to full transcriptome profiling. The analysis of gene ontology confirms that genes having their expression altered by treatment of MCF-7 cells with 10,000 µg/L glyphosate were involved in cell cycle regulation, as well as in stimulation by steroid hormones. The transcriptome of glyphosate-treated cells was also reflective of cell death through apoptosis. Roundup ProBio was assessed at the glyphosate equivalent concentrations of 1 µg/L (environmental level), 100 µg/L and 1000 µg/L (showing a cell proliferative trend). The statistical analysis of differential expression showed that genes having their function altered by Roundup or POEA had low fold changes). No genes whose expression was increased or repressed by POEA showed fold changes higher than 2. The study of transcriptome profiles shows that POEA alone is unlikely to have estrogenic effects. This was confirmed in that none of the treatments exhibited statistically significant correlations to the ER biomarker

RNA-sequencing gene expression profiling - In order to confirm endocrine disturbances provoked by glyphosate, RNA extracted from MCF-7 cells treated with glyphosate (10,000 µg/L), estradiol (0.27 µg /L) or bisphenol A (80 µg/L) for 48 hours were subjected to a full RNA-Seq analysis using the Illumina sequencing platform. Although their gene expression profile were different, MCF-7 cells treated by these 3 chemicals presented alterations reflecting a response to steroid hormones and a modulation of cell proliferation, although the significance of the overlapping genes and those in these pathways was lower for glyphosate than the other compounds. Overall, the RNA-seq method was more sensitive and identified 2-3 times more genes whose expression was significantly altered compared to the microarray approach. A total of 5102, 2939 and 1083 genes had their expression significantly disturbed by estradiol, BPA and glyphosate, respectively. Afterwards the ER gene expression biomarker was applied to see if ERα agonist effects could be detected after transcriptome profiling using RNA-Seq platforms. The results were similar to those obtained using the microarray data. Glyphosate failed to pass the threshold of significance for ERα activation. Although the RNA-seq platform was able to identify more statistically significant genes than microarrays, the genes altered differed between the two methods.

Molecular dynamics simulation and ONIOM binding energy calculations - The ability of glyphosate to bind to ERα was evaluated by molecular dynamics simulations and ONIOM calculations. Results from the molecular dynamics simulations of glyphosate-ER interactions reveal that glyphosate enters the active site with a large number of water molecules. The glyphosate phosphonate group interacts with ARG 394 by creating hydrogen bonds. It was noted that glyphosate is unlikely to interact with HIS 524, a residue having a pivotal role in maintaining protein structure in the biologically active agonist conformation. The results of the ONIOM binding energy assessment strongly imply that the binding of glyphosate at the active site of the receptor is weak and unstable, suggesting that glyphosate is unlikely to bind to ERα.

Discussion and Conclusions

The results of this study suggest that glyphosate is a weak activator of ERα in hormone-dependent human breast cancer cells. The glyphosate intake necessary to reach a systemic concentration representative of the estrogenic effects shown in this study would only be encountered in cases of extreme exposures (incidental ingestion, mishandling). An evaluation of the glyphosate binding energy confirmed that this compound is unlikely to activate the ER. The presence of glyphosate-associated cytotoxic effects could explain the discrepancies between the results we obtained with the ERα biomarker and those from the cellular assays. It is thus plausible that glyphosate is activating ERα through a ligand-independent mechanism albeit at high concentrations. To determine any estrogenic potential of adjuvant co-formulants, a number of glyphosate-based formulations were tested and no estrogenic effects could be demonstrated. However, cytotoxicity was observed at glyphosate-equivalent concentrations lower than those required to elicit a proliferative response to glyphosate alone.

Conclusion
This study has demonstrated that glyphosate activates ERα in breast cancer cells but only at relatively high concentrations, and that this activation happens through a ligand-independent pathway. These results suggest that humans exposed to glyphosate would not exhibit ER activation at typical exposure levels.

3. Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to evaluate the possible estrogenicity of glyphosate and glyphosate-based formulations and their adjuvants. The tests performed were the E-screen using different cell lines, the ERE-luciferase reporter gene assay, microarray gene expression profiling and RNA-sequencing gene expression profiling. An increase in cell proliferation was observed in human breast cancer cells (MCF-7) at 10,000 µg/L and reached a maximum response at 1,000,000 µg/L. Similar but less pronounced results were observed with the T47D cell line. Glyphosate stimulated ERE-mediated transcription of the luciferase reporter gene starting at a concentration of 1,000 µg/L. The analysis of gene ontology confirms that genes having their expression altered by treatment of MCF-7 cells with glyphosate were involved in cell cycle regulation, stimulation by steroid hormones and cell death through apoptosis. ONIOM binding energy assessment strongly implies that the binding of glyphosate at the active site of the estrogen receptor is weak and unstable, suggesting that glyphosate is unlikely to bind to ERα.

This study has demonstrated that glyphosate activates ERα through a ligand-independent pathway only at high concentrations that are not encountered at typical exposure levels. This publication is considered relevant for glyphosate risk assessment and reliable without restrictions.

Reliability criteria for *in vitro* toxicology studies

Publication: Mesnage *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 98.0%. Source: Sigma-Aldrich (UK).
Only glyphosate acid or one of its salts is the tested substance	Y	Also glyphosate based formulations and surfactants

		were tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This study has demonstrated that glyphosate activates ERα through a ligand-independent pathway only at high concentrations that are not encountered at typical exposure levels. This publication is considered relevant for glyphosate risk assessment and reliable without restrictions.		

1. Information on the study

Data point:	CA 5.3
Report author	Milic M. *et al.*
Report year	2018
Report title	Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate
Document No	Arh Hig Rada Toksikol (2018) Vol. 69, 154-168
Guidelines followed in study	None
Deviations from current test guideline	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

In this 28 day-study, the effects of herbicide glyphosate administered by gavage to Wistar rats at daily doses equivalent to 0.1 of the acceptable operator exposure level (AOEL), 0.5 of the consumer acceptable daily intake (ADI), 1.75 (corresponding to the chronic population-adjusted dose, cPAD), and 10 mg kg-1 body weight (bw) (corresponding to 100 times the AOEL) were evaluated. At the end of each treatment, the body and liver weights were measured and compared with their baseline values. DNA damage in leukocytes and liver tissue was estimated with the alkaline comet assay. Oxidative stress was evaluated using a battery of endpoints to establish lipid peroxidation via thiobarbituric reactive substances (TBARS) level, level of reactive oxygen species (ROS), glutathione (GSH) level, and the activity of glutathione peroxidase (GSH-Px). Total cholinesterase activity and the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were also measured. The exposed animals gained less weight than control. Treatment resulted in significantly higher primary DNA damage in the liver cells and leukocytes. Glyphosate exposure significantly lowered TBARS in the liver of the AOEL, ADI, and cPAD groups, and in plasma in the AOEL and cPAD group. AChE was inhibited with all treatments, but the AOEL and ADI groups significantly differed from control. Total ChE and plasma/liver ROS/GSH levels did not significantly differ from control, except for the 35 % decrease in ChE in the AOEL and ADI groups and a significant drop in liver GSH in the cPAD and 100xAOEL groups. AOEL and ADI blood GSH-Px activity dropped significantly, but in the liver it significantly increased in the ADI, cPAD, and 100xAOEL groups vs. control.

Materials and methods

Chemicals - Glyphosate (purity of ≤100 %), purchased under the brand name PESTANAL®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany.

Animals - Thirty male Wistar rats were obtained from the breeding unit at IMROH and were kept under pathogen-free, steady-state microenvironmental conditions in polycarbonate cages with 40-60 % humidity at 22 °C and normal 12-hour light/dark cycle. The animals had free access to standard Good Laboratory Practice-certified food and tap water. At 3 months of age, the rats were weighted, inspected, and judged to be healthy and fit for the experiment by a licensed veterinarian at IMROH.

Experimental design - Rats were randomly divided into 6 dose groups of 5 animals each and treated orally by gavage for 28 days. Glyphosate was tested at 0.1, 0.5, 1.75 and 10 mg/kg bw/day, doses corresponding respectively with the AOEL from EFSA, the ADI, the chronic population-adjusted dose (cPAD) from EPA and 100 times the AOEL. Ethyl methanesulfonate (EMS) dissolved in PBS served as the positive control and was administered at 300 mg/kg bw over the last 3 days of the experiment. PBS served as the negative control. Body weight was monitored once a week and the glyphosate doses

adjusted accordingly. Survival and clinical signs of toxicity were monitored on a daily basis. All animals were humanely sacrificed and dissected on day 29, 24 hours after the last dose. All animals were subjected to necropsy and examined for gross pathological changes.

Sample collection and preparation - Liver was excised, rinsed with cold PBS (pH 7.4) and weighed. Livers were then washed in cold TBS buffer (pH 7.5) to remove as much blood as possible, homogenized in a 50 mM potassium PBS (pH 7.4) with 1 mM EDTA, and centrifuged at 20,000 x g at 4°C for 30 minutes to obtain the supernatant. In a separate procedure, a small portion of the liver tissue was minced in a chilled mincing solution to obtain a cellular suspension. The cell suspension was left a few seconds for large clumps to settle, and the supernatant was used to prepare comet slides. This was performed within 60 minutes from sacrifice to avoid confounding necrotic changes. Blood samples were collected directly from the heart into heparinized vacutainers with an extra addition of Li-heparin and mixed vigorously. Samples were then kept at 4°C pending further processing. Plasma for the biochemical assays was prepared by centrifugation of heparinized blood at 976 x g at 4°C for 10 minutes and kept at -20°C pending analysis.

Alkaline comet assay in leucocytes and liver cells - Two microgels were prepared per tissue per animal. Slides were marked with a randomly generated code. For each slide, an aliquot of 10 µL of the cell suspension was mixed with low melting point agarose (LMPA, 0.5 %) dissolved in TBS buffer. "Sandwich" agarose microgels made of four layers were prepared on microscopic slides. Slides were pre-coated with 1 % normal melting point agarose (NMPA) and air-dried. The second gel layer of 0.6 % NMPA was then applied. The third layer consisted of 0.5 % LMPA mixed with heparinized whole blood (10 µL per slide) or 10 µL of liver cell suspension per slide. Finally, 0.5 % LMPA was applied as the top layer over the gel-embedded cells. After solidification of the gel on ice-cold metal tray, the slides were submerged in freshly prepared cold lysing solution (pH 10) at 4°C overnight. The slides were quickly washed with distilled water and left in a vertical Coplin jar with chilled electrophoresis buffer (pH >13) at 4°C for 10 minutes. The slides were then transferred into a horizontal electrophoresis unit. After electrophoresis, the slides were washed 3 times with neutralization buffer (pH 7.5). All gels were dehydrated with 70 % and 96 % ethanol, respectively, air dried, and stored at room temperature. Before scoring, the slides were stained with ethidium bromide (20 µg/mL) and analyzed with a fluorescent microscope at 200x magnification using the Comet Assay IV image analysis system equipped with appropriate filters. Three hundred cells (2 x 150 nucleoids) were scored in total for each animal and sample. Medium-sized cells (parenchymal cells or hepatocytes, between 30 and 40 µm of head length) and small-sized cells (non-parenchymal cells, <30 µm of head length) were recorded separately. Areas near slide margins were not scored. DNA damage was measured as comet DNA tail intensity (% of DNA in tail) and tail length (TL, expressed in µm, measured from the estimated edge of the comet head). Comets with small or non-existing head and large, diffuse tails (cells with >80 % DNA in the tail) were excluded from analysis. The frequency of such comets ("hedgehogs" or "clouds") was determined based on visual scoring among 100 nucleoids per sample.

Determination of ROS levels in plasma and liver - ROS levels in plasma and liver homogenates were measured using 2′,7′-dichlorofluorescein diacetate (DCF-DA). The acetate group of DCFH-DA gets DCFH-DA into the cells or organelles, and once inside, it is removed by cellular esterases, producing reduced DCFH which then can be oxidized by peroxides to form fluorescent oxidized DCF that can be measured spectrophotometrically. Plasma samples and 1 % liver tissue homogenate were prepared by dilution with ice-cold PBS (pH 7.4). Black 96-well plates were filled with 0.07 mL of PBS, and 0.03 mL of 1 % liver tissue homogenate or with 0.1 mL of 10 % plasma in quintuplicate for each glyphosate concentration and sample type. 20 µL of 0.12 mM DCFH-DA dye in PBS was then added to each well, and the plates were incubated at 37°C for 30 minutes. Control for dye autofluorescence was prepared without the addition of dye. Control samples were included in each experiment. Samples were analyzed using a Victor3™ multilabel plate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The readings were expressed as relative fluorescence units (RFU).

Determination of lipid peroxidation in plasma and liver - The concentration of thiobarbituric reactive substances (TBARS), as a measure of lipid peroxidation, was determined. 5 µL of butylated hydroxytoluene (BHT, 0.2 % w/v) and 750 µL of phosphoric acid (1 % v/v) were added to 50 µL of

sample. After mixing, 250 µL of thiobarbituric acid (TBA, 0.6 % w/w) and 445 µL of water were added, and the reaction mixture was incubated at 90°C for 30 minutes. The mixture was cooled, and absorbance measured at 532 nm using a Shimadzu UV probe spectrophotometer. TBARS concentrations were calculated using the standard curves for 1,1,3,3-tetramethoxypropane, obtained by increasing its concentrations, and expressed as µM.

Determination of GSH in plasma and liver - GSH levels were analyzed with a fluorogenic bimane probe using monochlorobimane (MBCl), which reacts specifically with GSH to form a fluorescent adduct. Plasma samples and liver tissue homogenates were prepared as previously described for ROS determination. 20 µL of 0.24 mM MBCl dye in PBS was then added to react at 37 °C for 20 minutes. The amount of GSH in the samples was analyzed using a Victor3™ multilabel plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Control samples were included in each experiment. The readings were expressed as relative fluorescence units (RFU). Each sample analysis was performed in quintuplicate.

Determination of GSH-Px activity in whole blood and liver - GSH-Px activity in whole blood and in the supernatant of liver homogenate were determined in accordance with the European standardized method. The amount of GSH oxidized by t-butyl hydroperoxide was determined based on the decrease in β-NADPH absorbance at 340 nm, measured by spectrophotometry. One unit of GSH-Px was the number of micromoles of β-NADPH oxidized per minute. Its activity in whole blood was expressed per gram of haemoglobin (U g_{Hb}^{-1}), and in liver homogenate per gram of total protein (U $g_{protein}^{-1}$).

Protein quantification - Proteins were quantified according to the method of Bradford, using bovine serum albumin as the standard.

Cholinesterase activity in plasma - Plasma samples were analyzed for total ChE, AChE, and BChE activities in a 0.1 M sodium phosphate buffer (pH 7.4) at 25°C using ATCh (1.0 mM) and DTNB (0.3 mM) as described by Ellman *et al*. To distinguish between AChE and BChE activities the BChE-selective inhibitor ethopropazine (20 µM) was used. Increase in absorbance was monitored at 412 nm over 4 minutes by means of a Cecil 9000 spectrophotometer. Enzyme activities were expressed as IU g/protein.

Statistical analysis - Statistical analysis was run on Dell Statistica software STATISTICA, version 13.2. The data were first evaluated with descriptive statistics. The results were expressed as means ± standard deviation, and for the comet assay also medians and ranges (min-max) were used. Relative liver weights were logarithmically transformed and analyzed with one-way ANOVA. For pairwise organ comparison the *post-hoc* Tukey's HSD test was used. Normality was tested with the Levene's test. Since the results of the alkaline comet assay were not normally distributed even after logarithmic transformation, the non-parametric Mann-Whitney U test was used. For multiple comparisons of cholinesterase activities, TBARS, and GSH-Px activities between the glyphosate and control groups the Kruskal-Wallis test was used. ROS and GSH levels were compared between the groups using the non-parametric Mann-Whitney U test. P values of ≤ 0.05 were considered statistically significant.

Results and Discussion

Changes in body and liver weight - No premature death or any clinical signs of systemic toxicity in male adult Wistar rats was observed in any of the groups dosed with glyphosate for 28 days. Gross necropsy did not reveal any treatment-related findings. All glyphosate-treated animals showed a similar weight gain throughout the 28-day treatment period, with significant difference between the day before treatment and at the end of treatment. There were no statistically significant changes in relative liver weight.

Alkaline comet assay in leucocytes and liver cells - Glyphosate-treated rats had higher primary DNA damage in leukocytes when compared to control in tail length and tail intensity. While tail length was significantly greater at all dose levels, only the lowest dose tested resulted in significantly higher mean tail intensity. One reason for that could be high standard deviations. It is worth noting that glyphosate

caused greater DNA damage in liver cells than in the leukocytes. The greatest liver cell DNA damage (as tail intensity) occurred at the lowest and the highest dose both in parenchymal and non-parenchymal liver cells. In fact, the group exposed at 10 mg/kg bw/day suffered even greater damage in medium-sized liver cells than the positive control.

ROS levels in plasma and liver - No statistically significant difference was found in ROS levels in plasma between the glyphosate dose groups and control. A small decrease in ROS of around 7 % was seen in the groups with the highest glyphosate doses. Liver tissue showed a similar pattern with a decrease in ROS at the highest dose levels which was statistically significant at 10 mg/kg bw/day. ROS levels in the liver were 100 times greater than in the plasma.

Lipid peroxidation in plasma and liver - TBARS concentrations in plasma and liver were decreased in all glyphosate-dosed groups when compared to control.

GSH levels in plasma and liver – In plasma, no statistically significant difference was evident between the glyphosate-dosed groups and the control group. In the liver, however, a statistically significant decrease in GSH was seen in the two highest dose groups.

GSH-Px activity in whole blood and liver - GSH-Px activity in whole blood was statistically significantly decreased at 0.5 and 1.75 mg/kg bw/day but not at 10 mg/kg bw/day. In contrast, GSH-Px activity in the liver was statistically significantly increased at 0.5, 1.75 and 10 mg/kg bw/day.

Cholinesterase activity in plasma - Glyphosate did not significantly affect total cholinesterase (ChE) activity, even though there was a decrease about 35 % at 0.1 and 0.5 mg/kg bw/day when compared to control. Acetyl cholinesterase (AChE) activity in turn, was statistically significantly decreased at 0.1 and 0.5 mg/kg bw but not at the higher dose levels. There was no statistically significant change in activity of butyl cholinesterase in the glyphosate dose groups when compared to the control group.

Discussion and conclusion

This study suggests that sub-chronic exposure to glyphosate mostly affects DNA in the liver and white blood cells. General oxidative stress was not confirmed, while total cholinesterase activity showed some, but inconsistent, changes from control. Exposure to environmentally relevant levels of glyphosate, presumably not harmful to humans, seems to have different effects from exposure to much higher doses, especially where oxidative stress is concerned. In this study it has been demonstrated that even without evidence of oxidative stress, small doses allowed for human exposure can produce significant primary DNA damage and inhibit AChE, which may both be related to indirect action through glycine substitution.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Glyphosate was orally administered to male rats at 0.1, 0.5, 1.75 and 10 mg/kg bw for 28 days to investigate its effect on DNA damage, oxidative stress and cholinesterase activity. The endpoints of this study were DNA damage as measured in the alkaline comet assay, ROS in plasma and liver, lipid peroxidation in plasma and liver, GSH in plasma and liver, GSH-Px activity in whole blood and liver and total cholinesterase, acetyl cholinesterase and butyl cholinesterase activity in plasma. The results of the alkaline comet assays revealed a statistically significant increase in tail length and tail intensity in leucocytes and small and medium sized liver nuclei. With the exception of tail length of small sized liver nuclei no dose effect relationship was evident. Tail intensity of the leucocytes could not be assessed because of the very high variability of the results. From the results of the oxidative stress markers in plasma and liver and cholinesterase activity in plasma it can be concluded that there was no dose related effect.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the high variability of the results of the comet assay, although the conduct of this assay was in general compliant with OECD test guideline 489.

