グリホサートカリウム塩

要旨及び評価結果

（ヒトに対する毒性）

検索期間：2020 年 1 月 1 日～2020 年 6 月 30 日

評価対象：適合性区分 a に該当する文献

シンジェンタジャパン株式会社

1. Information on the study

Data point:	CA 5.5/026
Report author	Crump, K. et al.
Report year	2020
Report title	Accounting for Multiple Comparisons in Statistical Analysis of the Extensive Bioassay Data on Glyphosate.
Document No	https://doi.org/10.1093/toxsci/kfaa039
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	-/Reliable without restrictions

2. Full summary of the study according to OECD format

Abstract

Glyphosate is a widely used herbicide worldwide. In 2015, the International Agency for Research on Cancer (IARC) reviewed glyphosate cancer bioassays and human studies and declared that the evidence for carcinogenicity of glyphosate is sufficient in experimental animals. We analyzed ten glyphosate rodent bioassays, including those in which IARC found evidence of carcinogenicity, using a multi-response permutation procedure that adjusts for the large number of tumors eligible for statistical testing and provides valid false-positive probabilities. The test statistics for these permutation tests are functions of p-values from a standard test for dose-response trend applied to each specific type of tumor. We evaluated three permutation tests, using as test statistics the smallest p-value from a standard statistical test for dose-response trend and the number of such tests for which the p-value is less than or equal to 0.05 or 0.01. The false-positive probabilities obtained from two implementations of these three permutation tests are: smallest p-value: 0.26, 0.17, p-values ≤ 0.05: 0.08, 0.12, p-values ≤ 0.01: 0.06, 0.08. In addition, we found more evidence for negative dose-response trends than positive. Thus, we found no strong evidence that glyphosate is an animal carcinogen. The main cause for the discrepancy between IARC's finding and ours appears to be that IARC did not account for the large number of tumor responses analyzed and the increased likelihood that several of these would show statistical significance simply by chance. This work provides a more comprehensive analysis of the animal carcinogenicity data for this important herbicide than previously available.

Materials and methods

Analysis of bioassay data - Of the bioassays identified by U.S. EPA, EFSA, or IARC, 10 glyphosate cancer studies of sufficient quality were selected which allowed the analysis of individual animal data. The characteristics of the selected studies are summarized in Table 1.

Table 1. Characteristics of the bioassays selected for analysis.

Bioassay	Species	Strain	# Dose Groups/ Sex	Animals/Dose	Maximum Dose[a] (mg/kg/d)		Maximum Weeks on test	Sites Where Histopathology was Conducted in All Dose Groups[b]
					Males	Females		
Atkinson et al 1993b[c]	Mouse	CD-1	4	50	988	1000	105	Kidney, Liver, Lung, Vascular System
Knezevich and Hogan 1983[c]	Mouse	CD-1	4	50	4841	5873	102	(all)
Wood et al 2009b	Mouse	CD-1	4	51	810	1081	81	Kidney, Liver and Lung
Sugimoto 1997	Mouse	CD-1-ICR	4	50	4348	4116	78	(all)
Atkinson et al 1993a[c]	Rat	SD[d]	5	50	1007	1018	105	Kidney, Liver, Lung and Salivary Glands: Parotid, Mandibular and Sublingual
Lankas 1981[c]	Rat	SD	4	50	31.49	34.02	111	(all)
Stout and Ruecker 1990[c]	Rat	SD	4	60	940	1183	105	(all)
Brammer 2001[c]	Rat	Wistar	4	64	1214	1498	104	(all)
Suresh 1996	Rat	Wistar	4	50	595.2	886	107	(none)
Wood et al 2009a	Rat	Wistar	4	51	1077	1382	105	Kidney, Liver, Lung and Bone Marrow

[a] All doses in each bioassay are listed in Table 2.
[b] Systemic tumors are assumed to have been searched for if at least one tissue in an animal was given a histopathological examination.
[c] These six studies were evaluated by IARC. IARC (2015) also reviewed two additional studies in which they identified shortcomings, but which they did not claim were "inadequate": Chruscielska et al (2000) and JMPR (2006). No glyphosate-related tumor responses were noted in either of these studies.
[d] SD = Sprague Dawley.

Eight of the 10 studies were assigned a Klimisch code 1 (reliable without restrictions). The Knezevich and Hogan (1983) study was assigned a Klimisch code 2 (reliable with restrictions) mainly because this study was initiated before the implementation of the OECD test guidelines and GLP, and the study of Lankas (1981) also a pre-GLP and pre-guideline study was assigned a Klimisch code 3 (not reliable) mainly because of the low power of the study due to the low dose range selected.

Statistical tests - The statistical tests applied in the analysis were functions of p-values obtained from conventional continuity-corrected poly-3 tests (survival-adjusted Cochran-Armitage test) for trend applied to each type of tumor or combination of tumor types in each bioassay. In the present analysis, the continuity-corrected version of the poly-3 test used was copied from a key portion of the computer program used by the NTP. Direct comparisons have shown that the author's implementation gives the same results as the version used by the NTP. Throughout this paper, all implementations of the poly-3 test, are one-sided, as are the NTP implementations of the test.

Results from 3 multi-response permutation tests are presented. In the simplest test, referred to as the "min-test", the test statistic is the smallest p-value obtained from applying the poly-3 test to all tumor types in all of the 10 bioassays. In the simplest implementation of this test, animals are randomly reassigned to dose groups (permuted among dose groups) in a Monte Carlo analysis, keeping the total numbers of animals in each dose group equal to the number in the original data. The tumors in each such reassignment are analyzed using the poly-3 test in exactly the same way as in the original data. Males and females are permuted separately. The false positive rate is the proportion of random reassignments that result in a smallest poly-3 p-value that is smaller than or equal to the smallest poly-3 p-value obtained from the original data. In addition to the min-test, two additional permutation tests were computed. The test statistics for these tests were the number of poly-3 tests of tumors in the original data for which the p-value is less than or equal to the critical value of 0.05 (05-test) or 0.01 (01-test). The false positive rates for these tests are the proportion of random permutations of the data for which the number of poly-3 p-values from the permuted data that are less than or equal to the critical value equal or exceed the number from the original data. The min-test could have enhanced power in a situation in which a test agent causes cancer at a single site, whereas the 05-test could have enhanced power when a test agent causes detectable cancer of several types. The permutation tests described above are conditional, not just on the total number of tumors, but also on the patterns of tumors occurring in individual animals.

In addition to conducting conventional poly-3 tests on specific types of tumors, tests were also conducted on combinations of tumor types thought to have a common origin such as liver adenomas and carcinomas. Since including these combinations resulted in the same tumors being present in multiple analyses, it was decided to perform two analyses, one (primary analysis) that included all of the individual tumors and combinations, and one (reduced analysis) in which individual tumors and combinations of tumors were removed from the analysis if they were part of a more inclusive tumor combination (e.g. the individual tumor categories of liver adenoma and liver carcinoma were removed and only the combination was used in the analysis).