Reliability criteria for *in vivo* toxicology studies

Publication: Milic *et al*., 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	*In vivo* Comet assay was compliant with OECD TG 489.
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 ≤100 %). Source: PESTANAL®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	0.1, 0.5, 1.75 and 10 mg/kg bw/day, positive and negative control included.
Number of animals used per dose level reported	Y	5 males per 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	Limited to body weight, organ weight, DNA damage, oxidative stress and ChE activity.
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the high variability of the results of the comet assay, although the conduct of this assay was in general compliant with OECD test guideline 489.		

1. Information on the study

Data point:	CA 5.4
Report author	Nagy K. *et al.*
Report year	2019
Report title	Comparative cyto- and genotoxicity assessment of glyphosate and glyphosate-based herbicides in human peripheral white blood cells
Document No	Environmental Health (2018) Vol. 17, pp. 52/1-52/13
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

This study investigates the cyto- and genotoxic potential of the active ingredient glyphosate and GBHs in human mononuclear white blood (HMWB) cells. HMWB cells were treated for 4 h at 37 °C with increasing concentrations (1–1000 μM) of glyphosate alone and in three GBHs (Roundup Mega, Fozat 480 and Glyfos) to test cytotoxic effect with fluorescent colabelling and genotoxic effect with comet assay. In addition, each concentration was tested with and without metabolic activation using human liver S9 fraction.

Materials and methods

Chemicals; Analytical-grade glyphosate (N-(phosphonomethyl) glycine, CAS No: 1071-83-6) was purchased from VWR International Kft (Debrecen, Hungary) and samples of three GBHs, i.e.

• Roundup Mega containing 551 g/L or 42% (w/w) potassium salt of glyphosate (CAS No: 70901-12-1; equivalent to 450 g/L glyphosate) and 7% (w/w) ethoxylated etheralkylamine (CAS No: 68478-96-6);

• Fozat 480 containing 480 g/L or 41% (w/w) isopropylammonium salt of glyphosate (CAS No: 38641-94-0; equivalent to 360 g/L glyphosate) and < 5% (w/w) hygroscopic substances;

• Glyfos containing 480 g/L or 42% (w/w) isopropylammonium salt of glyphosate (equivalent to 360 g/L glyphosate) and 9% (w/w) polyethoxylated tallow amine (CAS No: 61791-26-2);

were kindly provided by pesticide applicators. Composition data for each formulation were retrieved from the material safety data sheets (MSDS). Chemicals used for the assays and human liver-derived metabolic activation system (S9 fraction) were obtained from Sigma-Aldrich Chemie GmbH (Heidelberg, Germany). Cell culture medium and its supplements were obtained from Biowest (Nuaillè, France). The acetomethoxy derivative of calcein (Calcein AM) and propodium iodide (PI) fluorescent dyes were purchased from Biotium (Hayward, CA, USA). Heparin-containing vacutainers were purchased from BD Vacutainer Systems (Plymouth, UK).

Cell cultures; Human peripheral whole blood samples were obtained by venipuncture and collected into heparin-containing vacutainer tubes from four non-smoking, healthy volunteers (three males and one female, aged 20–40 years) without known previous contact with pesticides, mutagens or carcinogens. Cultures were prepared within 1 h of phlebotomy. Human mononuclear white blood (HMWB) cells were prepared from erythrocytes by density-gradient centrifugation over Histopaque-1077 gradient. The buffy coat was aspirated and re-suspended in RPMI 1640 medium containing 10% foetal calf serum (FCS).

Cell treatment; HMWB cells were treated in the cell-culture medium with increasing concentrations (1 μM, 10 μM, 100 μM, 250 μM, 500 μM, 750 μM and 1 mM) of glyphosate alone and in three GBHs in

a way that the concentrations of glyphosate in GBHs were equivalent. Concentrations were chosen based on the results from previous in vitro studies performed on human lymphocytes. The stock solutions and the dilution series were made in phosphate-buffered saline (PBS) and adjusted with 1 M NaOH to pH 7.2. Aliquots of different concentrations of glyphosate and GBH solutions, as well as PBS as negative control, were added to the cell cultures and incubated for 4 h at 37 °C. The PBS content was always < 10% (v/v) in the cell culture medium. The experiments were conducted in the presence and absence of S9 fraction. 100 µL of the working S9 mix containing 10% (v/v) of S9 fraction was composed of 8 mM MgCl2, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate, and 4 mM NADP was added to the S9+ samples. 100 µM hydrogen peroxide was used as a positive control.

Cytotoxicity assay; After treatment, aliquots of samples were immediately subjected to cytotoxicity test. Calcein AM and propidium iodide (PI) fluorescent dyes were used to colabel the cells. Calcein AM is a non-polar compound that passively crosses the plasma membrane of living cells, where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm. PI is a DNA intercalating dye, which is able to permeate membranes of dead and dying cells but cannot penetrate plasma membranes of live healthy cells. Both fluorescent dyes were dissolved in PBS (pH 7.2) to a final concentration of 2 µM each. 200 µL of this working solution added to the cell pellets (1 × 105 cells), and incubated for 30 min at 4 °C, protected from light. The labelled cells were washed and re-suspended in ice-cold PBS buffer. 40 µL of the cell suspension was put on a microscope slide for immediate microscopic examination at 100x magnification using a Zeiss Axioplan epifluorescent microscope. FITC filter for Calcein AM and TRITC filter for PI was applied to excite the colabelled cells. Survival rate was determined by visual examination of 10 randomly selected non-overlapping fields per slide. Each field contained 10 to 30 images. Cell viability was expressed as the mean of the percentages of living cells from repeated experiments. The proportions of living cells observed in technical replicates were subjected to statistical analysis.

Genotoxicity assay; The alkaline version of the comet assay was performed according to the methodology of Collins (2004). Following treatment, samples were centrifuged and HMWB cells were resuspended in serum-free medium at a cell density of 2000 cells/µL. Degreased frosted slides were coated with two layers: 1% normal melting point agarose (NMA) covered with 0.75%, low melting point agarose (LMA) containing the cells (~2 × 105 per slide). After solidification, the embedded cells were lysed (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris base, pH 10, 1% sodium N-lauroyl sarcosinate and 1% Triton X-100 added fresh) at 4 °C for 18–20 h, shielded from light. After lysis, the DNA was allowed to unwind for 20 min in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA, pH 13) and subjected to electrophoresis in the same buffer for 20 min at 0.8 V/cm and 300 mA in a horizontal electrophoresis tank (Cleaver Scientific, Rugby, UK). Finally, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris base-HCl, pH 7.5) to remove excess alkali and detergent. After drying, each slide was stained with ethidium bromide (20 µg/ml) and stored in a humidified container at 4 °C until analysis. The fluorescence signal was detected at 400x magnification using a Zeiss Axioplan epifluorescent microscope equipped with a CCD camera connected to an image analysis system. The Comet Imager v.2.2.1. Software (MetaSystems GmbH, Germany) was used to analyse 2 × 50 randomly captured comets from duplicate slides and compute the DNA damage parameters. Percentage of DNA in the tail (tail DNA%) and tail length in µm (TL) were measured to quantify DNA damage. The results are presented as mean of the median values of DNA damage parameters from repeated experiments. The medians of technical replicates were subjected to statistical analysis.

Data analysis; Experiments were independently performed three times from three different donors. The rate of cell viability and the central values of DNA damage parameters in the comet assay induced by various concentrations of the xenobiotics in repeated experiments were statistically compared to that of untreated cells using ANOVA with Dunnett's post hoc test. To statistically analyse the effect of metabolic activation, DNA damage values were pooled from three repeated experiments to compare results from S9-treated and S9-untreated samples at each exposure concentration by using Mann-Whitney test because pooled data sets followed non-normal distribution. Statistically significant difference was accepted at 5% significance level.

Results

Cytotoxicity; The viability of HMWB cells treated with glyphosate alone was found to be over 86% in the absence and presence of S9 over the entire concentration range (Fig. 1). Regardless of metabolic activation, GBHs induced a significant decrease in the proportion of living cells from 250 μM of Round Mega and Glyfos whereas from 500 μM of Fozat 480.

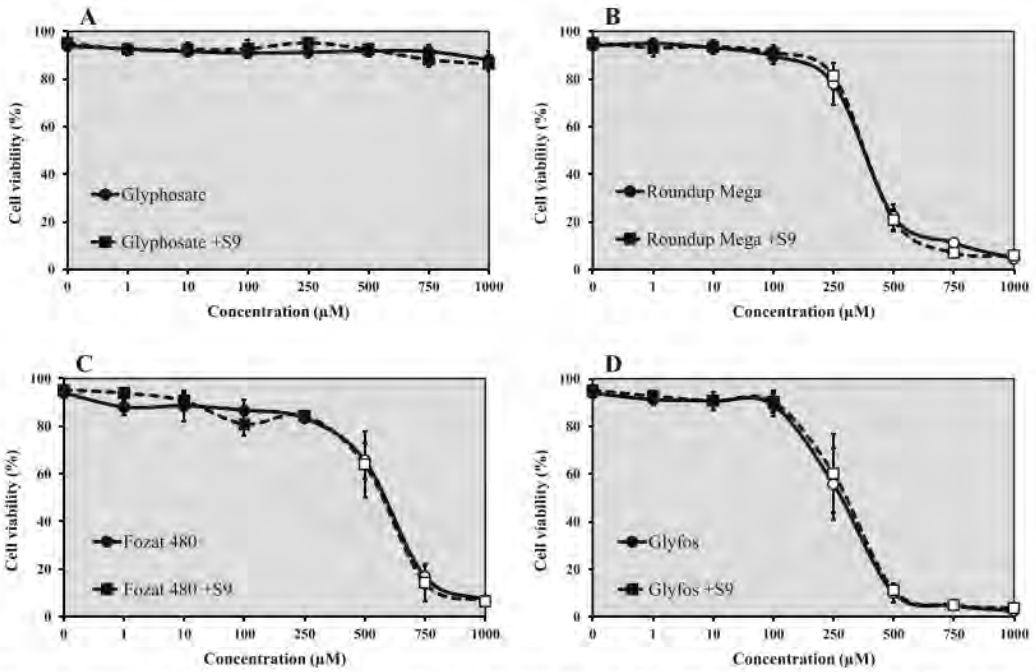

Fig. 1. Effect of 4-h exposure to increasing concentrations of glyphosate (A), Roundup Mega (B), Fozat 480 (C) and Glyfos (D) on cell viability in the absence and presence (+S9) of metabolic activation system. The data points indicate the means ± stand error of the mean (SEM) of three repeated experiments. Statistically significant decrease of cell viability, indicated by empty data points, was determined by comparing the values induced by various doses of glyphosate or GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.

DNA damage; Exposure of HMWB cells to glyphosate in the 0–1000 μM concentration range did not result in dose-dependent increase of DNA damage measured by the comet assay parameters. Unlike the active principle, GBHs induced statistically significant increase of both DNA damage parameters from 500 μM (Roundup Mega and Glyfos) and 750 μM (Fozat 480) compared to untreated cells. S9 treatment did not influence the effect of active ingredient glyphosate over the whole concentration range. On the contrary, addition of S9 to the assay modified the effects of GBHs in a non-consistent manner. The presence of metabolic enzymes significantly decreased the DNA damage induced by Roundup Mega and Fozat 480 at 1000 μM and from 10 μM, respectively. Metabolic activation could be observed only in samples exposed to 250 μM or higher concentrations of Glyfos indicated by the statistically significant differences in the tail DNA% and TL values between the S9-treated and S9-untreated cells (Tables 1 and 2).

Table 1
DNA damage induced by 4-h exposure to glyphosate and GBHs with and without metabolic activation system (S9) in HMWB cells measured as tail DNA%.

Concentration (μM)	Tail DNA% ± SEM							
	- S9				+ S9			
	Glyphosate	Roundup Mega	Fozat 480	Glyfos	Glyphosate	Roundup Mega	Fozat 480	Glyfos
0	1.51 ± 0.05				1.39 ± 0.18			
1	2.81 ± 0.30	2.61 ± 0.39	2.27 ± 0.60	1.51 ± 0.41	2.90 ± 0.49	1.91 ± 0.60	1.60 ± 0.05	3.64 ± 2.33††
10	2.86 ± 0.28	2.97 ± 0.87	8.39 ± 5.37	2.08 ± 0.69	2.03 ± 0.38	1.77 ± 0.82	2.19 ± 1.10†††	0.99 ± 0.10†††
100	2.51 ± 0.52	2.49 ± 0.94	13.09 ± 9.67	1.87 ± 0.80	2.06 ± 0.57	2.23 ± 0.77	1.99 ± 0.76†††	1.24 ± 0.33
250	2.42 ± 0.18	(3.47 ± 0.55)	6.59 ± 1.52	(6.11 ± 2.58)	3.01 ± 0.45	(3.58 ± 0.07)	2.97 ± 0.47†††	(22.84 ± 14.24†††)
500	2.60 ± 0.84	(16.88 ± 3.54***)	(7.47 ± 0.69)	(17.69 ± 2.06*)	2.98 ± 0.24	(13.71 ± 3.09)	(4.59 ± 0.65†††)	(36.81 ± 15.40†††
750	3.64 ± 1.00	(24.67 ± 2.56***)	(28.58 ± 1.93*)	(37.81 ± 5.92***)	3.46 ± 0.67	(25.64 ± 6.53**)	(24.20 ± 1.20***†	(50.47 ± 10.64*†††)
1000	1.57 ± 0.24	(44.75 ± 0.46***)	(45.94 ± 3.74***)	(54.58 ± 2.74***)	1.51 ± 0.16	(31.45 ± 4.14***†††)	(22.28 ± 2.40***†††)	(62.38 ± 2.99**†††)

Data are means of median values of three repeated experiments.
Data in parentheses refer to samples with significant cytotoxic response.
Statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) increase in DNA damage was determined by comparing the values of DNA damage induced by various doses of glyphosate and GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.
Statistically significant ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$) difference in DNA damage levels between S9-treated and S9-untreated cells induced by the same concentration of glyphosate and GBHs was determined by Mann-Whitney test.

Table 2
DNA damage induced by 4-h exposure to glyphosate and GBHs with and without metabolic activation system (S9) in HMWB cells measured as tail length (μm).

Concentration (μM)	Tail length (μm) ± SEM							
	- S9				+ S9			
	Glyphosate	Roundup Mega	Fozat 480	Glyfos	Glyphosate	Roundup Mega	Fozat 480	Glyfos
0	0.47 ± 0.19				0.35 ± 0.06			
1	0.66 ± 0.06	0.52 ± 0.08	0.39 ± 0.08	0.11 ± 0.08	0.60 ± 0.08	0.34 ± 0.14	0.18 ± 0.04	0.56 ± 0.45††
10	0.67 ± 0.07	0.77 ± 0.27	1.77 ± 1.09	0.29 ± 0.15	0.65 ± 0.12	0.40 ± 0.17	0.57 ± 0.26†††	0.05 ± 0.04†††
100	0.64 ± 0.13	0.56 ± 0.27	2.70 ± 1.92	0.27 ± 0.15	0.68 ± 0.15	0.42 ± 0.18	0.41 ± 0.17†††	0.17 ± 0.10
250	0.99 ± 0.22	(1.09 ± 0.14)	1.51 ± 0.12	(1.27 ± 0.38)	0.90 ± 0.25	(1.03 ± 0.25)	1.00 ± 0.32†††	(4.41 ± 2.42†††)
500	0.93 ± 0.35	(3.93 ± 1.41*)	(1.62 ± 0.12)	(5.26 ± 1.37**)	0.81 ± 0.20	(2.87 ± 0.37*)	(1.19 ± 0.23†††)	(7.31 ± 2.27*†††)
750	1.05 ± 0.22	(5.40 ± 0.76**)	(6.57 ± 0.11**)	(8.91 ± 0.97***)	0.99 ± 0.21	(4.51 ± 1.07***)	(4.61 ± 0.19***†††)	(9.55 ± 0.50**†)
1000	0.31 ± 0.08	(6.77 ± 0.41***)	(8.13 ± 0.61**)	(10.09 ± 0.39***)	0.28 ± 0.07	(5.62 ± 0.32***†††)	(3.90 ± 0.31***†††)	(10.41 ± 0.22***†)

Data are means of median values of three repeated experiments.
Data in parentheses refer to samples with significant cytotoxic response.
Statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) increase in DNA damage was determined by comparing the values of DNA damage induced by various doses of glyphosate and GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.
Statistically significant ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$) difference in DNA damage levels between S9-treated and S9-untreated cells induced by the same concentration of glyphosate and GBHs was determined by Mann-Whitney test.