For 5 of the glyphosate bioassays, all tissues listed for histopathological examination were scheduled for a histological examination in all animals of all dose groups. In the remaining 5 bioassays, control and high dose animals were all given a complete histopathological examination, along with the animals that died before the final sacrifice in the intermediate dose groups. In addition, certain tissues in all animals were scheduled for a histopathological examination regardless of when they died.

Simple randomization suffers from a potential bias due to dose-related differential survival, and, for studies with incomplete histopathology, a problem of data comparability.

In each of the 10 bioassays, dose-related effects on survival were tested using a Cochran-Armitage test for negative trend on the proportions of animals surviving to final sacrifice in the various dose groups. Regardless of the outcome of this test, to control for potential dose-related differences in survival each randomization of the data maintained the same number of survivors and non-survivors in each group as was seen in the actual data.

For other tissues than the mandatory tissues in the studies with incomplete histopathology, only the non-survivors in the intermediate dosed groups could be used in the trend analyses. For mandatory tissues, the survivors and non-survivors were separately permuted. Mandatory tissues and other tissues had to be separately randomized in studies with incomplete histopathology to make sure that all pathological information routinely collected in this type of studies is included.

In all applications of the poly-3 test, the test is applied only to data from one sex in a single study and the p-values from the poly-3 tests of all the studies are combined to create the “global” tests (min-test, 05-test and 01-test) to give the correct false positive rates. In addition to the randomization procedures for testing for positive dose-response trends in tumor incidence, the same procedures were repeated after reconfiguring the poly-3 test for negative trends.

Results

When the frequency of poly-3 p-values for positive trend computed from all tumors in all 10 bioassays in which at least two tumors occurred are considered there is an excess of large p-values (close to 1.0) compared to small p-values (close to 0.0). Since the version of the poly-3 trend test applied is a one-sided test for a positive trend, p-values close to 1.0 would translate into p-values near 0.0 for one-sided trend tests for anti-carcinogenicity.

Results of tests for a dose-related decrease in survival in each study show that in none of the bioassays analyzed this test was statistically significant. Moreover, 4 of the datasets had p-values in excess of 0.95 which indicates a significant positive trend in survival with increasing dose.

The 24 tumors for which the poly-3 test for a positive dose-related trend was significant at the 0.05 level in the primary analysis are shown in Table 2. Pancreatic islet-cell adenoma in male rats reported in the Stout and Ruecker (1990) study is not listed because it did not have a significant dose-related trend. In an identical analysis with the poly-3 test configured to test for a negative dose-related trend, there were 26 tumors for which the dose-response trend was significantly negative at the 0.05 level.

Table 2. Tumors with a significant positive trend (poly-3 p ≤ 0.05)

Bioassay	Species/ Sex	Tumor	Summary Tumor Incidence					Poly-3 p-value	Cited by IARC[a]
Atkinson et al. 1993b	M/M	Haemangiosarcoma	0/50	0/50	0/50	4/50		0.0013	IARC
Lankas 1981	R/F	Thyroid: C-cell Carcinoma	1/47	0/49	2/50	6/47		0.0015	
Sugimoto 1997	M/F	Hemangioma	0/50	0/50	2/50	5/50		0.0028	
Sugimoto 1997	M/F	Hemangioma, Hemangiosarcoma	0/50	0/50	3/50	5/50		0.0062	
Stout and Ruecker 1990	R/F	Adrenal: Cortical Carcinoma	0/60	0/60	0/60	3/60		0.0072	
Sugimoto 1997	M/F	Osteoma, Osteosarcoma	0/50	0/50	0/50	3/50		0.0074	
Wood et al. 2009b	M/M	Lymphoma	0/51	1/51	2/51	5/51		0.0076	
Brammer 2001	R/M	Liver: Hepatocellular Adenoma	0/64	2/64	0/64	5/64		0.014	
Lankas 1981	R/M	Testis: Interstitial Cell Tumor	0/50	3/50	1/50	6/50		0.021	
Stout and Ruecker 1990	R/M	Liver: Hepatocellular Adenoma	3/60	2/60	3/60	8/60		0.022	IARC[b]
Atkinson et al. 1993a	R/F	Lipoma	0/50	0/50	0/50	0/50	2/50	0.022	
Wood et al. 2009b	M/M	Lung: Adenocarcinoma	5/50	5/51	7/51	11/51		0.025	
Knezevich and Hogan 1983	M/M	Kidneys: Renal Tubal Adenoma	0/49	0/49	1/50	3/50		0.034	IARC
Lankas 1981	R/F	Lipoma	0/50	0/50	0/50	2/50		0.036	
Sugimoto 1997	M/M	Malignant Lymphoma	2/50	3/50	0/50	6/50		0.038	
Knezevich and Hogan 1983	M/F	Lymphoblastic Lymphosarcoma	0/50	1/50	0/50	3/50		0.041	
Sugimoto 1997	M/F	Osteosarcoma	0/50	0/50	0/50	2/50		0.041	
Sugimoto 1997	M/M	Kidney: Adenoma	0/50	0/50	0/50	2/50		0.042	
Sugimoto 1997	M/M	Hemangiosarcoma	0/50	0/50	0/50	2/50		0.043	
Stout and Ruecker 1990	R/M	Neurofibroma, Neurofibrocarcinoma	0/60	0/60	0/60	2/60		0.045	
Sugimoto 1997	M/F	Harderian Gland: Adenoma	1/50	3/50	0/50	5/50		0.046	
Stout and Ruecker 1990	R/F	Thyroid Gland: C-cell Adenoma	2/60	2/60	6/60	6/60		0.047	IARC
Suresh 1996	R/M	Lymphoma	0/50	0/50	0/50	2/50		0.049	
Stout and Ruecker 1990	R/F	Thyroid Gland: C-cell Adenoma or Carcinoma	2/60	2/60	7/60	6/60		0.049	

[a] Indicates tumor responses cited by IARC (2015) as evidence of carcinogenicity. Pancretic islets in male rats in Stout and Ruecker (1990) was also cited by IARC (1/58, 8/57, 5/60 and 7/59) but this response did not give a p-value ≤ 0.05 by the Poly-3 trend test.

[b] IARC (2015) resported tumor responses of 2, 2, 3, 7.

The most significant poly-3 trend in all 10 bioassays was for hemangiosarcoma in male mice in the Atkinson *et al*. (1993) study with a p-value of 0.0013. The actual significance of this smallest p-value, which is the false positive rate for the min-test, was 0.26 based on the primary analysis, rather than the naive value of 0.0013. This means that 26 % of the randomizations of the 10 datasets gave a smallest p-value less than or equal to the smallest p-value obtained from the original data.

The false positive rate for the 05-test was 0.08, which means that 8 % of randomizations of the 10 datasets found at least 24 sites for which the poly-3 p-value was ≤ 0.05. The results from all permutation tests based on the reduced data were similar to those based on the primary data. The false positive rate for the 01-test was 0.06 in the primary analysis and 0.08 in the reduced analysis. Overall, these findings suggest that, after accounting for the number of statistical tests performed, there was no clear evidence of a positive dose-related trend in tumor occurrence.