Discussion

The use of glyphosate containing products both for agricultural and residential purposes continues to rise since their first introduction to the market in 1974. Today, uncountable GBHs are registered in more than 130 countries worldwide. The unrestricted application of GBHs in the past few decades has resulted in the accumulation of glyphosate residues in environmental media exposing – and also impairing the health of – aquatic and terrestrial animals, as well as humans. In this study, we measured potential differences in the cyto- and genotoxicity between the declared active ingredient glyphosate and three marketed GBHs in HMWB cells in vitro with the use of the comet assay. To examine the toxicity of potential metabolites of the selected herbicides, human liver-derived metabolic enzyme system (S9) was also applied. The cytogenetic side effects of glyphosate and GBHs in humans have received pronounced scientific coverage in the last decade, resulting in numerous in vivo and in vitro human studies, which demonstrated that glyphosate alone or GBHs have detectable genotoxic potential, while others have reached opposite conclusions. Our results indicate that glyphosate alone could not induce DNA strand breaks in HMWB cells up to 1000 μM measured with the comet assay even in the presence of metabolic activation. This finding contrasts with previous studies employing isolated human blood mononuclear cells treated with glyphosate concentrations corresponding to ours, but using different experimental settings. A study showed with comet assay that glyphosate at 0.7 μM (in vitro) caused a statistically significant increase of DNA damage in human peripheral blood lymphocytes; however, they applied longer (20 h) exposure time than used in this study, and, unlike in our experiment, cells were incubated on microscope slides embedded in agarose gel at 25 °C. A further study also observed DNA damage in the above cell type from 20.7 μM (3.5 μg/ml) and from 250 μM, respectively. Although they incubated the cell cultures under the same conditions as used in our study (for 4 h at 37 °C), they applied LMA at lower concentration (0.5%), as well as lower electrophoretic voltage (0.7 V/cm) and time (15 min). The execution of comet assay by them applying 24 h exposure time and lower electrophoretic voltage was also different from the method we used. These discrepancies in the experimental design along with the possible inter individual variability in response to genotoxic insults may explain the contradictory findings. The cytotoxic potential of the active ingredient glyphosate in the 0–1000 μM concentration range was found to be minimal, whereas formulations showed to have pronounced cell-killing activity to HMWB cells in our study. All the three GBHs resulted in substantially decreased cell viability (< 23%) from 500 μM (Roundup Mega and Glyfos) and from 750 μM (Fozat 480) concentrations. Exposure to GBHs also caused statistically significant ($p < 0.05$) increase of DNA damage from 500 μM (Roundup Mega and Glyfos) and 750 μM (Fozat 480). However, this observation cannot be explained by the direct genotoxic potential of GBHs, rather due to the high level of cytotoxic activity of the formulations, because cell death mechanisms, both apoptosis and necrosis, can produce spontaneous DNA fragmentation that can act as a confounder in the assessment of primary DNA damage in the comet assay. The high cytotoxic potential of GBHs may be attributed to the presence of other ingredients in the formulations, which has been reported in previous publications. POEA, the declared co-formulant in Glyfos, is more than 1000 times more cytotoxic than glyphosate alone, and concerns were also raised for its genotoxic potential at concentrations not causing cytotoxicity. Thus, EFSA concluded that POEA is clearly more toxic than glyphosate when tested in GBHs, and in response to this, Glyfos was withdraw from the Hungarian market in 2017. The ethoxylated etheralkylamine added to Roundup Mega has very similar toxicological properties as POEA, which is supported by the identical dose-response relationships of Roundup Mega and Glyfos, both of them containing surfactants at roughly the same concentration. The adjuvant content of Fozat 480 (< 5% hygroscopic substances) could not be determined exactly from the MSDS; however, we can suspect from the path of the cell viability dose-response that it may also contain ethoxylated surfactants similarly to the other two GBHs. It is proven that ethoxylated adjuvants can be embedded into the cell membrane disrupting its integrity and permeability and therefore increasing the bioavailability of glyphosate. In this sense, surfactants in GBHs have an indirect synergistic effect with glyphosate. Researchers concluded that DNA damage observed in HMWB cells after exposure to glyphosate, Roundup 360 PLUS or the metabolite of glyphosate (aminomethylphosphonic acid, AMPA) was not due to direct interaction of these compounds with DNA as no DNA adducts formation has been observed, but rather ROS-mediated effects induced by the chemicals leading to cell death and indirect DNA damage. Previous studies have not been able to clearly demonstrate the oxidative DNA damaging potential of glyphosate; however, GBHs produced

an increase in reactive oxygen species in both in vitro and in vivo test systems highlighting the role of adjuvants in the cytotoxic effects observed in our experiments. Despite the definite cytotoxic effect, Roundup Mega and Fozat 480 are still commercially available not only in Hungary, but also in many other European countries under various brand names. In our study, the presence of metabolic activation did not alter the cytotoxic potential of either the active ingredient glyphosate or the GBHs. By contrast, we noted decreased DNA damages in the comet assay with Roundup Mega and Fozat 480, but increased damage with Glyfos as a result of S9 treatment. In humans, the only metabolite of glyphosate is the AMPA. It has been shown that AMPA is not able to induce DNA damage below a relatively high (4.5 mM) concentration, which is in agreement with our data. The diverse response of GBHs to metabolic activation observed in the comet assay may be explained by the presence of variable adjuvants in the formulations that underwent different metabolic modifications. Our results may indicate that the metabolite(s) of POEA in Glyfos can be more toxic than the parent compound in the comet assay, but not in the cell viability assay. There is no evidence in the published literature that POEA or other surfactants, or even products which contain these adjuvants, might undergo metabolic activation, and afterwards, become able to cause cytogenetic effects. The potential metabolic transformation of adjuvants can be hypothesized only from animal experiments; however, these studies have provided contradictory findings so far. This can still be considered as a critical knowledge gap to clarify the genotoxic potential of adjuvants in common commercial formulations of glyphosate under in vivo circumstances. A complex long-term experimental animal study has recently been initiated to assess possible risks resulting from the ubiquitous exposure to GBHs, and has already provided preliminary data on Roundup-induced endocrine effects and altered reproductive developmental parameters in male and female Sprague Dawley rats at a dose level considered as “safe”.

Conclusion

This is the first study that compared toxic effects of various glyphosate-based herbicide formulations to each other and with the declared active ingredient glyphosate in isolated human mononuclear white blood cells. GBHs caused much stronger cytotoxic effect on HMWB cells in comparison to glyphosate that may be attributed to the effect of various surfactants added to the formulations or their interaction with the active ingredient glyphosate and/or with other components of GBHs. Therefore, the GBHs-induced DNA damage observed in the comet assay could be most likely explained by non-genotoxic mechanisms and cannot indicate direct DNA damaging effects of glyphosate-based herbicide formulations. Nevertheless, by applying extended exposure durations and/or other test systems, such as the cytokinesis-block micronucleus cytome assay would allow for the discrimination between the cyto- and genotoxic effects and for the determination of the possible permanent genotoxic effect of these herbicides. Furthermore, this study, for the first time, pointed out the possibility that POEA containing formulation can undergo metabolic activation which draws attention to the need for comprehensive investigation of the toxicity of formulations to confirm our results and to assess the true health risks of environmental and occupational exposures.

3. Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes a well conducted comet assay using human lymphocytes as the test system to examine the genotoxicity and cytotoxicity of glyphosate and 3 commercial products containing glyphosate. No DNA damage was induced by analytical grade glyphosate. The 3 glyphosate products induced an increase in tail intensity in the comet assay only at highly cytotoxic concentrations, non-toxic concentrations induced no DNA damage.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and no positive control was used.

Reliability criteria for *in vitro* toxicology studies

Publication: Nagy *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity, content and storage conditions are not reported. Source: VWR International Kft, Debrecen, Hungary.
Only glyphosate acid or one of its salts is the tested substance	N	Also 3 GBH tested: Roundup Mega, Fozat 480, Glyfos.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Human mononuclear white blood cells.
Test conditions clearly and completely described	Y	Comet selection criteria were not stated. It is not stated if the slides were coded prior to scoring
Metabolic activation system clearly and completely described	Y	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	1, 10, 100, 250, 500, 750 and 1000 μM.
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	No HCD so it is unknown what degree of background variation is apparent in this test system. This is exacerbated by the use of a single set of control cultures
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and no positive control was used.		

1. Information on the study

Data point:	CA 5.5
Report author	Pahwa M. *et al.*
Report year	2019
Report title	Glyphosate use and associations with non-Hodgkin lymphoma major histological sub-types: findings from the North American Pooled Project
Document No	Scandinavian Journal of Work, Environment & Health. 2019;45(6):600–609
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Not applicable for epidemiologic studies
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

In this paper a pooled reanalysis of the data from 2 published non-Hodgkin's lymphoma (NHL) case control studies was conducted: McDuffie et al. 2001 and DeRoos et al. 2003. The reanalysis sought to evaluate associations for glyphosate use and NHL overall and by histological sub-type. In addition, the pooled analysis implemented more extensive control of confounding factors than in the original publications and considered the impact of excluding pesticide information provided by next-of-kin or proxy respondents. The OR for NHL overall for ever using glyphosate was 1.4 (95% CI 1.1, 1.8). After adjustment for other pesticides, the OR was reduced to 1.1 (95% CI 0.8, 1.5). ORs for ever using glyphosate, adjusted for other pesticides, by NHL sub-type were 0.7 (95% CI 0.4, 1.2) for FL, 1.2 (95% CI 0.8, 1.9) for DLBCL, 1.8 (0.9, 3.7) for SLL, and 1.5 (95% CI 0.9, 2.6) for other NHL sub-types. Adjusted ORs excluding data from proxy respondents [supplemental table 1 in the article] were: 0.95 (95% CI 0.7, 1.3) for NHL overall, 0.7 (95% CI 0.4, 1.1) for FL, 1.1 (95% CI 0.7, 1.8) for DLBCL, 1.8 (0.0, 3.8) for SLL, and 0.96 (95% CI 0.5, 1.9) for other NHL sub-types. ORs for NHL overall were not elevated in the higher categories for years of use (> 3.5 years OR 0.9, 95% CI 0.6, 1.4) or lifetime days of use (> 7 days OR 1.1, 95% CI 0.7, 1.8) though there was an elevated OR for the category of more than 2 days of use/year (OR 1.7, 95% CI 1.0, 2.9). For NHL subtypes, consistent patterns of association across exposure metrics were not seen, with the possible exception of SLL, though SLL findings were not statistically significant. In general, exclusion of proxy respondents reduced ORs to a minor extent with the exception of the analyses for other NHL subtypes. For NHL overall, ever use and analyses that considered duration of use per se or lifetime days of use did not show a relationship with glyphosate. There was a moderate association seen for glyphosate use for the metric > 2 days/year. There was some limited evidence of an association between glyphosate use and SLL across exposure metrics, but the small number of SLL cases resulted in very imprecise OR estimates as evidenced by wide 95% CIs. The major limitation of the study is possible case recall bias in the reporting of prior pesticide use.

Materials and methods

Study population and exposure assessment

Pahwa et al. pooled data from case control studies in the US and Canada. For NHL specifically, this study is essentially a reanalysis of the published studies by McDuffie et al. (2001) in Canada and DeRoos et al. (2003) in the US. Case identification in the US was through cancer registries and hospitals during the 1980s in four US states (Iowa/Minnesota, Kansas, and Nebraska) and between 1991 and 1994 in six Canadian provinces (Quebec, Ontario, Manitoba, Saskatchewan, Alberta, and British Columbia). Methods for each study have been previously described. For this pooled analysis, the original histology

codes were revisited and classified according to a single scheme [International Classification of Diseases for Oncology version 1 (ICD-O-1)].

Participants, or their proxies, provided information about demographic characteristics, pesticide use, agricultural exposures, and exposure to other known or suspected NHL risk factors, including lifestyle and medical and occupational history. Self-reported glyphosate use was examined using several exposure metrics: ever/never, duration (years used), frequency (days/year handled), and lifetime-days (number of years used multiplied by number of days/year handled). Categories were created for duration, frequency, and lifetime-days analyses based on the median of glyphosate used/handled among controls. Some participants had missing data for duration and frequency of glyphosate use despite reporting that they had ever used glyphosate. In duration and frequency analyses, values for missing data were assigned to cases and controls based on the median duration or frequency of reported glyphosate use among controls by state/province and 10-year age group (simple imputation) and were used for the main analyses. Ordinal analyses and associated trend tests were conducted to determine possible changes in association for increasing increments of every five years, five days/year, and ten lifetime-days of glyphosate use.

Statistical analyses

Unconditional multiple logistic regression was performed using the LOGISTIC procedure of the SAS 9.4 statistical software package (SAS Institute, Cary, NC, USA) to calculate ORs and 95% CIs for associations between glyphosate exposure metrics (ever/never, duration, frequency, lifetime-days, and as ordinal variables) and NHL overall and by histological sub-type [diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), small lymphocytic lymphoma (SLL), and other]. Initial logistic regression models (OR) contained the following variables: age [age at diagnosis (cases); age at interview or death (controls)], state/province, sex, lymphatic or hematopoietic cancer in a first-degree relative, response by a proxy, and use of any personal protective equipment (PPE). Pesticides that were correlated with glyphosate use in the pooled data and that had previously been associated with NHL based on the individual case-control studies, specifically 2,4-dichlorophenoxyacetic acid, dicamba and malathion, were included in the more fully adjusted logistic regression models (OR). The former models will be referred to as crude and the latter models as adjusted.

Trends for duration, frequency, and lifetime-days of glyphosate use and NHL ORs were assessed by the asymptotic Cochran-Armitage trend test. Subjects who never used glyphosate were the reference group for all analyses. There was a small proportion of subjects (N=175, 2.6% of all participants) with missing age values. These were imputed using simple imputation based on state/province- and case/control-specific means of age rounded to the nearest whole number. Sensitivity analyses were conducted by excluding proxy respondents from the main analyses.

Ethics approval and consent to participate

Ethics approval for the pooled analysis was obtained from the University of Toronto Health Sciences Research Ethics Board (#25166) and an exemption was obtained from the US National Institutes of Health Office of Human Subjects Research (#11351). Investigators of individual studies received human subjects' approval from their institutions for each study prior to collection of data.

Results

Characteristics of NHL cases and controls

A total of 1690 NHL cases and 5131 controls was available for analysis – 69.6% of the cases and 70.6% of the controls were from the US studies. All NHL cases and controls, including those with proxy respondents, were included in analyses of ever/never glyphosate use. For assessments involving duration of use, 1520 cases and 4183 controls were included. For frequency and lifetime-days analyses, 898 cases and 2938 controls were included. The numbers of cases and controls available for the sensitivity analysis excluding proxy respondents were smaller (Figure 1). Characteristics of NHL cases and controls, including histological sub-types, are presented in Table 1.

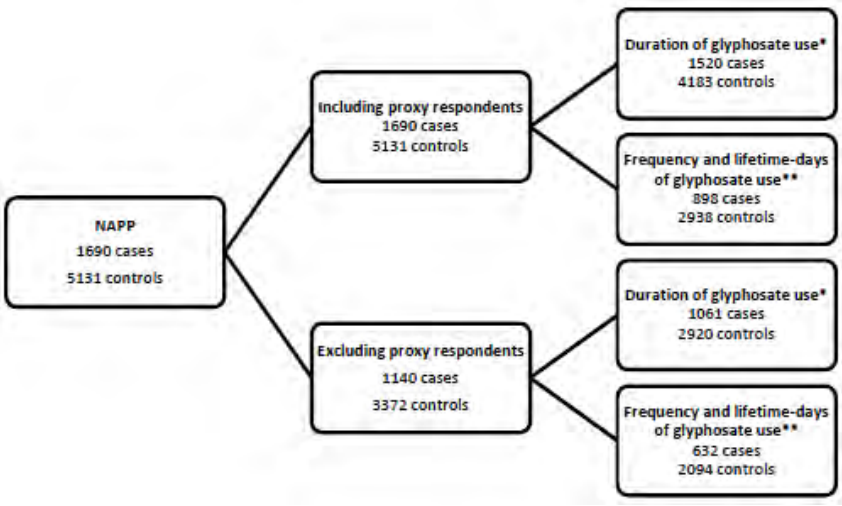

Figure 1. Subjects in main and proxy respondent analyses of glyphosate use and NHL in the North American Pooled Project (NAPP). * Duration (years) information was not collected in Kansas, ** Frequency (days/year) information was not collected in Iowa, Minnesota, and Kansas.

Table 1. Characteristics of non-Hodgkin lymphoma (NHL) cases and controls in the North American Pooled Project (NAPP). [OR=odds ratio; CI=confidence interval].

Characteristics	Cases (N=1690)		Controls (N=5131)			
	N	%	N	%	OR[a]	95% CI
Histological sub-type						
Diffuse large B-cell lymphoma (DLBCL)	647	38				
Follicular lymphoma (FL)	468	28				
Small lymphocytic lymphoma (SLL)	171	10				
Other	404	24				
State/Province U.S						
Nebraska	385	22	1432	28		
Minnesota	329	19	642	13		
Iowa	293	17	603	12		
Kansas	170	11	948	18		
Canada						
Ontario	142	8	585	11		
British Columbia	126	7	230	4		
Quebec	117	7	291	6		
Alberta	65	4	196	4		
Manitoba	34	2	113	2		
Saskatchewan	29	2	91	2		
Age (years)[b]						
≥19–≤29	26	2	277	5		
≥30–≤39	97	6	445	9		
≥40–≤49	159	9	514	10		
≥50–≤59	288	17	726	14		
≥60–≤69	564	33	1264	25		
≥70–≤79	402	24	1189	23		
≥80–≤89	137	8	610	12		
≥90	17	1	106	2		
Sex						
Male	1506	89	4424	86	1.00	ref
Female	184	11	707	14	0.94	0.75–1.17
Respondent type						
Self	1140	67	3372	66	1.00	ref
Proxy	533	32	1692	33	1.03	0.90–1.17
Unknown/missing	17	1	67	1		
Lymphatic or hematopoietic cancer in a first-degree relative						
No	1493	88	4790	93	1.00	ref
Yes	139	8	202	4	2.13	1.69–2.67
Unknown/missing	58	3	139	3		
Ever diagnosed with selected medical conditions[c]						
No	1011	60	3346	65	1.00	ref
Yes	545	32	1389	27	1.12	0.92–1.37
Unknown/missing	134	8	396	8		
Ever used any type of personal protective equipment						
No	374	22	1127	22	1.00	ref
Yes	105	6	310	6	1.12	0.86–1.45
Unknown/missing	1211	72	3694	72		

[a]Adjusted for age and state/province.

[b]Cases - mean 62.72 (SD 13.78) years; Controls - mean 61.66 (SD 17.13) years.

[c]Ever diagnosed with ≥1 of the following select medical conditions: allergies (any, food, or drug), asthma, hay fever, infectious mononucleosis, rheumatoid arthritis, tuberculosis, or received chemotherapy or radiation therapy.

Glyphosate use and associations with NHL overall and by major histological sub-type

Overall, 113/1690 cases (7%) and 244/5131 (5%) controls reported having used glyphosate at any point in their lifetime. In crude analyses, there was a significant association between ever use of glyphosate and NHL overall (OR1.4, 95% CI 1.1–1.8) that was attenuated appreciably when adjusted for ever use of the pesticides 2,4-D, dicamba, and malathion (OR1.1, 95% CI 0.8–1.5) (Table 2).[1] Adjusted ORs by for NHL subtypes were: 0.6 (95% CI 0.4 – 1.2) for FL, 1.2 (95% CI 0.8–1.9 for DLBCL, 1.8 (95%CI 0.9 – 3.7) for SLL, and 1.5 (95% CI 0.9 – 2.6) for other NHL subtypes.

When ORs for NHL and glyphosate use were examined by duration, there were lower ORs with longer use for NHL overall and for subtypes except SLL (see table 2). Conversely, there were higher ORs for those who reported use for more than 2 days per year verses those who reported use ≤ 2 days per year. Analyses by lifetime days of use – the metric used in most studies – showed near null results for all subtypes in the higher lifetime days category, except for SLL. It bears noting that the analyses by duration included 90% of cases and controls (Kansas subjects did not have the required data), while the analyses of days/year and lifetime days included only 53% of subjects (cases and controls from Kansas, Iowa, and Minnesota did not have the required data and were excluded). As such, these latter analyses may not be representative of results for the entire pooled study population.

Table 2. Adjusted Odds Ratios (95% confidence intervals) by Various Glyphosate Exposure Metrics

metric	NHL overall	FL	DLBCL	SLL	Other NHL
ever use	1.1 (0.8, 1.5)	0.6 (0.4, 1.2)	1.2 (0.8, 1.9)	1.8 (0.9, 3.7)	1.5 (0.9, 2.6)
duration					
≤ 3.5 years	1.3 (0.9, 1.8)	0.7 (0.3, 1.3)	1.6 (0.97, 2.7)	1.4 (0.6, 3.7)	1.8 (0.95, 3.5)
> 3.5 years	0.9 (0.6, 1.4)	0.6 (0.3, 1.3)	0.9 (0.5, 1.7)	1.9 (0.8, 4.8)	1.1 (0.5, 2.5)
days/year					
≤ 2	0.7 (0.5, 1.2)	0.4 (0.2, 1.2)	0.7 (0.4, 1.4)	1.3 (0.4, 4.3)	1.1 (0.5. 2.7)
≥ 2	1.7 (1.0, 2.9)	1.3 (0.6, 3.2)	2.1 (1.1, 4.3)	2.3 (0.6, 8.8)	1.6 (0.6, 4.5)
lifetime days					
≤ 7	0.9 (0.5, 1.5)	0.6 (0.3, 1.6)	0.8 (0.4, 1.7)	1.0 (0.2, 4.8)	1.4 (0.6, 3.5)
> 7	1.1 (0.7, 1.8)	0.8 (0.3, 1.8)	1.1 (0.6, 2.2)	2.2 (0.7, 6.9)	1.3 (0.5, 3.3)

Sensitivity analyses excluding proxy respondents

A sensitivity analysis was performed by excluding cases and controls whose data were provided by proxy respondents. The overall pattern of OR estimates were generally similar to the main analyses. However, for SLL, ORs were slightly higher in the > 2 days/year subgroup OR 2.3 (95% CI 0.6, 8.8) with proxies compared to OR 2.6 (95% CI 0.7, 10.1)) without proxies. For other NHL subtypes, ORs were slightly to appreciably lower for ever use with proxies OR 1.5 (95% CI 0.9, 2.6) compared to OR without proxies of 1.0 (95% CI 0.5, 1.9); OR for > 3.5 years of use 1.1 (95% CI 0.5, 2.5) compared to OR without proxies 0.5 (95% CI 0.2, 1.6)); and OR for > 7 lifetime days with proxies 1.4 (95% CI 0.6, 3.5) compared to OR without proxies of 1.1 (95% CI 0.4, 3.3)).

[1] Adjusting for other pesticides appreciably changed the NHL OR for glyphosate. Therefore, it seems most appropriate to focus on the ORs from the adjusted analyses.

Discussion & Conclusion

The objective of this study was to evaluate potential associations between glyphosate use and NHL in the NAPP, a pooled dataset that allowed for a more comprehensive analysis than previously possible in the individual studies. For NHL overall, the OR for ever use of glyphosate and NHL was near null when adjusted for reported use of 2,4-D, dicamba, and malathion (OR1.1, 95% CI 0.8, 1.5). Analyses by years of use and lifetime days of use showed near null results for NHL overall (OR 0.9 and 1.1, respectively), while the OR for > 2 days/year was elevated (OR 1.7, 95% CI 1.0, 2.9). It bears noting, however, that the analyses by days per year and lifetime days of use included only 50% of the pooled population – essentially the Canadian subjects and 1 of the 4 US case-control studies. It is uncertain, therefore, how representative these results are for the entire pooled population. The results of those analyses should be interpreted accordingly.

In analyses of NHL sub-types, there tended to be moderate positive associations between the various glyphosate exposure metrics and SLL. However, as there were only 15 SLL cases overall who ever used glyphosate and 14, 7, and 7 exposed SLL cases in the analyses by years of use, days per year, and lifetime days of use, respectively, the results were not statistically significant and the 95% CIs were imprecise. For other cell types, there were moderate positive relationships for glyphosate use of more than 2 days per year, though ORs were near null for these cell types for the higher category of years of use and lifetime days of use.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The main advantage of this pooled analysis compared with the previously published individual studies was to enable a more comprehensive analysis for glyphosate with regard to confounding factors and proxy respondents. In general, adjusting for use of 2,4-D, dicamba, and malathion reduced ORs for glyphosate. Analyses that excluded proxy respondents were generally similar to analyses that included them, though there were some instances, specifically for other NHL subtypes, where excluding proxies appreciably reduced the adjusted OR..

Left unaddressed in this pooled analysis is the intractable issue of case-recall bias in case control studies. Crump has shown in an analysis of all the case control studies that have reported ORs for glyphosate, including the studies in this pooled analysis, that results for all pesticides were markedly skewed toward positive associations (Crump K, Risk Analysis DOI: 10.1111/risa.13440). Crump noted particularly that the ORs for individual pesticides in the McDuffie et al. study (and 2 other studies not included in this pooled analysis) were nearly all greater than 1.0. He considered this evidence of case recall bias. Fundamentally, using self-reported exposure recollections from cases and controls violates the basic principle that data should be collected under equivalent circumstances for the groups to be compared (viz., cases and controls). That is impossible when pesticide recall is likely to be affected by their grievous illness for cases and not for controls. Accordingly, while this pooled analysis is an advance in understanding confounding by other pesticides and in assessing the impact of reporting by proxies (except in analysis where 50% of the subjects were excluded due to data limitations) in the 2 included studies, systematic error related to case recall bias remains an outstanding issue for interpreting the results for glyphosate.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because it concerns a pooled case control study which is subject to recall and selection bias. Notably, potential case-recall bias remains an unresolved issue in this pooled reanalysis.

Reliability Criteria: Epidemiology studies

Publication: Pahwa M. *et al.*, 2019	**Criteria met? Y/N/?**	**Comments**
Study Design		
Adequate study design given study objectives	Yes	Pooled reanalysis of 2 previously published studies.
Appropriate study population to address potential glyphosate-related health outcomes	Uncertain	Populations had very limited glyphosate exposure frequency.
Exposure studied		
Exposure to formulations with glyphosate as a.i.	Yes	
Exposure to formulations with other a.i.	Yes	
Exposure to other farm exposures	Uncertain	
Study Conduct/analysis		
Adequate description of study population	Yes	
Adequate description of exposure circumstances	Yes	
Comparable participation by groups being compared	No	Much less participation by controls: example: McDuffie study – participation 67% for cases, 48% for controls.
Information provided by proxy respondents	Yes, substantial	31% for cases, 40% for controls in DeRoos study; 21% for cases, 15% for controls McDuffie study (per Chang & Delzell 2016)
Adequate statistical analysis	Yes	More comprehensive than the original publications regarding confounding & proxy responses. Data for 47% of subjects were missing for analyses by days of use per year and

Publication: Pahwa M. *et al.*, 2019	**Criteria met? Y/N/?**	**Comments**
		lifetime cumulative days of use.
Adequate consideration of personal confounding factors	Yes	Better than original studies.
Adequate consideration of potentially confounding exposures	Yes	Better than original studies
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Yes	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because illustrates bias toward positive findings for glyphosate in the original publications due to confounding and, in part, due to proxy responses. Recall bias unresolved. Missing data for 47% of subjects for analyses by days/year of use and lifetime cumulative days of use hinders interpretation of related results.		

1. Information on the study

Data point:	CA 5.6
Report author	Panzacchi S. *et al.*
Report year	2018
Report title	The Ramazzini Institute 13-week study on glyphosate-based herbicides at human equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation
Document No	Environmental Health (2018) Vol. 17, 52
Guidelines followed in study	Based on the National Toxicology Program's (NTP) Modified One-Generation Reproduction Study 2011.
Deviations from current test guideline	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 current study represents the first phase of a long-term investigation of glyphosate-based herbicides (GBHs) that we are conducting over the next 5 years. In this paper, the study design, the first evaluation of in vivo parameters and the determination of glyphosate and its major metabolite aminomethylphosphonic acid (AMPA) in urine is presented.

Materials and methods

Chemicals - Glyphosate (purity of > 99.5%), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy). The representative formulated product, Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%), water (42.5%) and surfactant (16%)) was purchased from Consorzio Agrario dell'Emilia, Bologna, Italy.

Animals - Male and female SD rats were obtained from the colony used at the Cesare Maltoni Cancer Research Center laboratories of the Ramazzini Institute (CMCRC/RI). The animal room conditions were 22 °C ± 3 °C and 50 ± 20% relative humidity and a light/dark cycle of 12 hours. During the experiment the animals received standard pellet feed and tap water ad libitum.

Experimental design - Each of 8 virgin female SD rats (17 weeks old, 270-315 g) per dose group was placed individually in a polycarbonate cage with a single male rat of the same age and strain until evidence of copulation. Gestational day (GD) 0 was defined as the day on which sperm was found in vaginal smears. After mating, matched females were housed separately during gestation and delivery and pups were housed with the dams until weaning. The day on which parturition was completed was designated as lactating day (LD) 0 for the dam and postnatal day (PND) 0 for the offspring. On PND 28, the offspring were weaned and identified by ear punch. Sequentially, they were allocated in the same treatment group of their mother to obtain 18 animals (8 for the 6-week cohort and 10 for the 13-week cohort) per sex and for each dose group. No more than 2 males and 2 females from the same litter were included in the same cohort/treatment group. Altogether, 54 males and 54 females were enrolled in the post-weaning treatment phase. Rats were treated with glyphosate and MON 52276 at 1.75 mg glyphosate acid eq./kg bw/day in the drinking water. One group received only tap water as control. After weaning, until the end of the experiment (PND 73 ± 2 or 125 ± 2), glyphosate and MON 52276 were administered in the drinking water to F1 animals on the basis of the average body weight and average water consumption per sex and per experimental group. Males and females were considered separately because of their difference in weight gain, body weight and water consumption. Animals were checked 3 times daily on working days and 2 times daily on Sundays and non-working days. Clinical signs were checked before the start of the treatment, and at least every two days until the end of the experiment. The body weight of the dams was recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, the body weight of the dams was recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 and the

body weight of the pups by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. After weaning, body weight was measured twice a week until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifice. The mean individual body weights were calculated for each group and sex. Feed and water consumption of the dams were recorded twice weekly on GD 0, 3, 6, 9, 12, 15, 18, 21, and during lactation on LD 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28. After weaning the daily feed and water consumption per cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2. The mean individual feed and water consumption were calculated for each group and sex. The day before terminal sacrifice, all the animals were placed individually in metabolic cages and starved for around 16 hours during which the animals had free access to water alone or to the test compound solutions. In the morning of the following day, samples of at least 5 mL of urine collected from each animal were transferred to labelled tubes for analysis of glyphosate and AMPA. Samples from 3 dams/group and from 5 rats/sex/group belonging to the 6-week and 13-week cohorts were used for analysis.

Analysis of glyphosate and AMPA in urine - Analysis of glyphosate and AMPA in drinking water, feed and urine were performed by Neotron Laboratories. The specification and results are maintained in the experimental documentation. Analysis was performed using LC-MS/MS. The limit of quantification (LOQ) for glyphosate and AMPA was 0.10 µg/L in water, 50 µg/kg in feed, and 1 µg/L in urine.

Statistical analysis - Means ± standard deviations (SD), were calculated for continuous variables. For body weight, water and feed consumption over time multilevel mixed-effect linear regression models were used, to control for within subject correlation across time also considering the litter effect during the lactation period. Analysis of variance and Dunnett's test (when applicable) were also performed to compare body weight gain in different periods and consumption of food and water as mean consumption in several periods. All tests were two tailed with results considered as statistically significant if $p < 0.05$. Statistical analyses were performed by using STATA version10.

Results

Mortality, body weight, water and food consumption, clinical signs and litter data - All 24 dams and 108 rats from the 6-week and 13-week cohorts survived until sacrifice. Body weight and body weight gain of the dams during gestation and lactation were not statistically different among groups. Post-weaning body weight of female and male offspring was homogeneous and no statistically significant differences in body weight gain were observed among groups. Water and feed consumption during gestation and lactation were not different across groups. Litter sizes were fully comparable among groups, with mean number of live pups of 13.6 (range 10–16) in the control group, 13.3 (range 11–17) in the glyphosate group and 13.9 (range 11–16) in the MON 52276 group. Post weaning water and feed consumption were not affected by treatment. There was no clinical evidence of alterations in activity or behavior, reflexes, eyes or skin, respiratory, gastrointestinal, genitourinary and cardiovascular systems.

Analysis of glyphosate and AMPA in urine – The results of the analysis are presented in the Table below.

Table: Glyphosate and AMPA concentration in urine. Results are reported as mean ± standard deviations

		Dams		Offspring (6-week cohort)		Offspring (13-week cohort)	
	Treatment	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)
Male	Control			0.012 ± 0.010	0.003 ± 0.003	0.011 ± 0.010	0.006 ± 0.004
	Glyphosate	–	–	0.938 ± 0.414	0.014 ± 0.007	1.684 ± 0.768	0.023 ± 0.012
	Roundup			1.174 ± 0.439	0.011 ± 0.005	2.280 ± 1.520	0.027 ± 0.016
Female	Control	0.009 ± 0.001	0.006 ± 0.002	0.013 ± 0.007	0.005 ± 0.001	0.008 ± 0.005	0.003 ± 0.005
	Glyphosate	0.480 ± 0.010	0.024 ± 0.002	0.938 ± 0.377	0.016 ± 0.010	1.354 ± 0.359	0.013 ± 0.006
	Roundup	0.700 ± 0.106	0.024 ± 0.001	0.910 ± 0.383	0.018 ± 0.007	1.524 ± 0.585	0.021 ± 0.007

The urinary concentrations of glyphosate and AMPA of rats treated with glyphosate at 1.75 mg/kg bw/day were comparable to those observed in rats treated with MON52276 at 1.75 mg glyphosate acid

eq. /kg bw/day, despite the limited sample size and the large standard deviations. Glyphosate and AMPA urinary levels were all below or close to the LOQ of 0.001 mg/kg in the control group. In the treated rats, the majority of glyphosate was excreted unchanged, with urinary levels about 100-fold higher than that of AMPA. For example, the mean urinary levels of glyphosate were 1.354 and 1.524 mg/kg bw in glyphosate and MON 52276 treated females in the 13-week cohort, respectively, while the corresponding AMPA levels were 0.013 and 0.021 mg/kg bw. In glyphosate and MON 52276 treated rats, a time-dependent increase in the mean urinary concentration of glyphosate was observed. In glyphosate and MON 52276 treated males, an increase of approximately 2-fold was observed of the mean urinary concentration of glyphosate in the 13-week cohort when compared to the 6-week cohort. In glyphosate treated females, the 6-week cohort showed a 2-fold higher mean urinary concentration of glyphosate than the dams after weaning, while the 13-week cohort showed a 1.5-fold increase compared to the 6-week cohort. In the MON 52276 group, the increase was less steep, but the time-dependent pattern was still evident. In glyphosate and MON 52276 treated rats, the levels of AMPA were comparable at the different time points in both males and females. Large variations were observed of the AMPA concentrations in urine, in particular those close to the LOQ as in the control groups.

Discussion

Survival, body weights, food and water consumption of rats were not affected by the treatment with glyphosate or MON 52276. No clinical changes were observed in the animals of the dosed groups. Overall, oral treatment of glyphosate and MON 52276 via the drinking water seemed to be well tolerated. Exposure to glyphosate and MON 52276 led to comparable concentrations of glyphosate and AMPA in urine, indicating that systemic exposure does occur at the selected exposure level of 1.75 mg/kg bw/day, corresponding to the US ADI. The bioavailability of glyphosate in this study is also supported by the evident increase of glyphosate concentration in urine in relation to the length of treatment. The adjuvants and the other substances present in MON 52276 did not seem to exert a major effect on the absorption and excretion of glyphosate, even though mean values of glyphosate seem to be somewhat higher in the formulation treated group. The levels in urine were also comparable between the two sexes, although a consistent inter-individual variability was observed. In rats, glyphosate in urine appears to be the most accurate biomarker of exposure to glyphosate based herbicides (GBHs). The results from this study confirm previous evidence that in rodents most of the administered dose of glyphosate (98%) is excreted as unchanged parent compound, whereas the metabolite AMPA in urine is at around 0.2–0.3% of the administered dose. Furthermore, with the level of exposure to glyphosate used in this pilot study, AMPA urinary values of treated animals (0.011–0.027 mg/kg) were already close to the LOQ (0.001 mg/kg) which might limit the reliability of the data. Glyphosate concentrations in urine of treated animals (0.480–2.280 mg/kg) were found to be 100-fold higher than the AMPA concentrations and at least 500-fold higher than the LOQ. Therefore, in order to assess exposure to glyphosate in rats, in particular at doses that are equal or lower than 1.75 mg/kg bw/day, glyphosate appears to be the biomarker of choice. The presence of negligible levels of glyphosate (0.003–0.013 mg/kg) in some of the urine samples of the control groups might reflect an ubiquitous environmental contamination at ultra-low doses of glyphosate, which is consistent with previous reports from other authors. As the current LOQ of glyphosate in HPLC for pelleted animal feed is 0.050 mg/kg, this represents a technical limiting factor for the testing ultra-low doses of glyphosate.

Conclusion

A pilot study was performed on the health effects of glyphosate and its formulation Roundup Bioflow (MON 52276) administered orally to rats at the US ADI of 1.75 mg/kg bw/day. Treatment with either glyphosate or MON 52276 seemed to be overall well tolerated, consistent with previous experiments performed by the US NTP. Both glyphosate and MON 52276 exposure led to comparable urinary concentrations of glyphosate and AMPA with an increasing excretion of glyphosate in urine with duration of treatment. This indicates the systemic bioavailability of glyphosate and a possible mechanism of bioaccumulaton. The adjuvants and the other substances present in the formulation did not seem to exert a major effect on the absorption and excretion of glyphosate when administered orally. The results of this study confirm that, in rodents, glyphosate is a much more relevant biomarker in urine than AMPA, in particular at doses that are equal or lower than 1.75 mg/kg bw/day.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this study the general toxicity of glyphosate was compared against that of its reference formulation MON 52276 in pregnant rats and their progeny. Also the urinary excretion of glyphosate and AMPA was investigated. The test compounds were administered via the drinking water resulting in a daily dose of 1.75 mg glyphosate acid eq./ kg bw. The endpoints investigated were mortality, body weight, water and food consumption, and clinical signs in dams and offspring and litter data. There was no mortality and no statistically significant differences were observed among control, glyphosate, and MON 52276 groups in any of the endpoints investigated. Urinary concentrations of glyphosate and AMPA of rats treated with glyphosate at 1.75 mg/kg bw/day were comparable to those observed in rats treated with MON 52276 at 1.75 mg glyphosate acid eq. /kg bw/day. This indicates that the co-formulants in this glyphosate formulation have little influence on the oral bioavailability of glyphosate. In the treated rats, the majority of glyphosate was excreted in urine unchanged at levels of about 100-fold higher than that of AMPA and the mean urinary concentration of glyphosate increased with the duration of treatment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only one dose level for glyphosate and MON 52276 was considered, only 8 animals were used per dose and per sex and the method of analysis of glyphosate and AMPA in urine and its validation were not fully reported.

Reliability criteria for *in vivo* toxicology studies

Publication: Panzacchi *et al*., 2018	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Only a part of the reproductive toxicology study was reported, one dose level of glyphosate or MON 52276 was considered and 8 females per dose group were used.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	For the part that was reported.
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate (purity of > 99,5%), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy).
Only glyphosate acid or one of its salts is the tested substance	N	The representative formulated product, Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5%),

		water (42.5%) and surfactant (16%) was purchased from Consorzio Agrario dell'Emilia, Bologna, Italy.
AMPA is the tested substance	Y	Determined in urine of rats treated with glyphosate and MON 52276.
Study		
Test species clearly and completely described	Y	Male and female SD rats.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	1.75 mg glyphosate acid eq./kg bw/day administered as glyphosate and as MON 52276.
Number of animals used per dose level reported	Y	8 virgin female SD rats per dose group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Method of analysis described for analysis of urine	N	No details on the conduct of the method of analysis and no complete validation data set.
Complete reporting of effects observed	Y	Limited to body weight,
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	Not possible with one dose level of each test item.
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 only one dose level for glyphosate and MON 52276 was considered, only 8 animals were used per dose and per sex and the method of analysis of glyphosate and AMPA in urine and its validation were not fully reported.		