The evidence for negative trends is greater than that for positive trends in all analyses. The smallest poly-3 p-value for a negative trend was 0.0008 whereas the smallest p-value for a positive trend was 0.0013. The 01-test for a negative trend was highly significant in both the primary and reduced analyses with a p-value of 0.002 for each. These findings suggest stronger evidence for negative rather than positive dose-response trends in tumor occurrence.

Discussion and conclusions

The highest doses given to any animal groups in the 10 bioassays analyzed were 5,873 mg/kg bw/day and 4,841 mg/kg bw/day in female and male mice, respectively. Despite the extremely high doses, there was no evidence of reduced survival in this study. On the contrary, there was a statistically significantly enhanced survival in male mice in this study, as well as in male animals in several other bioassays. The use of the individual animal data allowed the authors to distinguish between an adenoma and a carcinoma occurring in separate animals and both tumors occurring together in a single animal.

Knowledge of the age at death of each individual animal is required for the conduct of the poly-3 test. In addition to the application of the poly-3 test, which is an age-adjusted Cochran-Armitage test, age was also controlled by keeping the numbers of animals surviving to final sacrifice in each dose group the same in all permutations as in the original data.

In the primary analysis of all bioassays 525 poly-3 analyses were conducted of individual tumor responses, of which a total of 174 were on combinations of individual tumor types that may have similar etiologies. In the primary analysis, individual tumors can appear in more than one poly-3 analysis. Since this will happen in the original data and the permuted data with equal frequency, it will not bias the analysis. Also a reduced analysis was conducted in which individual tumors and combinations of tumors were removed from the analysis if they were part of a more inclusive tumor combination. This reduced analysis involved 304 poly-3 analyses. Results from these two analyses were quite similar.

The smallest poly-3 p-value (0.0013) found in the analysis of all the datasets was that for hemangiosarcoma in male mice in the Atkinson *et al*. study. The analysis showed that the actual false positive rate for this finding after accounting for multiple comparisons was 0.26 in the primary analysis and 0.17 in the reduced analysis. Neither the 05-test nor the 01-test gave a false positive rate that was clearly less than 0.05 in the primary and reduced analysis. The statistically significant ($p = 0.0013$) response of 8 % in CD-1 males in the Atkinson *et al*. study resulted from 4 hemangiosarcomas at a dose level of 1,000 mg/kg bw/day, with no hemangiomas or hemangiosarcomas reported at the 3 lower doses. The CD-1 male mice in the Knezevich and Hogan study were exposed to 4,831 mg/kg bw/day, a dose nearly 5 times that used in the Atkinson *et al*. study with no hemangiomas or hemangiosarcomas. Moreover, the incidence of 8 % was still within the historical control range reported for male CD-1 mice by Atkinson *et al*. (0-8 %) and Giknis and Clifford (2005) (0-12 %). The 10 % incidence in hemangioma/ hemangiosarcoma in female CD-1 mice reported in the Sugimoto (1997) study is within the historical control range of 0-12 % of hemangiosarcoma reported by Giknis and Clifford (2005). The lack of a consistent dose response in either males or females suggests that finding significant responses in hemangioma and hemangiosarcoma in both sexes of mice may be attributable to chance, especially considering that this represents the “worst case” of more than 100 tumor sites/types in these bioassays that could have shown evidence of carcinogenicity.

The only other tumor in the mouse studies that the IARC regarded as being clearly related to glyphosate exposure was the marginally significant increase (0/49, 0/49, 1/50, 3/50) in kidney adenoma in male mice observed in the Knezevich and Hogan study. However, additional step sectioning of kidneys in the dosed and control groups revealed one kidney adenoma in the control group, but no additional kidney tumors in the dosed groups. Since the new data provided did not identify this control animal, this additional tumor could not be taken into account in the analysis of this paper. When this tumor-bearing control animal is taken into account in the trend analysis the trend is not anymore statistically significant, adding to the evidence that the tumor increases reported in the glyphosate studies are due to chance.

Comparing the results of negative dose-related trends with those testing for a positive trend, the evidence for an effect was stronger for negative than for positive trends. The smallest p-value for a positive trend was 0.0013 versus 0.0008 for a negative trend, although the corresponding false positive rates after correcting for multiple comparisons were 0.26 and 0.11 demonstrating how adjusting for multiple comparisons can change the interpretation of analyses of individual tumors. The only clearly significant results for any of the 3 permutation tests were the highly significant 01-tests for negative trend in both the primary and reduced analysis.

In all 10 bioassays, the analysis made in this paper identified 24 tumors that exhibited a poly-3 positive trend with a p-value ≤ 0.05. Nevertheless, after accounting for the multitude of statistical tests this analysis did not find that number statistically significant ($p = 0.08$). The statistical analysis of 10 glyphosate bioassays presented in this paper found no strong statistical evidence that glyphosate is carcinogenic. The main cause for the discrepancy between the analysis made by IARC and that of the authors appears to be that IARC failed to consider the large number of statistical tests performed in the multiple bioassays they reviewed and the resulting multiple comparison problem. IARC and other organizations involved with interpreting results from large data sets to which a large number of statistical

tests have been applied should consider applying analyses of the type used in this paper to make informed and reasonable decisions. The present analysis provides new information on the potential carcinogenicity of glyphosate by being the first to provide results from statistical tests with correct false positive rates. These tests found no strong or convincing evidence that glyphosate is an animal carcinogen.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Ten cancer bioassays of sufficient quality and which allowed the analysis of individual animal data was selected for the application of a multi-response permutation procedure that adjusts for the large number of tumors eligible for statistical testing and provides valid false-positive probabilities. The statistical tests applied in the analysis were functions of p-values obtained from conventional continuity-corrected poly-3 tests for trend applied to each type of tumor or combination of tumor types in each bioassay. Results from 3 multi-response permutation tests are reported and discussed: the "min-test", the "05-test" and the "01-test". In the min-test, the test statistic is the smallest p-value obtained from applying the poly-3 test to all tumor types in all bioassays investigated. Animals are randomly reassigned to dose groups in a Monte Carlo analysis, keeping the total numbers of animals in each dose group equal to the number in the original data. The tumors in each such reassignment are analyzed using the poly-3 test in exactly the same way as in the original data. Males and females are permuted separately. The false positive rate is the proportion of random reassignments that result in a smallest poly-3 p-value that is smaller than or equal to the smallest poly-3 p-value obtained from the original data. The test statistics for the 05-test and the 01-test are the number of poly-3 tests of tumors in the original data for which the p-value is less than or equal to the critical value of 0.05 or 0.01, respectively. In all applications of the poly-3 test, the test is applied only to data from one sex in a single study and the p-values from the poly-3 tests of all the studies are combined to create the "global" tests (min-test, 05-test and 01-test) to give the correct false positive rates. In addition to the randomization procedures for testing for positive dose-response trends in tumor incidence, the same procedures were repeated after reconfiguring the poly-3 test for negative trends. When the frequency of poly-3 p-values for positive trend computed from all tumors in all 10 bioassays in which at least two tumors occurred are considered there is an excess of large p-values (close to 1.0) compared to small p-values (close to 0.0). Since the version of the poly-3 trend test applied is a one-sided test for a positive trend, p-values close to 1.0 would translate into p-values near 0.0 for one-sided trend tests for anti-carcinogenicity. Results of tests for a dose-related decrease in survival in each study show that in none of the bioassays analyzed this test was statistically significant. Moreover, 4 of the datasets had p-values in excess of 0.95 which indicates a significant positive trend in survival with increasing dose. The most significant poly-3 trend in all 10 bioassays was found in the Atkinson *et al.* (1993) study for hemangiosarcoma in male mice with a p-value of 0.0013. The actual significance of this smallest p-value, which is the false positive rate for the min-test, was 0.26 based on the primary analysis, rather than the naive value of 0.0013. This means that 26 % of the randomizations of the 10 datasets gave a smallest p-value less than or equal to the smallest p-value obtained from the original data. Besides, the incidence in hemangiosarcomas (8 %) remained within the historical control range and no such tumors were identified in another mouse study at a dose level nearly 5 times of that used in the Atkinson *et al.* (1993) study. Overall, these findings suggest that, after accounting for the number of statistical tests performed, there was no clear evidence of a positive dose-related trend in tumor occurrence. The 01-test for a negative trend was highly significant with a p-value of 0.002. These findings suggest stronger evidence for negative rather than positive dose-response trends in tumor occurrence. In all 10 bioassays investigated, the analysis made in this paper identified 24 tumors that exhibited a poly-3 positive trend with a p-value of less than or equal to 0.05. Nevertheless, after accounting for the multitude of statistical tests this analysis did not find that number statistically significant ($p = 0.08$). The statistical analysis of 10 glyphosate bioassays presented in this paper found no strong statistical evidence that glyphosate is carcinogenic. This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because state-of-the-art statistical methods were employed to a selected set of cancer bioassays to demonstrate false-positive probabilities.

Reliability criteria for *in vivo* toxicology studies

Publication: Crump *et al*., 2020.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N.A.	
Study performed according to GLP	N.A.	
Study completely described and conducted following scientifically acceptable standards	Y	Statistical re-analysis of 10 selected bioassays for which individual animal data are available.
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Provided in the original bioassays.
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	Provided in the original bioassays.
Test conditions clearly and completely described	Y	Provided in the original bioassays.
Route and mode of administration described	Y	Oral *via* the diet.
Dose levels reported	Y	Provided in the original assays. The maximum doses reported are 5,873 mg/kg bw/day and 4,841 mg/kg bw/day in female and male mice, respectively.
Number of animals used per dose level reported	Y	50 – 64 animals per dose group.
Method of analysis described for analysis test media	N	Should be provided in original bioassays.
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	N.A.	Statistical re-analysis of all tumor sites.
Statistical methods described	Y	Application of a multi-response permutation procedure providing valid false-positive probabilities.
Historical control data of the laboratory reported	Y	For some tumors.
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because state-of-the-art statistical methods were employed to a selected set of cancer bioassays to demonstrate false-positive probabilities.		

1. Information on the study

Data point:	CA 5.6
Report author	Ganesan S. *et al*.
Report year	2020
Report title	Absence of glyphosate-induced effects on ovarian folliculogenesis and steroidogenesis
Document No	Reproductive Toxicology, (2020) 96, 156-164 DOI: 10.1016/j.reprotox.2020.06.011
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

Glyphosate (GLY) is an herbicidal active ingredient and both in vitro and in vivo studies suggest that GLY alters ovarian function. To determine if a chronic GLY exposure model affected steroidogenesis or folliculogenesis in vivo, postnatal day 42 C57BL6 female mice were orally delivered vehicle control (saline) or GLY (2 mg/Kg) from a pipette tip five days per week for either five or ten weeks. Mice were euthanized at the proestrus stage of the oestrous cycle. GLY exposure did not impact body weight gain, organ weights, or healthy follicle numbers. In addition, GLY exposure did not affect abundance of ovarian mRNA encoding kit ligand (*Kitlg*), KIT proto-oncogene receptor tyrosine kinase (*c-Kit*), insulin receptor (*Insr*), insulin receptor substrate (*Irs1* or *Irs2*) and protein thymoma viral proto-oncogene 1 (AKT) or phosphorylated AKT. Ovarian mRNA or protein abundance of Star, 3β-hydroxysteroid dehydrogenase (*Hsd3b1*), *Cyp11a1* or *Cyp19a* were also not altered by GLY. Circulating 17β-estradiol and progesterone concentration were unaffected by GLY exposure. In conclusion, chronic GLY exposure for five or ten weeks did not affect the ovarian endpoints examined herein.

Materials and methods

Reagents

Glyphosate (CAS # 1071-83-6), 2-β-mercaptoethanol, Tris base, Tris HCL, Sodium chloride, EDTA, SDS, NaF, HEPES and Tween -20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Ponceau S, Invitrogen iBlot 2NC regular stacks, Pierce BCA protein assay kit, Glycerol, Citric acid, Saline, DAPI nuclear stain and Sodium citrate were obtained from Thermo Fisher Scientific. Mini-PROTEAN TGX™ precast protein gels and protein size markers were purchased from BioRad. RNeasy Mini kit, QIA shredder kit, RNeasy Min elute kit, and Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility (Ames, IA). Antibodies directed against AKT, phosphorylated AKT, CYP11A1, anti-mouse Alexa flour 488 and SignalFire ECL plus chemical luminescence detection kit were from Cell Signaling (Danvers, MA). Antibodies directed against ERα, STAR and donkey anti-rabbit IgG-FITC were from Abcam. Novus Biologicals (Centennial, CO) was the source of antibodies directed against HSD3β and CYP19A1. ELISA kits to measure E_2 and progesterone (P_4) were from DRG International Inc (Springfield, NJ).

Animals

The Iowa State University Institutional Animal Care and Use Committee approved all animal experiments in accordance with NIH guidelines. Postnatal day (PND) 42 C57BL/6 J female mice received vehicle control (saline; n = 20) or glyphosate (GLY; 2 mg/Kg/day; n = 20) per os for five days per week for a duration of 5 (n = 10 control; n = 10 GLY) or 10 weeks (n = 10 control; n = 10 GLY). Mice were housed in groups of 3-4 and were provided with ad libitum access to food and drinking water. Body weight was obtained weekly. Vaginal cytological analysis was performed for 10 days in the 5-week exposure study and for 21 days in the 10-week study prior to euthanasia and the length spent in each stage of the oestrous cycle calculated as a percentage of the time analysed. Mice were euthanized at the proestrus phase of their oestrous cycle. Weights of the ovaries, uterus, liver, kidneys and heart were recorded. Blood samples were collected post-euthanasia by cardiac puncture.