1. Information on the study

Data point:	CA 5.6
Report author	Perego M.C. *et al.*
Report year	2017
Report title	Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells *in vitro*
Document No	J. Appl. Toxicol. 2017; 37: 692–698
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restriction

2. Full summary of the study according to OECD format

The aim of this study was to determine the potential endocrine disruptor effects of glyphosate (GLY) on ovarian function evaluating cell proliferation, steroidogenesis and gene expression using bovine granulosa cells (GC) and theca cells as *in vitro* models. GC proliferation was impaired ($P< 0.05$) after exposure to GLY at 0.5, 1.7 and 5 μg ml^{-1}. GC progesterone production was not affected ($P\geq 0.05$) at all doses tested, while estradiol production was inhibited ($P< 0.05$) by GLY at 5 μg ml^{-1}. At the same concentration, GLY showed no effect ($P\geq 0.05$) on theca cell proliferation and steroidogenesis. At higher concentrations (0.01 and 0.3 mg ml^{-1}), GLY had no significant effect ($P\geq 0.05$) on GC proliferation and steroidogenesis. These studies, for the first time, suggest that GLY may affect the reproductive system in cattle via direct action on ovarian function; however, further studies will be required to understand better the mechanism of action and to determine the *in vivo* reproductive effects of GLY.

Materials and methods

Cell culture

Ovaries from non-pregnant beef heifers were collected from a local slaughterhouse and were treated as previously described (Lagaly *et al*., 2008; Langhout *et al*., 1991; Spicer & Aad, 2007). Thecal cells (TC) were collected from large (8–22 mm) follicles as previously described (Lagaly *et al*., 2008; Spicer & Chamberlain, 1998; Stewart *et al*., 1995). Follicular fluid was collected and final cell preparations were prepared in serum-free medium (Dulbecco's modified Eagle medium and Ham's F12) as previously described (Lagaly *et al*., 2008; Schreiber & Spicer, 2012). Trypan blue exclusion method was performed to determine viable cells (Langhout *et al*., 1991; Spicer *et al*., 1993; Tiemann *et al*., 2003a,b). Cells were then plated (2.5×10^5 in 20–80 μl of medium) on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1 ml of basal medium composed of a mixture of 1:1 Dulbecco's modified Eagle medium and Ham's F-12 containing glutamine, gentamicin and sodium bicarbonate (Sigma-Aldrich Co., St. Louis, MO, USA) as previously described (Schreiber & Spicer, 2012). Plates were maintained in a humidified 95% air and 5% CO_2 environment at 38.5 °C changing medium every 24 h. Cells were also kept in the presence of 10% fetal calf serum (FCS; Equitech-Bio, Inc., Kerrville, TX, USA) for the first 48 h of culture to procure an optimal attachment. After 48 h, cells were washed twice with serum-free medium and the different treatments were applied in serum-free medium containing 500 ng ml^{-1} of testosterone (as an estradiol [E_2] precursor; from Steraloids, Wilton, NH, USA) for 48 h. Ovine follicle-stimulating hormone (FSH; NIDDK-oFSH-20, activity: 175 × NIH-FSH-S1 U mg^{-1}, from the National Hormone and Pituitary Program, Torrance, CA, USA) was added to all treatments, because insulin-like growth factor (IGF) 1 alone does not have an effect on steroid production (Ranzenigo *et al*., 2008; Spicer *et al*., 2002).

After aspiration of follicular fluid, large follicles were bisected and GC were separated from the TC via blunt dissection and the theca interna was enzymatically digested as previously described (Aad *et al*., 2006; Spicer & Chamberlain, 1998; Stewart *et al*., 1995). The non-digested tissue was eliminated by using sterile syringe filter holders with metal screens of 149 μm mesh (Gelman, Ann Arbor, MI, USA) and filtered TC were then centrifuged at 50 g for 5 min. As described for granulosa cells (GC), TC were washed with serum-free medium and resuspended in serum-free medium containing collagenase and DNase. TC (2.0×10^5 viable cells per well) were plated and cultured as described for GC. Culture medium was also supplemented with 30 ng ml^{-1} of ovine luteinizing hormone (LH; LH activity; 2.3 × NIH-LH-S1 U mg^{-1}; from the National Hormone and Pituitary Program) because progesterone (P_4) and androstenedione (A_4) production are not induced by IGF1 in the absence of LH (Spicer & Stewart, 1996; Stewart *et al*., 1995).

Assays

Medium collected from individual wells was frozen at -20°C for subsequent steroid analyses. Radioimmunoassays (RIA) were performed to determine concentrations of P_4, E_2 and A_4 as previously described (Lagaly *et al*., 2008; Langhout *et al*., 1991; Spicer *et al*., 1993; Stewart *et al*., 1995). The intra- and inter-assay coefficients of variation were 7% and 13% for the P_4 RIA, and 8% and 17% for the E_2 RIA, respectively.

Determination of granulosa cell and theca cell numbers

The numbers of GC and TC, in the same wells from which medium was collected, were determined via a Coulter counter (model Z2; Beckman Coulter, Inc., Hialeah, FL, USA), and used to calculate steroid production on ng or pg per 10^5 cell basis. Cells were washed twice using 0.9% saline solution (500 μl), exposed to 500 μl of trypsin (0.25% wt/vol= 2.5 mg ml^{-1}) for 20 min at 37°C and then scraped from each well and enumerated as previously described (Lagaly *et al*., 2008; Ranzenigo *et al*., 2008).

RNA extraction and quantitative reverse transcription–polymerase chain reaction

At the end of the treatment period, medium was either aspirated or collected from each well depending on the experiment and cells from two replicate wells were lysed in 0.5 ml of TRI reagent solution (Life Technologies, Inc., Grand Island, NY) as previously described (Lagaly *et al*., 2008; Spicer & Aad, 2007). RNA samples were solubilized in DEPC-treated water (Life Technologies, Inc., Grand Island, NY), quantitated by spectrophotometry at 260 nm using a NanoDrop ND-1000 spectrophotometer, and stored at -80°C. Cholesterol side chain cleavage enzyme (*CYP11A1*) and aromatase (*CYP19A1*) primers and probes for quantitative reverse transcription–polymerase chain reaction were designed using Primer Express™ software (Foster City, CA, USA) as previously reported (Lagaly *et al*., 2008; Spicer & Aad, 2007)[11]. The bovine *CYP11A1* and *CYP19A1* primer and probe sequences and information are described by Lagaly *et al.* (2008). Relative quantification of target gene mRNAs were expressed using the comparative threshold cycle method as previously described (Lagaly *et al*., 2008; Spicer & Aad, 2007).

Experimental design

Experiment 1 was performed to evaluate the effects of glyphosate (GLY) on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng ml^{-1}), FSH (30 ng ml^{-1}) and IGF1 (30 ng ml^{-1}; recombinant human IGF1 from R&D Systems, Minneapolis, MN, USA) with or without various doses of GLY (i.e., 0, 0.5, 5 μg ml^{-1}; Sigma-Aldrich Co.). Only the 0 and 5 μg ml^{-1} doses of GLY were tested in the absence of IGF1 to determine any possible effect of GLY on FSH-stimulated steroidogenesis. After 48 h of treatment, cells were counted and medium was collected for E_2 and P_4 determinations. In a separate set of cells, the effect of 2-day treatment with 5 μg ml^{-1} GLY on GC viability was evaluated using the trypan blue exclusion method as previously described (Adashi *et al*., 1987; Spicer & Alpizar, 1994).

Experiment 2 was designed to evaluate the effects of higher doses of GLY on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng ml^{-1}), FSH (30 ng ml^{-1}) and IGF1 (30 ng ml^{-1}) with or without the various doses of GLY (i.e., 0, 0.01, 0.3 mg ml^{-1}). After 48 h of treatment, cells were counted and medium was collected for E2 and P_4 determinations.

Experiment 3 was designed to test the effect of GLY on *CYP11A1* and *CYP19A1* mRNA abundance in GC. Cells were cultured as previously described for experiment 1 except that no testosterone was included in the medium and treatments were only applied for 24 h: no additions, FSH (30 ng ml^{-1}) plus IGF1 (30 ng ml^{-1}), and FSH plus IGF1 plus GLY (5 μg ml^{-1}). After 24 h of treatment, cells were lysed for RNA extraction as described earlier. A combined treatment of FSH and IGF1 was selected to test the GLY effect because the inhibitory effect of GLY was seen with this treatment in experiment 1.

Experiment 4 was performed to study the effect of GLY on TC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing LH (30 ng ml^{-1}) and IGF1 (0 or 30 ng ml^{-1}) with or without GLY (*i.e.*, 0 and 5 μg ml^{-1}). After 48 h of treatment, cells were counted and medium was collected for P_4 and A_4 determinations.

Experiment 5 was designed to determine the effect of GLY on serum-stimulated GC proliferation. Cells were cultured for 4 days in 10% FCS. During the last 2 days of culture, cells were treated as follows: control (no additions) or GLY (1.7 μg ml^{-1}). At the end of treatment, cells were counted.

Statistical analysis

Experimental data are presented as the least squares means ± SEM of measurements from replicated culture wells. Each experiment was performed three times with different pools of GC collected from 10 to 20 ovaries for each pool and each treatment replicated three times within each experiment. Treatment effects and interactions were assessed using the GLM procedure of the Statistical Analysis System for Windows (version 9.2; SAS Inst. Inc., Cary, NY, USA). Main effects were treatment, experimental replicate (i.e., pool of cells) and their interaction. Steroid production was expressed as ng or pg per 10^5 cells per 24 h, and cell numbers determined at the end of the experiment were used for this calculation. Mean differences in cell growth and steroid production between treatments were determined using the Fisher's protected least significant difference procedure (Ott, 1977) $P< 0.05$ was considered statistically significant.

Results

Experiment 1: Dose–response of glyphosate on granulosa cell proliferation and estradiol and progesterone production in the presence of follicle-stimulating hormone with or without insulin-like growth factor 1

GLY at 0.5 and 5 μg ml^{-1} was found to decrease significantly the GC proliferation in the presence of FSH plus IGF1 (Fig. 1). Regarding steroid production, GLY at all tested concentrations (0.5 and 5 μg ml^{-1}) had no effect on GC P4 production (Fig. 2A). GLY had no effect on GC E2 production in the presence of FSH whereas GLY at 5 μg ml^{-1} decreased ($P< 0.05$) E2 production in the presence of FSH plus IGF1 (Fig. 2B). Cell viability was not significantly affected by 2-day treatment with 5.0 μg ml^{-1} GLY (91.6 vs. 88.6±5.0% for control and GLY-treated GC, respectively).

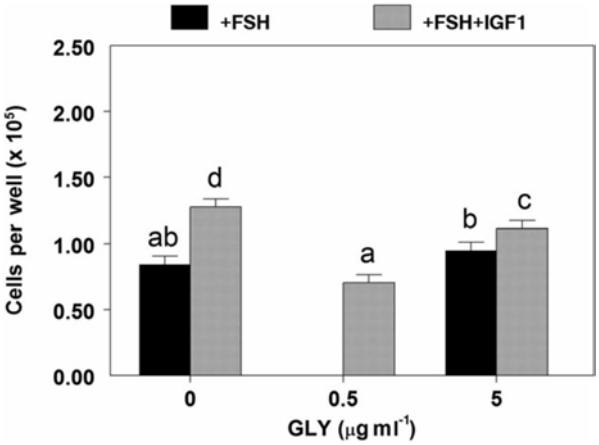

Figure 1. Effect of GLY on numbers of granulosa cells from bovine follicles (experiment 1). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h either with 30 ng ml^{-1} FSH alone and GLY at 0 or 5 μg ml^{-1}, or with 30ng ml-1 FSH and IGF1 (30 ng ml^{-1}) with GLY at 0, 0.5 or 5.0 μg ml^{-1}. All cells were treated concomitantly with 500 ng ml^{-1} of testosterone. Values are means ± SEM from three separate experiments (n = 9). Means without a common letter (a-d) differ (P< 0.05). FSH, follicle-stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.

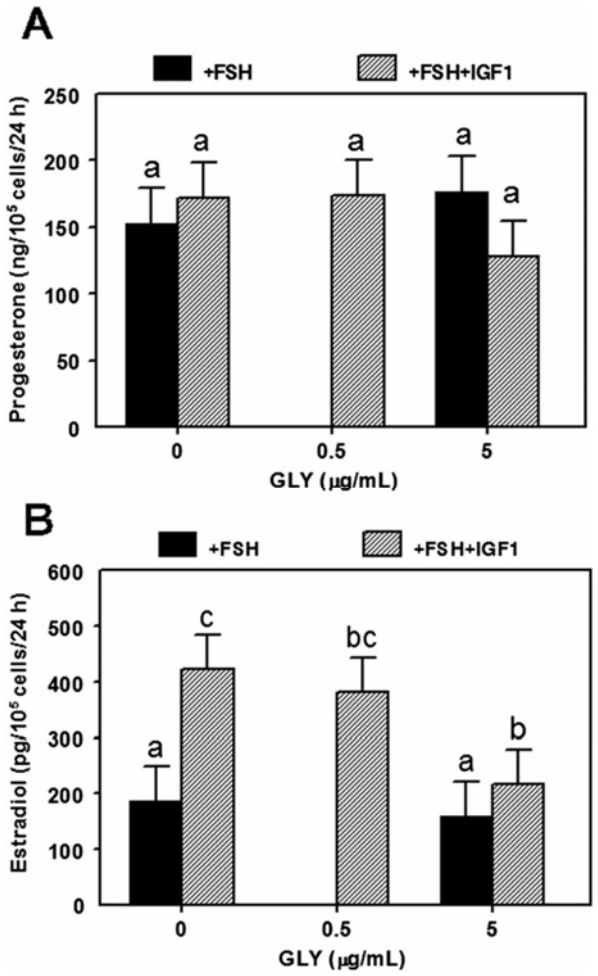

Figure 2. Effect of GLY on progesterone (A) and estradiol (B) production by granulosa cells from bovine follicles (experiment 1). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h either with 30 ng ml^{-1} FSH alone and GLY at 0 or 5 μg ml^{-1}, or with 30 ng ml^{-1} FSH and IGF1 (30 ng ml-1) with GLY at 0, 0.5 or 5.0 μg ml^{-1}. All cells were treated concomitantly with 500 ng ml^{-1} testosterone. Values are means +/-SEM from three separate experiments (n = 9). Within a panel, means without a common letter (a–c) differ (P< 0.05). FSH, follicle stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.

Experiment 2: Dose–response of glyphosate on granulosa cell proliferation and estradiol and progesterone production in the presence of follicle-stimulating hormone plus insulin-like growth factor 1

GLY at all tested concentrations (0.01 and 0.3 mg ml^{-1}) had no significant effect either on GC proliferation or steroid production. Cell numbers averaged 1.25, 1.47 and 1.00 ± 0.2 (×105 cells per well) for 0, 0.01 and 0.3 mg ml^{-1} GLY, respectively. P4 production averaged 99, 154 and 91 ± 23 ng 10-5 cells per 24 h for 0, 0.01 and 0.3 mg ml^{-1} GLY, respectively. E2 production averaged 130, 157 and 187 ± 28 pg 10-5 cells per 24 h for 0, 0.01 and 0.3 mg ml^{-1} GLY, respectively.

Experiment 3: Effect of glyphosate treatment on CYP19A1 and CYP11A1 mRNA in granulosa cells

The combined IGF1 plus FSH treatment increased (P< 0.05) CYP19A1 and CYP11A1 mRNA abundance by threefold and twofold, respectively, above untreated control GC (Fig. 3A,B). GLY (5µg ml^{-1}) had no significant effect on CYP19A1 or CYP11A1 mRNA in GC co-treated with FSH and IGF1 (Fig. 3A,B).

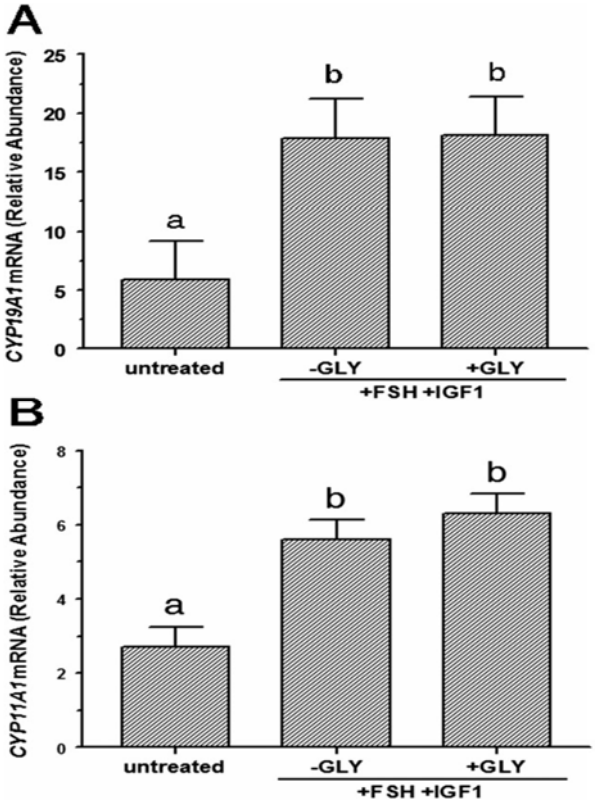

Figure 3. Effect of GLY on abundance of CYP19A1 (A) and CYP11A1 (B) mRNA in granulosa cells frombovine follicles (experiment 3). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 24 h either with either no additions (controls), or with FSH (30 ng ml^{-1}) and IGF1 (30 ng ml^{-1}) with GLY at 0 or 5.0 µg ml^{-1}. Values are means ± SEM from three separate experiments (n = 6). Within a panel, means without a common letter (a–b) differ (P< 0.05). FSH, follicle stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.

Experiment 4: Dose–response of glyphosate on theca cell proliferation and androstenedione and progesterone production in the presence of luteinizing hormone with or without insulin-like growth factor 1

GLY at the tested concentration of 5 µg ml^{-1} had no significant effect on TC proliferation and P4 or A4 production in the presence of LH either with or without IGF1. IGF1 significantly increased cell numbers

and steroid production. Cell numbers averaged 0.46, 0.53, 0.99 and 1.06 ± 0.05 for: LH alone; LH plus 5 µg ml^{-1} GLY; LH plus IGF1; and LH plus IGF1 plus 5 µg ml^{-1} GLY, respectively. P4 production averaged 40.5, 40.4, 70.3 and 78.3 ± 3.7 pg 10-5 cells per 24 h for: LH alone; LH plus 5 µg ml^{-1} GLY; LH plus IGF1 alone; and LH plus IGF1 plus 5 µg ml-1 GLY, respectively. A4 production averaged 1.45, 1.37, 2.75 and 2.47 ± 0.12 ng 10-5 cells per 24 h for: LH alone; LH plus 5 µg ml^{-1} GLY; LH plus IGF1 alone; and LH plus IGF1 plus 5 µg ml^{-1} GLY, respectively.

Experiment 5: Effect of glyphosate on serum-induced granulosa cell proliferation

Alone, GLY (1.7 µg ml^{-1}) increased (P< 0.05) GC proliferation (Fig. 4). Cell numbers were increased by 11% after 1 day and by 8% after 2 days of GLY treatment (Fig. 4).