Histological analysis

One ovary per animal (n = 10 per treatment) was fixed in 4 % paraformaldehyde for 24 h and transferred to 70 % ethanol and paraffin embedded. Ovaries were sectioned (5 μM thickness) and every 6th section was mounted and stained by hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 12th section. Unhealthy follicles were distinguished from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Follicles were classified and enumerated as previously described (Flaws *et al*., 1994).

Quantitative reverse transcriptase polymerase chain reaction

Approximately 25 % of each ovary was used for RNA isolation and two ovarian samples were combined (n = 10 ovaries; 5 RNA samples). RNA was isolated using a RNeasy Mini kit and the RNA concentration determined using a Nano Drop spectrometer (λ = 260/280 nm; ND 1000; Nanodrop Technologies Inc., Wilmington, DE). RNA (200 ng) was reverse transcribed to cDNA, followed by quantitative PCR. Primers for specific genes of interest were designed by Primer 3 Input Version (0.4.0) (listed in Table 1). The regular cycling program consisted of a 15 min hold at 95 °C and 45 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s at which point data were acquired. Each sample was normalized to 18 s RNA before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Table 1: Primer sequences

Gene	Forward Primer	Reverse Primer
c-Kit	ttctccttaggaagcagccc	cctgcttgaatgttggcctt
Kit-lg	tcagtcatagattggagtttgca	tgtatcaaaagggtcgggaca
Akt	tttgttgctgtgtcccatgc	caagtgctaggagaagggct
Star	atgttcctcgctacgttcaag	cccagtgctctccagttgag
Hsd3b1	gctggaaactgtgagcttcc	tgcttcctcccagttgacaa
Cyp11a1	aggtccttcaatgagatcccctt	tccctgtaaatggggccatac
Cyp19a1	atgttcttggaaatgctgaaccc	aggacctggtattgaagacgac
ERa	aattctgacaatcgacgccag	gtgcttcaacattctccctcctc
Erb	tgctccaagggtaggatggac	ctgtgcctcttctcacaagga
Insr	tgtcatcaatgggcagtttg	atcaggttccgaacagttgc
Irs1	ctatgccagcatcagcttcc	ggaggatttgctgaggtcat
Irs2	gaagcggctaagtctcatgg	gacggtggtggtagaggaaa

Western blot analysis

Ovaries (n = 10 per treatment) were homogenized in tissue lysis buffer (containing protease and phosphatase inhibitors) and centrifuged at 10,000 rpm twice for 15 min. The protein concentration of the supernatant was measured using a bicinchoninic acid assay and stored at -80 °C until further use. Protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5 % milk in Tris-buffered saline containing tween 20. Membranes were incubated in primary antibodies directed against protein kinase B (AKT; primary - 1:200; secondary - 1:500), phosphorylated AKT (pAKT; primary - 1:200; secondary - 1:200), estrogen receptor alpha (ERα; primary - 1:500; secondary - 1:500), estrogen receptor beta (ERβ; primary - 1:500; secondary - 1:200), steroidogenic acute regulatory protein (STAR; primary - 1:500; secondary - 1:200); 3-β hydroxysteroid dehydrogenase (HSD3β; primary - 1:1000; secondary - 1000), cytochrome P450 isoform 11A1 (CYP11A1; primary - 1:500; secondary - 1:500) and cytochrome P450 isoform 19A1 (CYP19A1; primary - 1:500; secondary - 1:500) overnight at 4 °C with rocking. Following three washes in TTBS (1X) membranes were incubated with species-specific secondary antibody for 1 h at room temperature. Autoradiograms were developed on X-ray films in a dark room following 10 min incubation of membranes with 1X SignalFire™ ECL reagent. Densitometry of the appropriate-sized bands was measured using Image Studio Lite Version 3.1 (LI-COR Biosciences, Lincoln, NE) which eliminates background noise. The sum of the gray values of all the pixels in the selection divided by the number of pixels, or mean grey value was quantified for each membrane using ImageJ software. Membranes were normalized to Ponceau S protein staining in which the entire lane of the transferred protein was quantified to account for loading variation. To ensure antibody specificity, negative control blots for each antibody used were performed in which the membranes were incubated with primary antibody only, secondary antibody only, or normal IgG in place of primary antibody with the inclusion of the appropriate secondary antibody. No protein bands were observed on these control blots indicating the specificity of the protein bands detected and analysed.

Immunofluorescence staining

Paraffin embedded ovaries (n = 5 per treatment) from the 10 week exposure group were serially sectioned (5 μM thick) and every 10^{th} section was mounted. Sections were deparaffinised in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1 M, pH 6.1). Sections were blocked in 5 % BSA for 1 h at room temperature. Sections were incubated with primary antibodies directed against pAKT (1:50 dilution), STAR (1:500 dilution), HSD3β (1:500 dilution), CYP11A1 (1:100 dilution), CYP19A1 (1:100 dilution) and ERα (1:200 dilution) overnight at 4 °C. After washing in 1 % PBS, sections were incubated with the appropriate donkey anti-rabbit IgG-FITC (1:200 to 1:500 dilutions) or anti-mouse Alexa flour 488 (1:200 dilution) secondary antibody for 1 h. Slides were counterstained with 4-6-diamidino-2-phenylindole (DAPI) for 5 min. Negative technical controls incubated the ovarian sections omitting both the primary and secondary antibody to ensure specificity of the antibodies. Images were captured using a Leica DMI3000 B fluorescent microscope. Raw internal density of staining was analysed using Image J software. For analysis, 5 ovaries per treatment were assessed with three sections per ovary and 7-10 large follicles per slide analysed.

Steroid hormone quantification

Serum (25 μL; n = 10 per treatment) was added in duplicate per sample to an ELISA plate to measure E_2 or P_4. Plates were incubated for 60-90 min after adding the enzyme conjugate (100-200 μl). The wells were rinsed with wash solution three times, substrate solution added (100-200 μL) and incubated for 15-30 min. The enzymatic reaction was stopped by adding stop solution (50-100 μL) and the signal determined within 10 min using a plate reader at 450 nm absorbance.

Sample identity blinding

The identity of samples used for ELISA and follicle counting were not known to the investigator performing the analysis. In addition, mRNA for PCR and protein samples from mice treated for 10-weeks for western blotting were double blinded - the identity of the samples was unknown to the investigator and after western blots were completed, they were then identified by group for the purpose of statistical analysis. Only after the statistical analysis was completed was group identity revealed.

Statistical analysis
Raw data were analysed by unpaired t-test. All statistical analysis was performed using Prism 5.04 software (GraphPad Software). Statistical significance (*) was defined as $P < 0.05$. A tendency for a statistically meaningful difference was considering if the *P*-value was between 0.05-0.1

Results

Impact of GLY exposure on body weight gain
Body weight gain was determined from the onset to the completion of the dosing period which was either a duration of 5 or 10 weeks. As anticipated, mice gained weight with aging, however, there was no impact of GLY exposure on body weight gain at either time point (Figure 1).

Effect of GLY exposure on the estrous cyclicity
In mice exposed to GLY for either duration, no impact of GLY exposure on the percentage time spent at any stage of the oestrous cycle was observed, though there was a tendency ($P < 0.1$) for increased time spent in estrus and reduced time spent in metestrus/diestrus after 5 weeks of GLY exposure (Figure 2).

Endocrinological impact of GLY exposure
Mice were euthanized at the proestrus stage of the oestrous cycle, thus E_2 (Figure 3A) levels were higher than P_4 (Figure 3B). There was no effect of 5 or 10 weeks of GLY exposure on circulating E_2 (Figure 3A). No impact of GLY exposure on serum P_4 was noted at either timepoint (Figure 3B).

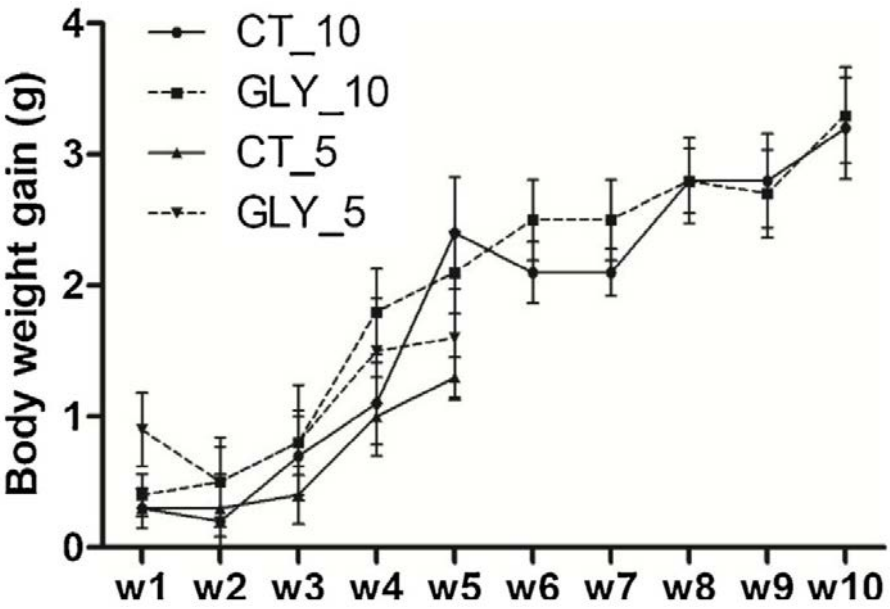
Relative organ weight effects of GLY exposure
There was no effect of either duration of GLY exposure on the relative weight of the heart (Figure 4A,F), liver (Figure 4B,G), kidneys (Figure 4C,H), or uterus (Figure 4D,I). In addition, neither 5 weeks (Figure 4E) or 10 weeks of GLY exposure (Figure 4J) affected ovarian weight, although a tendency ($P < 0.1$) towards a reduction in ovarian weight after 10 weeks of exposure was observed.

Impact of GLY exposure on ovarian follicle number
GLY exposure for 5 (Figure 5A) or 10 weeks (Figure 5B) did not influence the number of ovarian primordial, primary, secondary or antral follicles. There was also not a different between CT and GLY treated mice at either 5 or 10 weeks in total follicle number or the percentage of follicles per developmental stage within the ovary. Corpora lutea were evident, thus, there were not a defect in ovulation noted and there was no different in the number of CL per ovary due to GLY exposure (data not shown and Figure 5C-F).

Effect of GLY exposure on ovarian mRNA encoding genes involved in ovarian folliculogenesis and steroidogenesis
GLY exposure did not impact abundance of ovarian mRNA encoding the PI3K members kit ligand (*Kitl*), KIT proto-oncogene receptor tyrosine kinase (*c-Kit*), or protein kinase b (*Akt*) after 5 (Figure 6A) or 10 (Figure 6D) weeks. GLY exposure also did not alter ovarian mRNA abundance of steroidogenic acute regulatory protein (*Star*), 3β-hydroxysteroid dehydrogenase (*Hsd3b*), cytochrome P450 (*Cyp*) *11a1* and *Cyp19a* after 5 (Figure 6B) or 10 weeks (Figure 6E) of exposure. In addition, there was no effect of GLY exposure on mRNA encoding the ovarian insulin receptor (*Insr*), insulin receptor substrate 1 (*Irs1*) or *Irs2*, estrogen receptor (*Er*) alpha (*Erα*) or *Er* beta (*Erβ*) mRNA abundance at either 5-weeks (Figure 6C) or 10-weeks (Figure 6F) post-exposure.

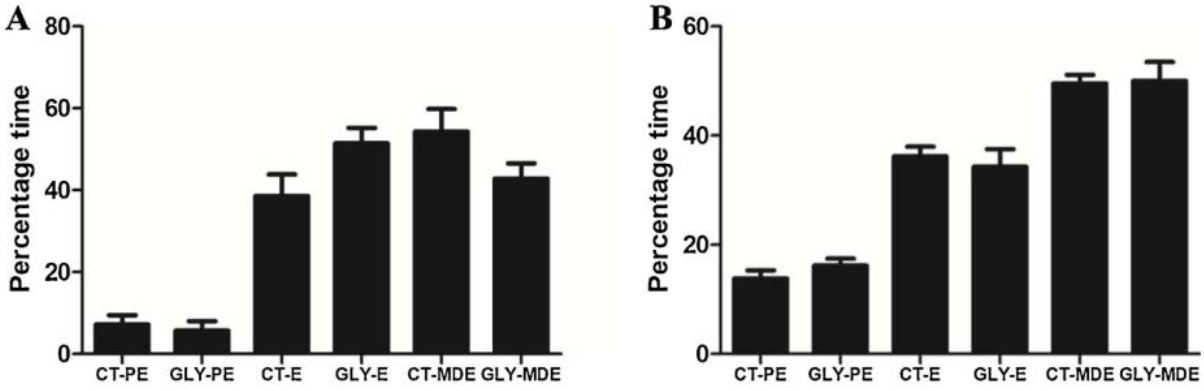
GLY exposure impact on ovarian folliculogenesis and steroidogenesis protein abundance
Using western blot analysis to interrogate changes to ovarian proteins involved in folliculogenesis or steroidogenesis, no impacts of 5 (Figure 7A,B) or 10 (Figure 7C,D) weeks of GLY exposure on ERα, ERβ, AKT, pAKT, STAR, HSD3β, CYP11A1, or CYP19A1 protein abundance were detected. Use of immunofluorescence staining in ovaries from the 10-week exposure group identified localization of pAKT (Figure 8A-C), STAR (Figure 8D-F), HSD3β (Figure 8G-I), CYP11A1 (Figure 8J-L), CYP19A1 (Figure 8M-O) and ERα (Figure 8 P–R) but, with the exception of a tendency for a reduction in HSD3B and CYP19A1 ($P < 0.1$); Figure 9A,B). there was no difference between saline- and GLY-treated mice in the internal density of immunostaining for these proteins.