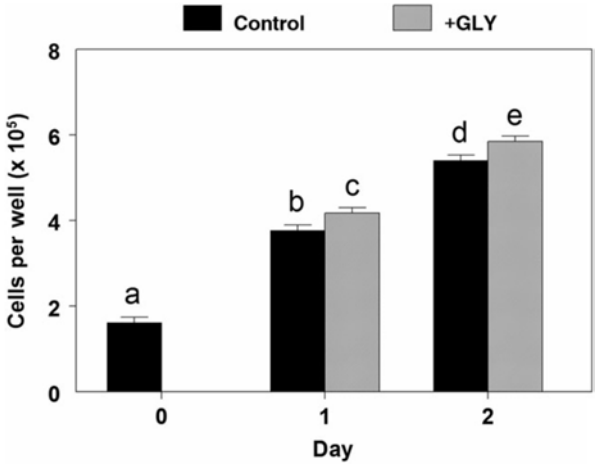

Figure 4. Effect of GLY on serum-induced proliferation of bovine granulosa cells (experiment 5). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 0, 1 or 2 days with 10% fetal calf serum and GLY at 0 or 1.7 µg ml^{-1}. Values are means ± SEM from three separate experiments (n = 9).Means without a common letter (a–e) differ (P< 0.05). GLY, glyphosate.

Discussion

In the present study, both cell proliferation and the production of P4, A4 and E2, hormones that are connected to normal ovarian cyclicity and establishment and maintenance of pregnancy (Wood & Strauss, 2002), were evaluated to determine if GLY has effects on bovine GC and TC. GLY was tested at low doses, from 0.5 µg ml^{-1}, very far below agriculture use and authorized residues in food or feed (approximately 7000 and 800 times lower, respectively) (Gasnier et al., 2009). No effects were observed on GC P4 production in the presence of FSH either with or without IGF1, whereas GLY at 5 µg ml^{-1} had inhibitory effects on E2 production in the presence of FSH plus IGF1, showing a potential impairment on GC function that is essential for oocyte survival (Petro et al., 2012). Results of our study also showed that GLY at 5 µg ml^{-1} had an inhibitory effect on GC proliferation in the presence of FSH plus IGF1, and this inhibitory effect of 5 µg ml^{-1} GLY on cell numbers was not associated with a change in cell viability.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In this *in vitro* study, glyphosate had minimal effects on granulosa cells (GC). In the presence of FSH only, glyphosate had no effect on GC cell viability or on progesterone or estradiol production. In the presence of FSH and IGF1, glyphosate reduced GC proliferation without a dose-response at 0.5 and 5 µg ml^{-1} but not at lower test concentrations (0.01 and 0.3 µg ml^{-1}) and did not affect progesterone

production or CYP19A1 and CYP11A1 mRNA expression; estradiol production was reduced at 5 µg ml^{-1} only (not at lower test concentrations). Without FSH or IGF1, 1.7 µg ml^{-1} of glyphosate slightly increased GC proliferation in response to serum (≤11%).

Glyphosate at 5 µg ml^{-1} had no effect on the theca cell (TC) proliferation or the production of progesterone or androstenedione.

Overall, with the exception of slight, non-dose-related alterations in GC proliferation under different test conditions, this study showed no effects of glyphosate on GC at physiologically relevant test concentrations. Glyphosate had no effect on TH.

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 used and the tests were conducted with only one or 2 test concentrations of glyphosate.

Reliability criteria for *in vitro* toxicology studies

Publication: Perego *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)	N	Purity not reported. Source: Sigma Aldrich.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Test system clearly and completely described	Y	Bovine granulosa and theca cells.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y (partly)	0, 0.5, 5, or 0, 10, 300 µg/mL (1.77 mM), or 0, 5 µg/mL.
Cytotoxicity tests reported	Y?	Viability tested at only one concentration of glyphosate.
Biochemical methods described	Y?	Some could be more detailed.
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	Limited since max. 2 test concentrations.

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 tested was not sufficiently characterized, no positive controls were used and the tests were conducted with only one or 2 test concentrations of glyphosate.		

1. Information on the study

Data point:	CA 5.6
Report author	Pham T. H. *et al.*
Report year	2019
Report title	Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice
Document No	Toxicological Sciences (2019) Vol. 169(1), 260–271
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

In this study, it was analyzed the effects of glyphosate, alone or in formula, on the male reproductive system. Pregnant mice were treated from E10.5 to 20 days postpartum by adding glyphosate or a GBH (Roundup 3 Plus) to their drinking water at 0.5 (the acceptable daily intake, ADI dose), 5 and 50mg/kg/day. Male offspring derived from treated mice were sacrificed at 5, 20, and 35 days old (d.o.) and 8 months old (m.o.) for analysis.

Materials and methods

Chemicals - Glyphosate (purity 99.2%) was purchased from Sigma-Aldrich, St Louis, USA.

Animals – Four-month-old outbred Swiss adult mice were exposed to glyphosate via the drinking water at concentrations corresponding with 0.5, 5 and 50 mg/kg bw/day from the day of vaginal plug detection (embryonic day 0.5) to 20 days post-partum (dpp). The control group received water alone. Control and treated mice of 5, 20, 35 days old and 8 months old were euthanized and the reproductive organs excised. At least 5 animals derived from at least 3 to 4 different litters in each group were used for this test.

Testosterone quantification - Serum was collected from ketamine/xylazine-anaesthetized adult animals by terminal cardiac exsanguination and aliquots were stored at -20°C. Testosterone levels in the serum were assayed in duplicate using a commercial radioimmunoassay based on competitive binding with I125-labeled testosterone according to the manufacturer's recommendations.

Epididymal sperm count - The mice were euthanized and the epididymis excised, rapidly frozen in liquid nitrogen and stored at -80°C pending sperm count. The tissue was first cut in pieces and homogenized in 6 mL of 0.15 M NaCl containing 0.005% (v/v) Triton X-100. After homogenization by sonication, an aliquot of the cell suspension was loaded onto a Malassez hemocytometer, and spermatozoa heads were counted. The average sperm count was calculated from at least 6 controls or treated animals.

Histology and numbers of germ cells and Sertoli cells - Testis samples were fixed in Bouin's solution and embedded in paraffin. Histological sections (5 µm thick) were stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC), only the adult animals were perfused and the testes were fixed for 24 hours in 4% (w/v) paraformaldehyde and then embedded in paraffin. Testis sections (5 µm thick) were incubated overnight at 4°C with a primary rabbit polyclonal antibody DDX4 and then with secondary Alexa-Chicken anti-rabbit 488 antibody during 1 hour to check the number of undifferentiated spermatogonia in testes of mice of 5 days old. For IHC on testes of mice of 35 days old, testis sections (5 µm thick) were incubated overnight at 4°C with goat anti-ZBTB16 and rat anti-GATA1 and then with secondary Alexa-chicken anti-goat 488 antibody and secondary Alexa-donkey anti-rat 594 during 1 hour. The sections were all counterstained with 0.001% (v/v) 4,6-diamidino-2-phenylindole dihydrochloride and mounted in Vectashield before microscopic analysis. To quantify the number of Sertoli cells (GATA1-positive cells) and undifferentiated spermatogonia (ZBTB16-positive

cells), the cells were manually counted in 30 sections on average of seminiferous epithelium at stage VII in controls and glyphosate treated groups. Cells in 3 different areas of the testis were analysed for each biological replicate

RNA extraction and quantitative PCR - Total RNA was extracted from testes of animals of 5 days old using the RNeasy plus mini kit according the protocol of the manufacturer and reverse transcription was performed with 1 mg of RNA using the iScript cDNA Synthesis Kit according to the manufacturer's instructions. The resulting cDNA was diluted 5 times and used for quantitative PCR. QPCR was performed using the iTaq Universal SYBR Green Supermix according to the manufacturer's instructions on a CFX384 Touch Real-Time PCR Detection system. The PCR amplification of the coding regions of Actb and Rplp0 was used for normalization. The data from at least 6 samples were analysed, compared, plotted, and expressed as a fold change in treated samples compared with controls.

Statistical analysis - Statistical tests were carried out using R software. For each experiment, the results were separated and compared by modality. First, the data were tested for normality by the Shapiro test and homoscedasticity by the Bartlett test. If the data distribution for each modality followed a normal distribution and if the variances were equal, the ANOVA test was performed, a parametric comparison test. If the data of at least 1 of the groups were not normally distributed or if the data of the groups were distributed normally but the variances were not equal, the Kruskal-Wallis test was carried out, a nonparametric comparison test of average. The differences were considered statistically significant when $p < 0.05$. In this case, the Tukey test was performed after the ANOVA test and the Mann-Whitney test after the Kruskal-Wallis test. In animals of 5 and 20 days old and 8 months old, data were collected by randomising in view of origin litter and pups were used as the experimental unit for statistical analysis. In animals of 35 days old, corresponding to the first complete wave of spermatogenesis, litters were used as the experimental unit for statistical analysis.

Results

Male reproductive parameters of prepubertal mice of 5 days old – No statistically significant change has been found in the number of spermatogonia of mice exposed to glyphosate at 0.5, 5 and 50 mg/kg bw/day. No statistically significant change has been found in RNA expression of Cyp11a1, Cyp19a1 and Ret at all dose levels tested. Statistically significant changes were observed in RNA expression of Bax (increase at 0.5 mg/kg bw only), Bcl2 (increase at all dose levels), Dazl (increase at 0.5 mg/kg bw only), Kit (decrease at 50 mg/kg bw only), Sall4 (decrease at all dose levels), Nanos3 (increase at 0.5 and 50 mg/kg bw but not at 5 mg/kg bw) and Foxo1 (increase at 0.5 mg/kg bw only).

Male reproductive parameters of mice of 20 days old - The histopathological analysis of testis sections revealed that glyphosate causes an adverse effect on testis morphology when compared with the control. An increase in vacuoles in the seminiferous epithelium was observed at all dose levels. Empty seminiferous tubules were seen at 5 mg/kg bw but not at 0.5 and 50 mg/kg bw glyphosate.

Male reproductive parameters of mice of 35 days old – No statistically significant change has been found in relative epididymis weight, relative seminal vesicles weight, epididymal sperm count and GATA1 positive cells. A decrease in relative testes weight was recorded at 0.5 mg/kg bw glyphosate but not at the other dose levels. Serum testosterone was found to be statistically significantly decreased at 0.5 and 50 mg/kg bw but not at 5 mg/kg bw glyphosate. The number of ZBTB16 positive cells were statistically significantly decreased at 5 mg/kg bw glyphosate but not at 0.5 and 50 mg/kg bw.

Male reproductive parameters of mice of 8 months old – No statistically significant changes were noted for relative epididymis weight, relative seminal vesicles weight and serum testosterone. A statistically significant decrease has been observed for relative testes weight of mice exposed to glyphosate at 0.5 mg/kg bw but not at the higher dose levels.

Discussion

Glyphosate did not have any effect on the body weight of animals tested in this study. The decrease in relative testicular weight observed at 0.5 (statistically significant) and 5 mg/kg bw (not statistically significant) in mice of at 35 days old and at 0.5 mg/kg bw (statistically significant) in mice of 8 months

old indicates that glyphosate, even at low doses, can significantly impair spermatogenesis, as testicular weight is a very reliable indicator of the normal function of male reproductive system. This fact was confirmed by measuring the level of testosterone in plasma after glyphosate exposure. In this study, serum testosterone levels were statistically significantly decreased in mice of 35 days old when exposed to glyphosate at 0.5 and 50 mg/kg bw and in mice of 8 months old when exposed to glyphosate at 0.5 mg/kg bw but not at higher dose levels. The treatment with different doses of glyphosate did not produce monotonic dose-responses. This is the case for relative weight of the epididymis and seminal vehicles, testosterone levels and sperm counts in mice of 35 days old. Glyphosate exposure led to a non-statistically significant decrease in epididymal sperm count in mice of 35 days old at 0.5 and 5 mg/kg bw leading to a non-statistically significant decrease in relative epididymis weight. This decrease in sperm count suggests that exposure to glyphosate could reduce male fertility.

The increase in the expression of key apoptosis genes (Bax, Bcl2) in mice of 5 days old exposed to glyphosate could influence the balance between Sertoli cells and spermatogonia. At 5 mg/kg bw a statistically significant decrease in spermatogonia (ZBTB16 positive cells) number was observed in animals of 35 days old. As the cell count of the pool of undifferentiated spermatogonia in the testes of mice of 5 days old confirmed that there were no changes in the number of germ cells in the glyphosate treatment groups, the decrease in spermatogonia number in mice of 35 days old may be the result of a modification of key genes involved in germ cell differentiation. In this study, there was a decrease in Sall4 and Kit mRNA expression in 5-day old mice exposed to glyphosate. Both factors are part of the retinoic acid (RA)-dependent signalling pathway involved in the differentiation of Aal spermatogonia into A1 which are irreversibly committed in the differentiation process. This result suggests that glyphosate is able to alter spermatogonial differentiation and maturation and this explains, at least in part, the molecular mechanism responsible for the morphological changes observed in 20-day old animals and the decrease in sperm count observed in adults.

Conclusion

This study shows that glyphosate at the ADI dose of 0.5 mg/kg bw/day could have endocrine disrupting effects which could impair the male reproductive system in mice.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The effect of glyphosate exposure from the day of vaginal plug detection to 20 days post-partum via the drinking water at concentrations corresponding with 0.5, 5 and 50 mg/kg bw/day on male reproductive parameters in mice of 5, 20 and 35 days old and 8 months old was investigated. The parameters measured were the number of spermatogonia and expression of genes important to testicular function in 5-day old mice, testicular histopathology in 20-day old mice, relative weight of testes, epididymis and seminal vesicles, epididymal sperm count, serum testosterone levels, GATA1 positive cell count and ZBTB16 positive cell count in 35-day old mice, and relative weight of testes, epididymis and seminal vesicles and serum testosterone levels in 8-month old mice. No statistically significant change was found for the number of spermatogonia in 5-day old mice. The only genes of which the expression was statistically significantly changed in a dose-related fashion were Bcl2 and Kit. In 20-day old mice, sperm depleted seminiferous tubules were noted at 5 mg/kg bw but not at 0.5 and 50 mg/kg bw glyphosate. In 35-day old mice there was no statistically significant change in the relative weight of the epididymis and the seminal vesicles, epididymal sperm count and GATA1 positive cell count. No dose-effect relationship could be established for relative weight of testes, serum testosterone levels and ZBTB16 positive cell count. In 8-month old mice no statistically significant change could be observed for relative weight of epididymis and seminal vesicles and serum testosterone levels. No dose effect relationship could be established for the decrease in relative testes weight. From these data it can be concluded that there is no evidence that glyphosate dosed orally to mice up to 50 mg/kg bw/day during the perinatal period is an endocrine disruptor and has an adverse effect on testicular function and development. This has been corroborated by regulatory reproduction toxicology studies with rats at much higher dose levels.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test conditions were not clearly described and the number of animals tested per dose level is too limited.

Reliability criteria for *in vivo* toxicology studies

Publication: Pham *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 ≥ 99.2%. Source: Sigma-Aldrich.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup 3 plus.
AMPA is the tested substance		
Study		
Test species clearly and completely described	Y?	
Test conditions clearly and completely described	Y?	Not completely described. Control and treated young prepubertal or adult mice were euthanized, and reproductive organs were dissected in 5, 20, 35 days old (d.o.), and in 8 months old (m.o.) mice. Only male mice were analysed.
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	0.5, 5, 50 mg/kg bw/day.
Number of animals used per dose level reported	Y?	5 animals derived from at least 3 to 4 different litters in each 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?	Not always presented in tables.
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	

Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test conditions were not clearly described and the number of animals tested per dose level is too limited.		

1. Information on the study

Data point:	CA 5.5
Report author	Presutti R. et al.
Report year	2016
Report title	Pesticide exposures and the risk of multiple myeloma in men: An analysis of the North American Pooled Project
Document No	International Journal of Cancer (2016) Vol. 139, 1703–1714
Guidelines followed in study	None
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Three case-control studies in the United States and Canada were pooled to create the North American Pooled Project (NAPP) to investigate associations between pesticide use and haematological cancer risk. This analysis used data from 547 MM cases and 2700 controls. Pesticide use was evaluated as follows: ever/never use; duration of use (years); and cumulative lifetime- days (LD) (days/year handled 3 years of use). Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using logistic regression adjusted for age, province/state of residence, use of proxy respondents and selected medical conditions.

Materials and Methods

Study population - The North American Pooled Project (NAPP) is comprised of three population-based incident case-control studies conducted by the U.S. National Cancer Institute in Kansas, Iowa/Minnesota and Nebraska in the 1980s, and the Cross Canada Study of Pesticides and Health (CCSPH), a population-based incident case-control study that was conducted in Quebec, Ontario, Manitoba, Saskatchewan, Alberta and British Columbia in the early 1990s. All 4 studies aimed to investigate the effects of pesticides and other agricultural exposures on the risk of lymphatic and hematopoietic cancers. The present analysis is restricted to a subset of three NAPP studies conducted in Iowa, Nebraska and Canada (CCSPH) where multiple myeloma (MM) cases were recruited. The study design and data collection in the CCSPH were modeled after the U.S. studies, making the data amenable to pooling. Eligible participants included white men aged 30 years or older in Iowa, white men and women aged 21 years or older in Nebraska, and men aged 19 years or older in Canada. Deceased participants were considered eligible in Iowa and Nebraska, but not in the Canadian study. Proxy respondents were used on behalf of deceased subjects in Iowa and Nebraska, and were permitted in Iowa, Nebraska, and Canada for participants requiring assistance due to illness or disability. Incident MM cases were identified using state and provincial cancer registry records, with the exception of Nebraska and Quebec, where cases were recruited from hospitals. Population-based controls were identified using random digit dialing (all studies), Medicare records and state mortality files (Iowa and Nebraska), health insurance records (Alberta, Saskatchewan, Manitoba and Quebec), telephone listings (Ontario) and voter lists (British Columbia). Cases and controls were frequency-matched to the overall case distribution by age (62 years in Nebraska and Canada, 65 years in Iowa), vital status and year of death (if applicable), sex (Nebraska) and province of residence (Canada). The participation rates in the MM subset of NAPP studies were modest for Canadian controls (48%) and higher in Iowa (78%) and Nebraska (85%). Participation rates were higher for cases in Canada (58%), Iowa (84%) and Nebraska (88%).

Exposure assessment - A set of *a priori* pesticides to be investigated in this analysis included agents that showed positive (significant or nonsignificant) associations in the earlier U.S. and Canadian studies. Pesticides that met these criteria were 2,4-D, captan, carbaryl, chlordane, DDT, glyphosate, lindane, malathion, methoxychlor, permethrin and the pyrethrins. Self-reported information on pesticide use,

farming activities and demographic characteristics were obtained through standardized interviews with participants. Individuals who provided an affirmative answer to general questions about pesticide use or exposure to substances within broad groups (i.e. insecticides, herbicides, fungicides) were subsequently asked more detailed follow-up questions regarding specific agents, including the frequency and duration of exposure. Participants who did not report any pesticide use were classified as unexposed. Among individuals reporting pesticide use, missing information for duration or frequency of exposure was treated as missing or unknown. Information on duration of pesticide use (years) was collected in all studies, whereas frequency information (days per year) was only collected in Canada and Nebraska. The data from Nebraska were excluded from the analysis since the number of exposed cases for pesticides of interest was low and the proportion of missing data was prohibitively high (>40%).

Exposure metrics - Associations were examined for dichotomous exposure (ever/never pesticide use) and by major chemical classes i.e. phenoxy herbicides, and organochlorine, organophosphate and carbamate insecticides. The duration of exposure was evaluated for each individual pesticide using years of self-reported use. Cumulative exposure was investigated using a composite lifetime days (LD) metric, defined as: LD = years of pesticide use x days/year of pesticide use. Analyses of cumulative exposure were restricted to the Canadian subset of the NAPP data, where sufficient information about both years and days/year was available. For subjects with missing information for the duration of pesticide use, simple conditional imputation was carried out. Age- and state/province-specific median values for years and days/year were assigned to participants classified as exposed based on the ever/never metric. Imputed values were only assigned if <35% of exposure duration data were missing among cases, and if the proportions of missing data differed by <20% between cases and controls.

Statistical analysis - Descriptive analyses were performed on potential confounders identified from the literature including age, province/state of residence, use of a proxy respondent, farming history (ever lived or worked on a farm) and personal medical history. Covariates that were significantly ($p < 0.05$) associated with MM or those that produced meaningful changes (≥5%) in the OR estimates were retained in the final models. Unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CI) for pesticide exposure variables with adjustment for age, province/state of residence, use of proxy respondent, and ever being diagnosed with any allergy, hay fever, or rheumatoid arthritis. For all analyses of individual pesticides, the referent population consisted of subjects who did not report any pesticide use, or those who indicated that they had not used that specific agent. Duration of pesticide exposure and cumulative LD were modeled as ordinal variables and linear trends were examined (*p*-trend). Cut-offs for categories were based on the median duration and LD among cases and controls for each pesticide. The use of proxy respondents was also considered as an effect modifier and sensitivity analyses were conducted excluding information provided by proxy respondents All analyses were performed using SAS version 9.3 .

Results

The analysis included a total of 587 MM cases and 3,588 controls from Iowa, Nebraska and six Canadian provinces. Female cases ($N = 40$) and female matched controls ($N = 707$) contributed by the Nebraska study were excluded due to the very low prevalence of pesticide use among females. The youngest MM case in the NAPP dataset was 31 years old and, therefore, the controls of an age of 30 years and younger were excluded ($N = 181$) to maintain a comparable distribution of age between cases and controls. The final analysis included 547 male cases and 2,700 male controls. Among the participants, cases were older than controls. This was expected since a common age-matched control group was used for all cancer sites in the NAPP and the majority of MM cases are typically diagnosed at slightly older ages (>65 years) than non-Hodgkin lymphoma and Hodgkin lymphoma cases. Proxy respondents were used for 35% of the cases and 28% of the controls overall. Associations of demographic characteristics and medical history covariates with MM were modeled using logistic regression, with adjustment for age (in years) and province or state of residence. A history of any type of cancer among first-degree relatives was significantly associated with MM risk. However, having a first-degree relative with any lymphatic or haematopoietic cancer, including MM, was not associated with MM. A number of conditions associated with stimulation of the immune system showed statistically significant inverse associations with MM risk. In this summary only data on glyphosate are reported.

Pesticide exposure (ever/never in the NAPP) - For the "never" use of glyphosate 502 (91.8%) cases and 2,504 (92.7%) controls and for the "ever" use of glyphosate 45 (8.2%) cases and 196 (7.3%) controls were identified. The adjusted OR was 1.29 (95% CI 0.9-1.85). When proxy respondents were excluded then the adjusted OR was 1.07 (95% CI 0.69-1.66).

Pesticide exposure relative to the years of exposure (Iowa and Canada subset of the NAPP) – For no exposure to glyphosate 471 (91.4%) cases and 1,832 (91.7%) controls, for up to 3 years of exposure to glyphosate 22 (4.3%) cases and 87 (4.3%) controls and for more than 3 years 22 (4.3%) cases and 80 (4.0%) controls were identified. The adjusted OR was 1.30 (95% CI 0.79-2.16) for up to 3 years of exposure and 1.34 (95% CI 0.80-2.23) for more than 3 years of exposure. The trend was not statistically significant ($p = 0.16$). When the adjusted OR was taken with exclusion of proxy respondents it was 1.19 (95% CI 0.67-2.11) for up to 3 years of exposure and 0.95 (95% CI 0.50-1.83) for more than 3 years exposure. The trend was not statistically significant ($p = 0.90$).

Pesticide exposure relative to the number of lifetime days (LD) (Canadian subset of the NAPP) - For no exposure to glyphosate 310 (90.6%) cases and 1,228 (91.0%) controls, for up to 6 LD of exposure to glyphosate 18 (5.3%) cases and 65 (4.8%) controls and for more than 6 LD 14 (4.1%) cases and 56 (4.2%) controls were identified. The adjusted OR was 1.35 (95% CI 0.76-2.40) for up to 6 LD of exposure and 1.11 (95% CI 0.59-2.07) for more than 6 LD of exposure. The trend was not statistically significant ($p = 0.48$). When the adjusted OR was taken with exclusion of proxy respondents it was 1.43 (95% CI 0.76-2.70) for up to 6 LD of exposure and 0.94 (95% CI 0.44-1.99) for more than 6 LD of exposure. The trend was not statistically significant ($p = 0.74$).

Discussion and Conclusions

Despite the increase in the overall sample size resulting from pooling data from the CCSPH and U.S. NCI studies, the numbers of exposed participants were still low for some pesticides, and information for duration or frequency was sparse and not collected in all MM studies. Our ability to investigate the effects of high levels of exposure was further limited, since few participants reported frequent and long-term pesticide use. Exposure misclassification due to the use of proxy respondents may also influence results. Studies have shown that farmers may be able to recall pesticide use better compared to nonfarmers, and certain types of proxy respondents, such as friends and family members who also work in agriculture, may be more likely to recall the use of certain specific pesticides. It should be recognized that a large number of comparisons were made and some of the effect estimates were based on small numbers of exposed cases and controls. Therefore, it cannot be excluded that some of the observed associations may represent chance findings. Despite these limitations, this study has several important strengths. The NAPP is one of the largest pooled case-control studies of agricultural exposures and haematopoietic cancers. This analysis was the first to investigate the association between pesticide exposure and MM risk in a pooled sample of Canadian and American participants. Since similar pesticides were used in both Canada and the United States, it was possible to investigate the effects of these exposures in the NAPP overall. Furthermore, similarities in the design of the case-control studies facilitated successful pooling of these datasets, which afforded a larger sample size for more comprehensive and powerful analyses. Specifically, the investigation of different aspects of pesticide exposure, such as duration of use and cumulative lifetime exposure provided an informative and novel addition to this analysis of pesticide use alone. A further advantage of this study was the extensive medical history information that was collected in the Canadian and U.S. studies. This allowed us to take into account the influence of several conditions that result in sustained stimulation of the immune system, such as rheumatoid arthritis, systemic lupus erythematosus, certain viral infections and allergies, which were inversely associated with MM in our data.

No statistically significant increases in risk of multiple myeloma (MM) associated with self-reported exposure to glyphosate were observed.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Four population-based incident case-control studies (3 US studies and one Canadian study) pooled in the North American Pooled Project (NAPP) aimed to investigate the effects of pesticides and other agricultural exposures on the risk of lymphatic and hematopoietic cancers. The present analysis is restricted to a subset of three NAPP studies (Iowa, Nebraska and Canada) where multiple myeloma (MM) cases were recruited. Self-reported information on pesticide use, farming activities and demographic characteristics was collected and the odds ratios (OR) were calculated for "ever/never" exposure, years of exposure and cumulated lifetime days of exposure to glyphosate with and without exclusion of proxy respondents. The result is that no statistically significant increases in risk of multiple myeloma (MM) associated with self-reported exposure to glyphosate were observed.

This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because it concerns pooled case control studies which are subject to recall bias and in which confounding factors could not be ruled out.

Reliability criteria for epidemiology studies

Publication: Presutti *et al*., 2016	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines/practices.	Y	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Exposure to formulations with only glyphosate as a.i.		
Exposure to formulations with glyphosate combined with other a.i.		
Exposure to various formulations of pesticides	Y	
Study		
Study design – epidemiological method followed	Y	Pooled case control studies
Description of population investigated	Y	
Description of exposure circumstances	Y	May be subject to recall bias
Description of results	Y	
Have confounding factors been considered	N	Confounding factors cannot be ruled out
Statistical analysis	Y	
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 it concerns pooled case control studies which are subject to recall bias and in which confounding factors could not be ruled out.		

1. Information on the study

Data point:	CA 5.6
Report author	Ren X. *et al*.
Report year	2019
Report title	Effects of chronic glyphosate exposure to pregnant mice on hepatic lipid metabolism in offspring
Document No	Environmental pollution (2019) Vol. 254, pp. 112906.
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 present study aims to investigate the toxic effects of prenatal exposure to pure glyphosate or Roundup on lipid metabolism in offspring. During gestational days (GDs), ICR mice (from Institute of Cancer Research) were given distilled water, 0.5% glyphosate solution (w/v, 0.5 g/100 ml) or 0.5%-glyphosate Roundup solution orally. The livers and serum samples of the offspring were collected on gestational day 19 (GD19), postnatal day 7 (PND7) and PND21. The results showed a significant decrease in the body weight and obvious hepatic steatosis with excessive lipid droplet formation in offspring. Moreover, the concentrations of lipids such as triglycerides (TGs), total cholesterol (T-CHO), and low-density lipoprotein cholesterols (LDL-C) increased to a significant extent in both the serum and livers. Furthermore, there were significant differences in the expression levels of the genes SREBP1C, SREBP2, Fasn, Hmgcr, Hmgcs and PPARa, which are related to lipid biosynthesis or catabolism in the liver.

Materials and methods

Animals; Ten-week-old female and male ICR mice were purchased from Nanjing Qinglongshan Experimental Animal Center (Nanjing, China). After one week of adaptation, one male and two female mice were housed in each cage from 5.00 p.m. to 8.00 a.m. daily to obtain pregnant mice. Pregnant mice were placed into separate cages once the pregnancy was confirmed by a vaginal smear the following morning. This day was defined as the first day of gestation. Animals were fed with water and feed ad libitum. The temperature and relative humidity in the animal house were controlled at 23 ± 2 C and 50 ± 10%, respectively, and the animals were kept on a 12-h light/dark cycle. The animal experiments were approved by the Animal Welfare Committee of Nanjing Agricultural University (Nanjing, China) and implemented in accordance with the National Institutes of Health Guidelines for Animal Care and the Committee of Animal Research Institute.

Chemicals and treatment; Pure glyphosate (N-(phosphonomethyl)glycine) and Roundup (as the isopropylamine salt) were provided by Shanghai Ryon Biological Technology Co., Ltd. (Shanghai, China) and Sinochem Crop Protection Products Co., Ltd. (Shanghai, China), respectively. Glyphosate and Roundup were diluted with distilled water to obtain 0.5% active ingredient solutions (w/v, 5 g glyphosate/1 L solution). Then, the subjects were administered the 0.5% glyphosate or Roundup solution through the drinking water (pH was controlled at 7.2 ± 0.2).

Animal treatment and sampling; A total of 30 pregnant mice were randomly divided into three groups: CON (control, n = 10), GLP (0.5% glyphosate treated, n = 10), and RU (0.5% Roundup treated, n = 10). Half of the pregnant mice (five from each group) were exposed throughout the first 19 days of pregnancy and were sacrificed on GD19. The other half of the pregnant mice were exposed throughout the pregnancy period and given distilled water after giving birth. Weekly body weights of the offspring were recorded, and their anogenital distances were measured separately to identify their sexes. Seven and 21 days after birth, the prenatally exposed offspring were sacrificed (preferably two females and two males

per mother) for blood and tissue analysis. The water consumption of the pregnant mice was measured, and the real exposure dose of glyphosate in both GLP and RU groups was approximately 7 ml (Table 1). The serum was extracted through centrifugation (3500 rpm, 15 min, 4 C) and was used to assay the biochemical indexes. Parts of the livers were stored at 80°C for lipid concentration determination and reverse transcriptionpolymerase chain reaction (RT-PCR). Liver samples were either fixed in a 4% paraformaldehyde solution or embedded in optimal cutting temperature compound (O.C.T. compound) provided by Sakura Finetek Japan Co., Ltd. (Tokyo, Japan) prior to frozen sectioning for the histological observation of tissue sections.

Histological preparation; Some parts of the liver tissue were fixed in 4% paraformaldehyde solution for 24 h and then dehydrated, clarified, embedded with paraffin and sectioned. Tissue sections (5 mm) were used for hematoxylineeosin (H&E) staining. The remaining liver tissue was embedded with O.C.T. compound and sectioned using a microtome cryostat manufactured by Thermo Fisher Scientific Instrument Co., Ltd. (Shanghai, China) for Oil Red O staining.

Serum biochemical and liver lipid concentration assays; To preliminarily diagnose the liver injury and lipid content of the organisms, the following serum biochemical indexes were determined: aspartate transaminase (AST), alanine transaminase (ALT), triglyceride (TG), total cholesterol (T-CHO), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). Additionally, liver homogenate was centrifuged to obtain the supernatant (3500 rpm, 15 min, 4 °C) to measure the TG, T-CHO, LDL-C and HDL-C content. Both the serum biochemical indexes and hepatic lipid content were assayed with commercial reagent kits purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China).

Analysis of gene expression; Total RNA was extracted from liver tissue with the ISOGEN 2 reagent kit (from NIPPON GENE CO., LTD.) (Tokyo, Japan) according to the manufacturer's instructions. The concentration of the obtained RNA was determined by a spectrophotometer, and the purity was measured using a NanoDrop® 8000. Then, PrimeScriptTM RT Master Mix (from Takara Bio Inc.) was used to reverse transcribe RNA to cDNA, which acted as a template for the SYBR® Premix Ex Taq™ PCR kit (from Takara Bio Inc.) for real-time PCR. The expression levels of the genes SREBP1C (Sterol Regulatory Element Binding Protein 1C), SREBP2 (Sterol Regulatory Element Binding Protein 2), Fasn (Fatty acid synthase, which catalyzes fatty acid synthesis), Scd (Stearoyl-CoA Desaturase 1), Acc (Acetyl-CoA Carboxylase), Hmgcr (3-hydroxy-3-methyl-glutaryl-CoA reductase), Hmgcs1 (3-hydroxy-3-methylglutaryl-CoA synthase 1), Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2) and PPARa (Peroxisome proliferator-activated receptor alpha) were determined. The relative expression levels of the above genes were normalized to b-actin expression. All primers were designed and supplied by GenScript Bio-Tech Co., Ltd. (Nanjing, China).

Data analysis; The software packages SPSS Statistics 20.0 and GraphPad Prism (GraphPad Software, San Diego, CA, USA) were utilized to analyse the data. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed. Values are expressed as the mean ± standard error of the mean (SEM), and statistical significance was set as $p < 0.05$.

Results

Table 1: Effects of chronic glyphosate exposure on the performance of pregnant mice.

Items	CON	GLP	RU	*P*
Water consumption (ml)	9.69 ± 0.76^{a}	7.88 ± 0.46^{b}	7.45 ± 0.34^{b}	0.023
Feed intake (g)	9.00 ± 0.16	8.32 ± 0.55	9.63 ± 0.72	0.261
Body weight gain (g)	32.60 ± 3.30	35.96 ± 2.92	29.84 ± 0.70	0.281
Number of fetuses (n)	10.80 ± 1.50	14.40 ± 1.57	12.60 ± 2.11	0.376
Average birth weight (g)	1.73 ± 0.13	1.65 ± 0.08	2.03 ± 0.72	0.220

Each value represents the mean ± SEM of the group (n = 5).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

Physical and organ development; From GD19 to PND21, the body weight in both GLP and RU groups decreased and finally saw a statistically significant reduction on PND21 ($p < 0.05$) (Fig. 1). When separated according to sex, offspring showed no significant differences in either body weight or weight gain among the three groups (Table 2).

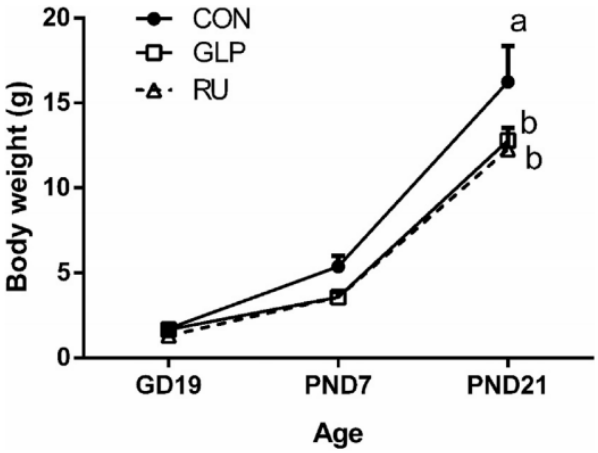

Figure 1: Effects of chronic glyphosate exposure to pregnant mice on the body weights of offspring at the ages of GD19, PND7 and PND21 (mean ± SEM). Different letters indicate statistically significant differences, $p < 0.05$.

Table 2: Effects of chronic glyphosate exposure to pregnant mice on the physical development of the offspring (g).

Items	Female				Male			
	CON	GLP	RU	*P*	CON	GLP	RU	*P*
PND7								
Body weight	5.72 ± 0.61	4.40 ± 0.28	4.29 ± 0.59	0.429	5.24 ± 1.00	4.80 ± 0.30	4.89 ± 0.88	0.918
Body weight gain	3.19 ± 0.80	2.74 ± 0.25	3.08 ± 0.50	0.222	3.51 ± 0.88	3.15 ± 0.29	2.87 ± 0.45	0.115
PND21								
Body weight	14.69 ± 2.44	13.63 ± 0.78	13.26 ± 1.06	0.786	15.79 ± 2.21	15.10 ± 0.85	13.86 ± 1.82	0.732
Body weight gain	12.04 ± 2.01	11.98 ± 0.76	11.24 ± 0.66	0.704	14.06 ± 2.09	13.45 ± 0.82	11.84 ± 1.33	0.243

Each value represents the mean ± SEM of the group (n = 7–10).

Liver histological observation; In both GLP and RU groups, relatively elevated numbers of vacuoles exhibiting hepatic lipid droplets were observed within the hepatocytes of both female and male offspring, when compared with the CON group. Additionally, the red areas observed in the Oil Red O stained sections represent lipid substances. In females, there tended to be more lipid droplets in the GLP group than in the other two groups. In contrast, in males, both the GLP and RU groups showed excessive lipid deposits. In addition, there were several clusters of monocytes in both the GLP and RU groups of PND7 females. It appears that glyphosate could cause inflammation in early-aged female mice.

Serum biochemical index; Compared with the CON group, TG levels showed a significant increase in the GLP group in both GD19 fetuses ($p < 0.01$) (Table 3) and PND21 female mice ($p < 0.05$) (Table 4). With respect to T-CHO levels, GLP mice showed a remarkable increase in both PND7 males ($p < 0.01$) and PND21 females ($p < 0.05$) compared with CON mice. LDL-C levels also increased in PND7 mice in both the GLP and RU groups ($p < 0.05$) (Table 4). The increased lipid content reflects the adverse effects of glyphosate on lipid metabolism, although this disturbed effect was not detected in every individual. Furthermore, significantly elevated AST levels in PND7 females in the RU group ($p < 0.01$) are theoretically considered to be a result of an injured liver.

Table 3: Effects of chronic glyphosate exposure to pregnant mice on the blood biochemical indexes in fetuses.