Figure 1: Effect of glyphosate on body weight



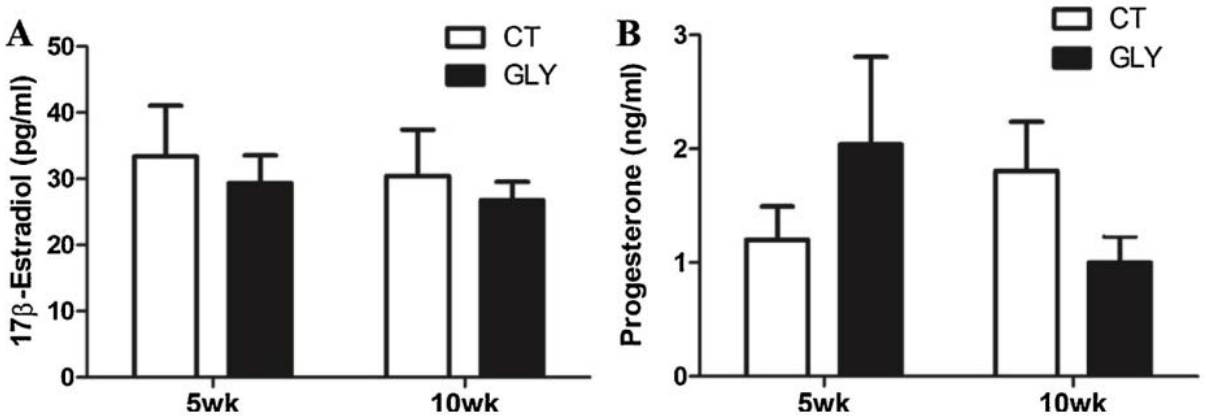
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for 5 or 10 weeks. Body weight was measured from day 0 of dosing to end of 5 or 10 weeks of dosing. Values are expressed as average body weight gained every week ± SEM; n = 10. Mice exposed to saline (CT) for 5 weeks indicated by solid line with triangle data point marker; Mice exposed to GLY for 5 weeks indicated by dashed line with triangle data point marker; Mice exposed to saline (CT) for 10 weeks indicated by solid line with circle data point marker; Mice exposed to GLY for 10 weeks indicated by dashed line with square data point marker.

Figure 2: Effect of GLY exposure on percentage time spent at stages of oestrous cycle



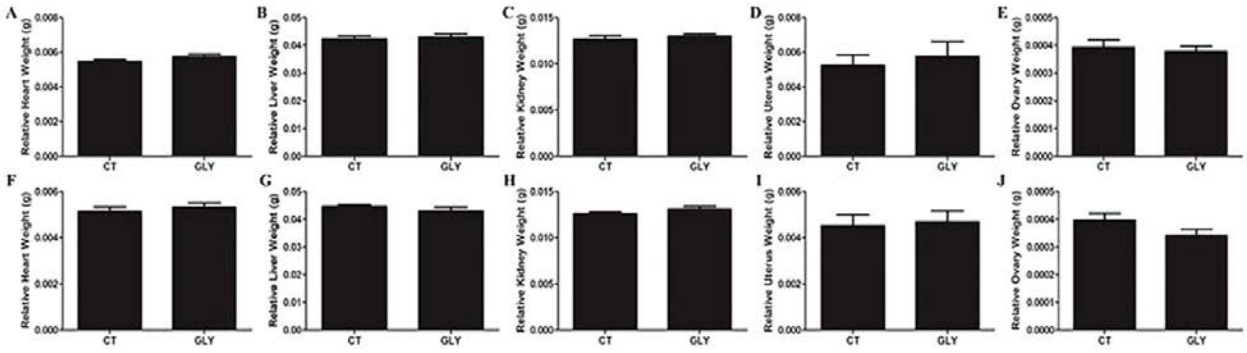
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A) 5 or (B) 10 weeks. The percentage time spent at stages of oestrous cycle were calculated: proestrus = PE; oestrus = E; metestrus and diestrus = MDE. Data points represent mean +/− SEM. Statistical analysis was performed on raw data.

Figure 3: GLY exposure effect on circulating E_2 and P_4



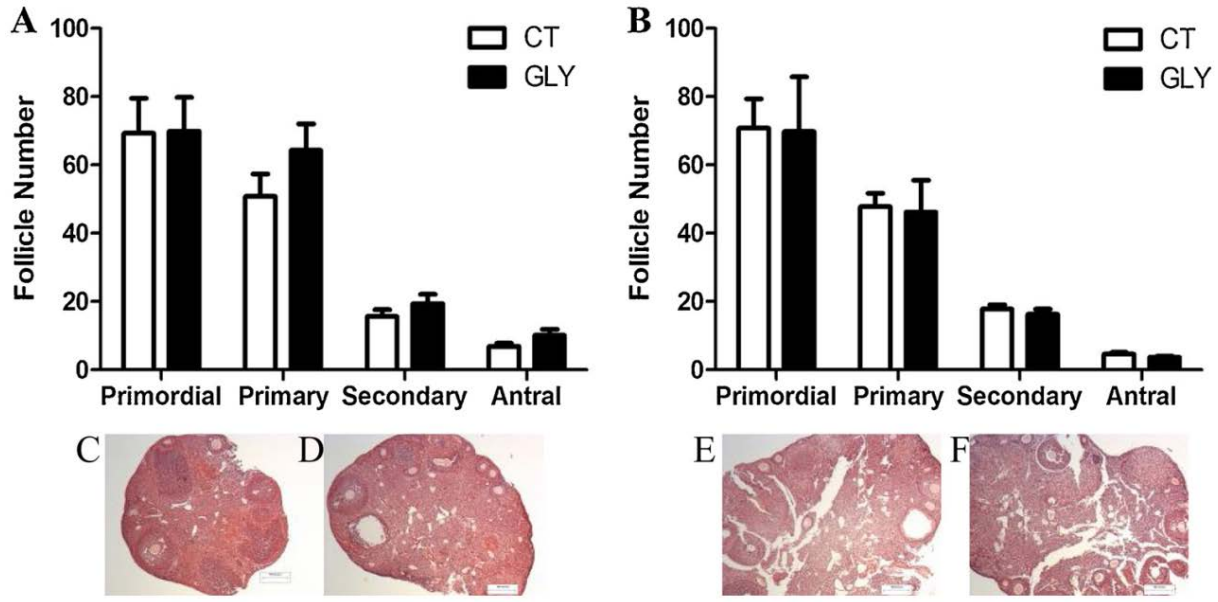
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for 5 or 10 weeks. Circulating (A) E_2 and (B) P_4 were measured by ELISA. Data points represent mean +/- SEM.