Items	CON	GLP	RU	*P*
TG (mmol/L)	0.15 ± 0.10^{b}	0.79 ± 0.21^{a}	0.30 ± 0.10^{b}	0.002
T-CHO(mmol/L)	2.15 ± 0.49	2.56 ± 0.52	1.22 ± 0.06	0.165
LDL-C (mmol/L)	1.03 ± 0.09	1.08 ± 0.04	0.91 ± 0.05	0.185
HDL-C (mmol/L)	0.14 ± 0.01	0.17 ± 0.02	0.17 ± 0.01	0.433
AST (IU/L)	67.88 ± 14.17	103.20 ± 10.30	76.21 ± 10.55	0.134
ALT (IU/L)	20.71 ± 1.02	47.54 ± 10.20	32.09 ± 2.35	0.091

Each value represents the mean ± SEM of the group (n = 20).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

Table 4: Effects of chronic glyphosate exposure to pregnant mice on the blood biochemical indexes of PND7 and PND21 offspring.

Items	Female				Male			
	CON	GLP	RU	*P*	CON	GLP	RU	*P*
PND7								
TG (mmol/L)	1.46 ± 0.07	1.79 ± 0.26	1.60 ± 0.08	0.324	1.11 ± 0.14	1.45 ± 0.14	1.45 ± 0.20	0.240
T-CHO (mmol/L)	1.46 ± 0.07	1.60 ± 0.27	1.42 ± 0.19	0.792	2.01 ± 0.23^{b}	2.92 ± 0.19^{a}	1.97 ± 0.11^{b}	0.007
LDL-C (mmol/L)	1.30 ± 0.18^{b}	1.73 ± 0.07^{a}	1.42 ± 0.07^{ab}	0.047	0.58 ± 0.07^{c}	0.99 ± 0.12^{b}	1.75 ± 0.11^{a}	0.000
HDL-C (mmol/L)	1.12 ± 0.11	1.18 ± 0.09	0.86 ± 0.04	0.074	0.86 ± 0.12	0.88 ± 0.04	1.13 ± 0.11	0.189
AST (IU/L)	28.03 ± 6.14^{b}	29.09 ± 6.22^{b}	63.84 ± 8.03^{a}	0.008	65.17 ± 7.65	72.54 ± 10.41	81.71 ± 10.52	0.503
ALT (IU/L)	30.93 ± 3.77	40.51 ± 4.86	39.89 ± 4.08	0.245	29.22 ± 2.39	32.01 ± 2.89	39.27 ± 2.29	0.058
PND21								
TG (mmol/L)	1.30 ± 0.18^{b}	2.41 ± 0.37^{a}	1.36 ± 0.15^{ab}	0.022	1.01 ± 0.11	1.10 ± 0.37	1.19 ± 0.38	0.741
T-CHO (mmol/L)	1.30 ± 0.18^{b}	2.06 ± 0.16^{a}	1.37 ± 0.15^{b}	0.028	0.92 ± 0.07	1.01 ± 0.06	1.05 ± 0.21	0.778
LDL-C (mmol/L)	2.26 ± 0.26	2.23 ± 0.12	2.65 ± 0.18	0.293	1.24 ± 0.19	1.08 ± 0.27	0.64 ± 0.13	0.115
HDL-C (mmol/L)	1.41 ± 0.28	1.65 ± 0.31	1.29 ± 0.10	0.570	1.56 ± 0.31	1.49 ± 0.17	1.22 ± 0.29	0.713
AST (IU/L)	41.14 ± 12.38	70.64 ± 9.87	50.04 ± 12.01	0.286	39.37 ± 9.28	54.06 ± 8.10	22.56 ± 8.90	0.091
ALT (IU/L)	19.02 ± 0.43	22.50 ± 4.61	19.48 ± 3.20	0.722	14.27 ± 4.06	16.01 ± 2.34	16.34 ± 3.04	0.890

Each value represents the mean ± SEM of the group (n = 7–10).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

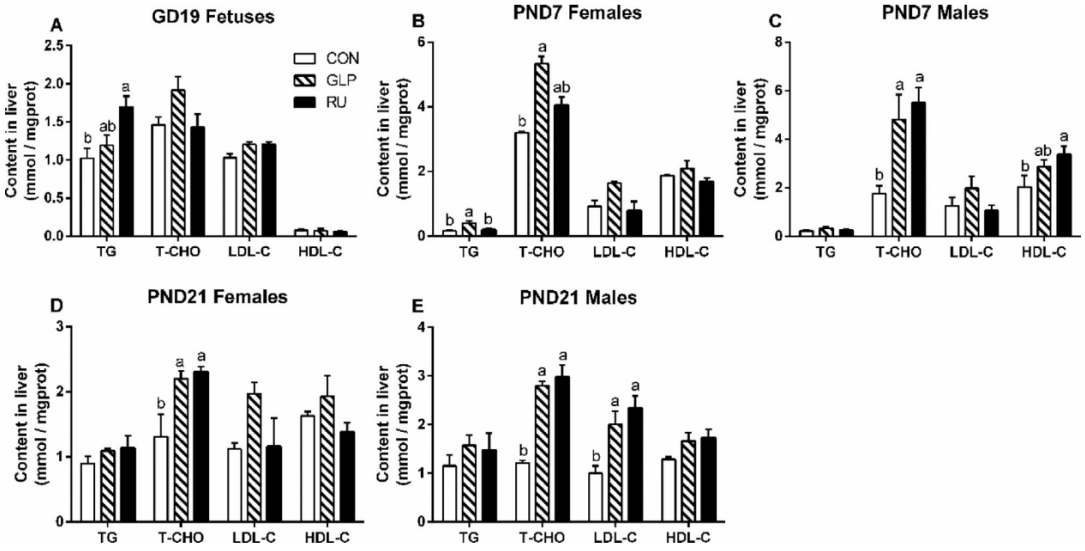

Figure 2: Effects of chronic prenatal glyphosate exposure on the lipid content in the livers of the offspring (mean ± SEM). (A) shows the TG, T-CHO, LDL-C and HDL-C content in livers of GD19 fetuses, and (B), (C), (D) and (E) show these parameters in PND7 and PND21 females and males, respectively. Different letters indicate statistically significant differences. a, b $p < 0.05$.

Lipid concentration in the liver; Compared with that in CON mice, TG levels in the RU group significantly increased in GD19 fetuses and PND7 female offspring ($p < 0.05$) (Fig. 2A and B). Moreover, T-CHO levels of both PND7 and PND21 offspring increased in the GLP or RU groups ($p < 0.05$) (Fig. 2B-E). Elevated TG and T-CHO levels in the liver can probably cause lipid deposits. The LDL-C levels of PND21 males showed a noticeable increase in both the GLP and RU groups ($p < 0.05$) (Fig. 2E), and the HDL-C levels in PND7 males were elevated in the RU group ($p < 0.05$) (Fig. 2C).

Both low-density and high-density lipoproteins can transport cholesterol in the extracellular environment. The elevated level of these proteins in serum is considered to be the result of increased cholesterol levels.

Expression levels of genes related to lipid metabolism in the liver; The relative expression levels of the genes SREBP1C, SREBP2, Fasn, Acc, Scd, Hmgcr, Hmgcs1 and Hmgcs2 in the GLP and RU groups showed a significant increase in GD19 fetuses and PND7 and PND21 offspring ($p < 0.05$) (Fig. 3). These genes are closely related to hepatic lipid production, so their elevation contributes to increased fat storage. However, this kind of increase does not match well to the trend in serum lipid content alteration. The levels of PPARa in PND7 males and PND21 females increased remarkably in both the GLP and RU groups, which is likely due to the growing demand for lipid catabolism caused by the increased lipid content.

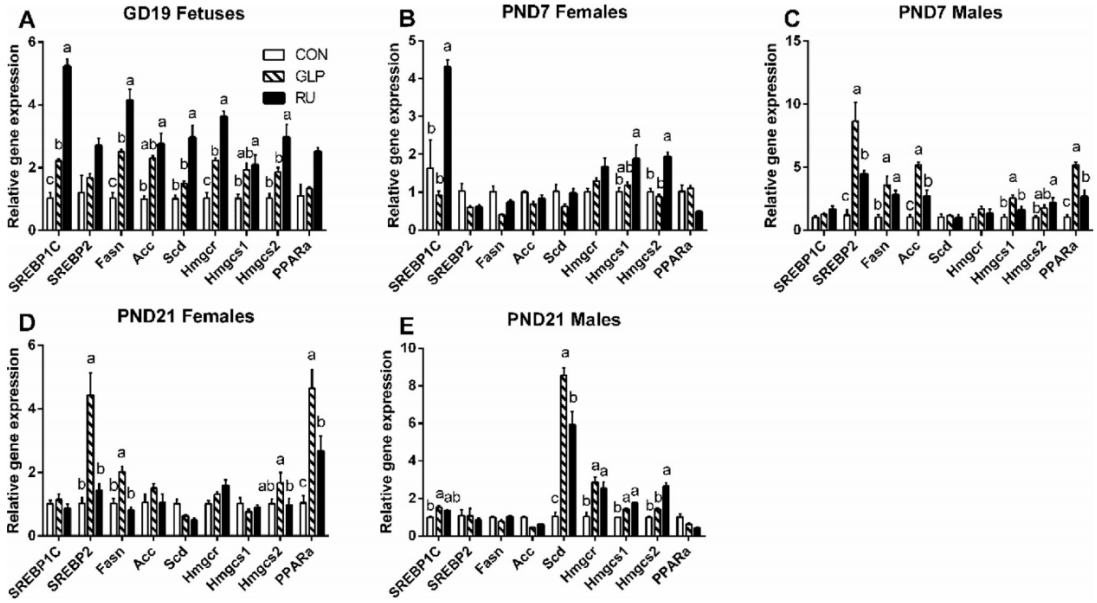

Figure 3: Effects of chronic prenatal glyphosate exposure on relative mRNA expression levels in the livers of the offspring (mean ± SEM). The relative expression levels of the genes SREBP1C, SREBP2, Fasn, Acc, Scd, Hmgcr, Hmgcs1, Hmgcs2 and PPARα genes in the livers of (A) fetuses, (B) PND7 female offspring, (C) PND7 male offspring, (D) PND21 female offspring, and (E) PND21 male offspring are shown. Different letters indicate statistically significant differences. a, b $p < 0.05$.

Discussion

Previous studies have found that glyphosate could cause fatty liver disease at the level of transcriptome and proteome. The transgenerational potential of glyphosate between dams and their litter successfully raises concerns about the hepatotoxicity of glyphosate in the progeny. The present study was designed to study the toxic effects of chronic prenatal glyphosate exposure on lipid metabolism in the livers of offspring. The results suggested that chronic maternal exposure to glyphosate can lead to the disruption of lipid metabolism in the next generation. In the present study, 19 or 21 gestational days was typically chosen as the exposure period, and offspring were collected on gestational day 19 and after birth on PND7 and PND21. The average water consumption in the GLP and RU groups was approximately 7 ml, which means that the pregnant mice took an average of 35 mg glyphosate per day. Thus, the real glyphosate exposure dose in fetuses was probably less than 35 mg per day. To our knowledge, the median lethal dose (LD50) of glyphosate in mice is 5000 mg/kg body weight (bw), and the non-observed adverse effect level (NOAEL) is 500 mg/kg bw. Although the true administered dose of glyphosate that the offspring received in the present study is far lower than the NOAEL, several phenomena still revealed excessive lipid production. Additionally, according to the classification criteria of the EU and the OECD Globally Harmonized System, glyphosate is not listed as an acute oral toxin based on 145 studies. In chronic exposure research, the “overall NOAEL” was assessed to be 100 mg/kg bw per day based on human beings, which is also higher than the present study's dose. Taken together, a dose lower

than overall NOAEL can still cause hepatic lipid metabolism disruption, although the consistency is not perfect among the tested parameters and groups in this study. Physical development is used as an important indicator of the health state of organisms. The body weights of the prenatal glyphosate-treated offspring showed a reduction compared to that of the CON group, which is in accordance with the previous results. The adverse effects on the physical development of offspring could be explained by the decreased body weight of the pregnant mice, while the feed intake of the pregnant mice showed no statistically significant changes. Therefore, the reduced body weight might be the result of energy consumption for the purpose of detoxification instead of for physical development. The liver is the most important detoxification organ that works to metabolize xenobiotics and can reflect the risk of the xenobiotics to the body system to some extent. To observe the liver state of the prenatally exposed offspring at the level of histology, H&E and Oil Red O staining were implemented in the present study. A relative increase in the number of fat vacuoles can be seen in several photomicrographs in the GLP or RU groups compared with the CON group, which is probably due to the elevated TG and T-CHO levels determined in both the serum and liver. Lipid production and storage in organisms are mainly controlled by liver lipogenesis and catabolism. We learned from the related gene expression level data that the genes related to these two biological processes showed increased expression levels in the GLP and RU groups compared with the CON group. SREBPs are essential activators for the synthesis of fatty acids and cholesterol once combined with a lipid synthesis promoter. SREBP1c, one of the isoforms of SREBP1, regulates the process of lipogenesis that increases from the early age of GD19, while SREBP2 is responsible for cholesterol biosynthesis. Furthermore, the increasing expression levels of downstream genes, such as Fasn, Scd, Acc, Hmgcr and Hmgcrs, contribute to higher fatty acid and cholesterol biosynthesis, which could result in inevitable hepatic fat storage. For lipid catabolism, PPARa plays a crucial role in mitochondrial b-oxidation and peroxisomal fatty acid oxidation, both of which act as important biological reactions in the degradation of liver lipids. Increased PPARa levels in the prenatally exposed offspring might be due to the growing demand for lipid catabolism caused by the rising TG or T-CHO levels. However, the alterations in gene expression are not completely consistent with TG or T-CHO synthesis proteins level or fat storage in the liver sections. This finding might be due to the limited number of subjects, and we will try to explore more detailed reasons and explain these reasons in our future studies. Taken together, we believe that glyphosate could affect lipid production by disturbing lipid metabolism-related gene expression. Previous research had detected a time-dependent enhancement in triglyceride and cholesterol levels as glyphosate-treated subjects aged, whereas there were contradictory results shown in bullfrog tadpoles. To explore more mechanistic insights, a lipidomic profiling experiment was conducted, and 62 distinct lipid species were identified as altered, including triglycerides and cholesteryl esters. Additionally, glyphosate is found to be metabolized into glyoxylate, which inhibits fatty acid oxidation enzymes, all of which are considered to be tightly associated with hepatic lipid dysregulation. Together with the reported impairment of mitochondrial oxidative phosphorylation and enzymatic activity after glyphosate treatment, the efficiency of the respiratory rate and tricarboxylic acid cycle will inevitably decline, which could further induce the diversion of redundant fatty acids into other lipid metabolism pathways. There have been many studies on glyphosate-induced chromosome and DNA aberrations spanning more than 20 years. A great variety of species as well as different doses of glyphosate were used in these studies. The reactive oxygen species and oxidative injury caused by glyphosate or its metabolites are commonly thought to be the reason for the genotoxicity. In the present study, glyphosate's genotoxicity was highly likely reflected in the disruption of lipogenesis. Glyphosate also caused obvious liver damage that appeared in the hematological parameters and histopathological alterations. The increased serum ALT and AST levels in prenatally exposed offspring demonstrated severe liver damage, similar to a reported study. The leakage of liver enzymes is a remarkable indicator of hepatic injury owing to xenobiotics. Additionally, the discovery of clusters of monocytes suggested the presence of inflammatory infiltration and immune responses, but it was not widely observed in all treatment groups. Apart from the liver lesions presented in our study, leukocyte infiltration, necrosis, blood congestion, hydropic degeneration and sinusoid dilation, which could enhance the risk of glyphosate-induced steatosis progression into fatty liver disease, has been observed in hepatocytes in other experiments. Therefore, it is probable that glyphosate-induced lipid metabolism disruption could progress into steatosis if no recovery or interference therapy was performed. Although significant differences were observed between some groups, the changes in the histopathological alterations, blood biochemical indexes and expression levels of lipid metabolism related genes among the groups showed imperfect consistency. This result is highly likely caused by the

limited quantity and individual variation of the subjects. Additionally, there are other factors that could have affected the results, such as sex, age and glyphosate source. Considering that the main aim of the present study was to explore the hepatotoxicity of glyphosate exposure, especially during pregnancy, on the lipid metabolism of offspring, these other factors will be future projects and will be studied in future research.

Conclusion

Chronic prenatal glyphosate exposure can probably cause lipid metabolism disruption in offspring, accompanied by an elevated lipid content in both serum and liver tissue. These alterations in hepatic lipid metabolism might result from rising lipogenesis in hepatocytes through increasing related gene expression.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The current study set out to examine any effects on lipid metabolism in fetuses and pups following prenatal exposure to glyphosate or the glyphosate formulation, Roundup™. Ten pregnant female rats per group were exposed from gestation day 1 through 19 to drinking water containing either 0.5% glyphosate, prepared using "pure" glyphosate (N-(phosphonomethyl) glycine), or 0.5% glyphosate using an appropriate dilution of Roundup™. A similar group of animals were given distilled water and served as the control group. Five females per group were terminated on gestation day 19 for examination of fetuses, while the remaining dams were allowed to litter and maintain their litters to postnatal day 21. Offspring (2/sex/litter where possible) were selected on postnatal days 7 and 21 for evaluation. Fetal and offspring evaluations included liver histology, serum biochemistry, liver lipid concentration and gene expression analysis of genes related to lipid metabolism in the liver.

The study is non-GLP and does not report the following information;

- Purity of test items
- Body weight and clinical signs for pregnant animals
- Clinical observations of offspring
- Achieved dose of glyphosate in treated animals in mg/kg bw/day.
- Measures to control inter animal and intergroup variability such as
    - Time of necropsy distributed equally across groups
    - Standardization of litter size on day 4 of lactation to mitigate variability caused by differences in liter size.
- More than a single dose level of glyphosate (0.5% solution); thus, preventing dose-response characterisation
- Liver weight of fetuses or offspring
- Normal physiological ranges for serum and liver biochemistry in this strain of rat at this laboratory
- Clear reporting of statistical evaluation and differences
- Thorough histological evaluations of the liver with incidence and severity of any recorded findings.

Although the authors concluded that there were treatment related effects on fetal and offspring body weight, there is no evidence from this study to suggest that glyphosate exposure has had any impact on fetal development or pup development postnatally. There was no effect on average birth weight of pups, the slight difference observed in the glyphosate treated group should be attributed to the slightly larger mean litter size observed (14.4 pups compared to 10.8 in the control group). Although figure 1 shows a reduction in mean pup weight in the glyphosate and Roundup™ treated groups (male and females combined). Group mean body weight of pups by sex showed no statistical differences from control.

Given the small group size, the large inter animal variability observed, the lack of consistency between the same parameter across the sexes, timepoints or sampling matrices, and the deficiencies

listed above, it not possible to clearly attribute any of the observed differences to glyphosate exposure. Therefore, the current study provides no evidence that glyphosate exposure causes lipid metabolism disruption in offspring following prenatal (in utero) exposure.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterized, only one dose level was tested, there was large inter animal variability observed and too few animals per dose level were analysed.

Reliability criteria for *in vivo* toxicology studies

Publication: Ren *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Shanghai Ryon Biological Technology Co, Shanghai, China.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup from Sinochem Crop Protection Products, Shanghai, China.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	?	Mice.
Test conditions clearly and completely described	?	
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	One dose level: 0.5% in drinking water.
Number of animals used per dose level reported	Y	10/dose.
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	
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 is not sufficiently characterized, only one dose level was tested, there was large inter animal variability observed and too few animals per dose level were analysed.