Figure 4: Relative organ weight impacts of GLY exposure



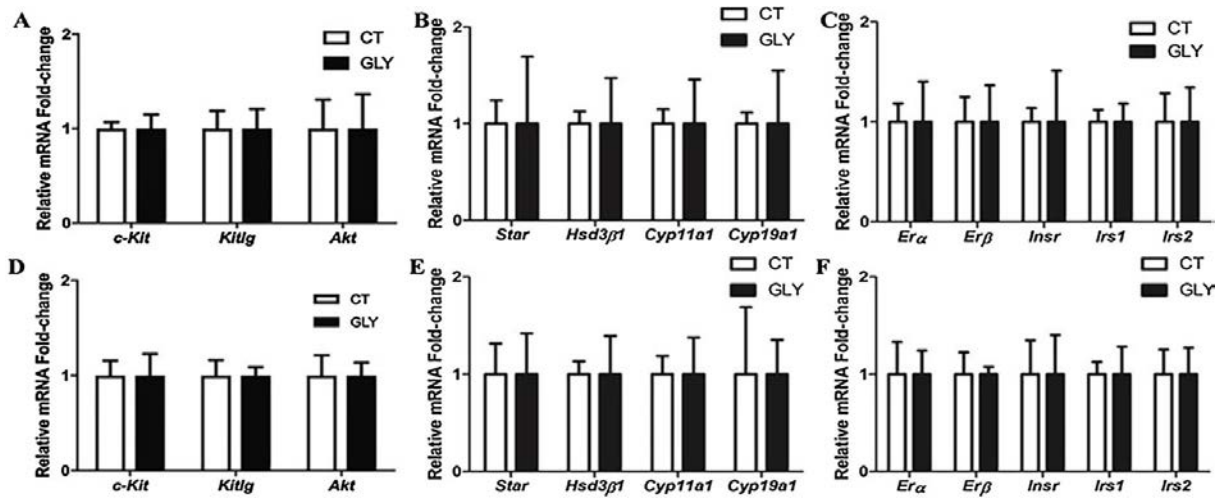
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A-E) 5 or (F-J) 10 weeks. Organ weights (g) were collected post-euthanasia and normalized to body weight: (A,C) Heart, (B,G) Liver, (C,H) Kidney, (D,I) Uterus and (E,J) Ovary weight. Data points represent mean +/- SEM.

Figure 5: Effect of GLY exposure on ovarian follicle number



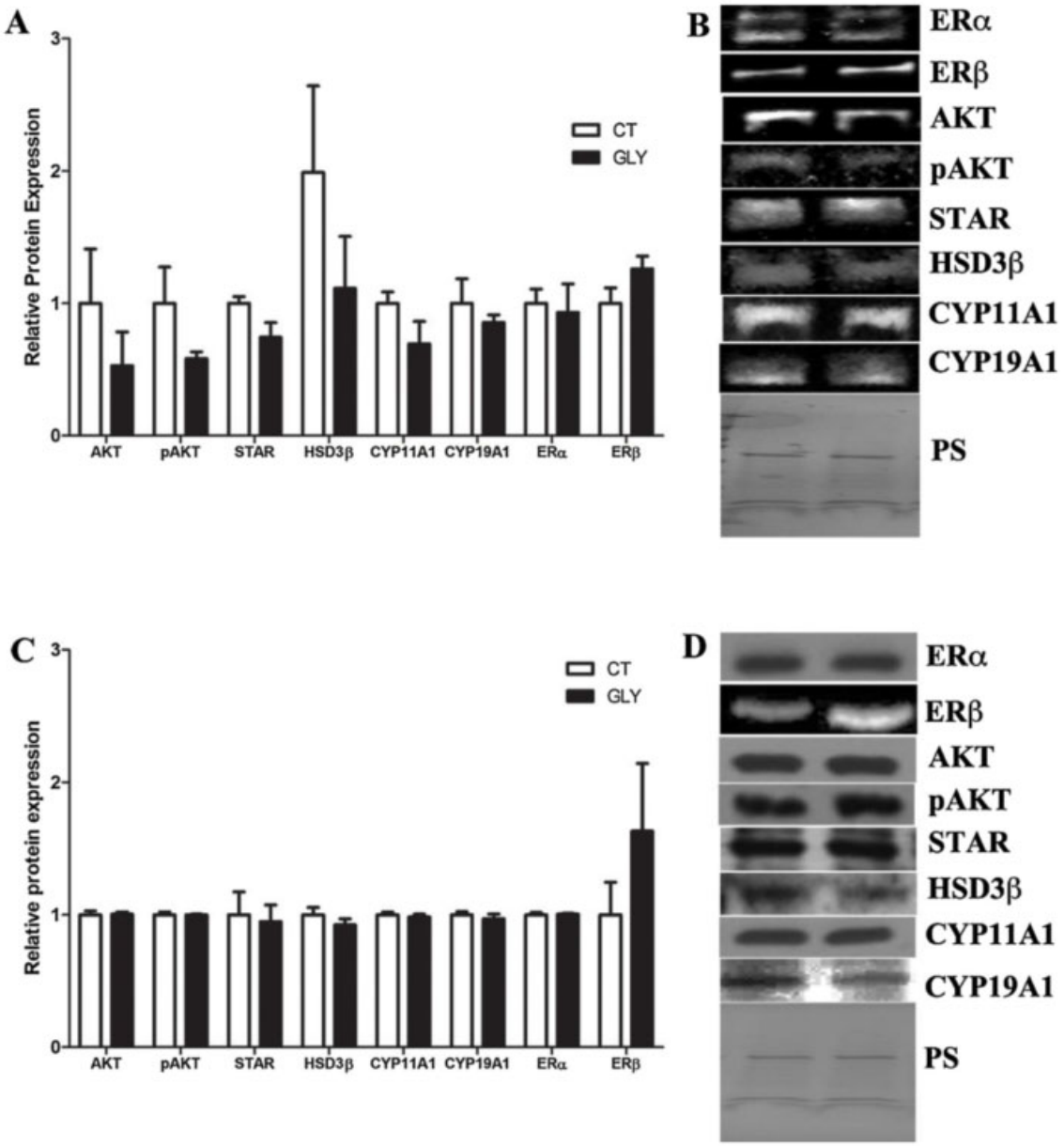
Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A) 5 or (B) 10 weeks. Ovaries were sectioned, stained with haemotoxylin and eosin and follicular stages classified and counted as primordial, primary, secondary, and antral. Data points represent mean +/- SEM. Representative (C) CT 5 week, (D) GLY 5 week, (E) CT 10 week and (F) GLY 10 week ovaries are provided.

Figure 6: Impact of GLY exposure on ovarian mRNA abundance



Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A-C) 5 or (D-F) 10 weeks. Ovaries were homogenized and mRNA isolated for qRT-PCR to analyse relative abundance of genes involved in (A,D) PI3K signaling; (B,E) Steroidogenesis; and (C,F) Endocrine signaling. Data points represent fold change relative to CT treated ovaries +/- SEM.

Figure 7: Consequence of GLY exposure on ovarian protein abundance



Female C57Bl/6 mice were exposed to saline vehicle control (CT) or GLY (2 mg/Kg) per os from a pipette tip five days per week for (A,B) 5 or (C,D) 10 weeks. Ovaries were homogenized and western blotting performed to quantify protein abundance of AKT, pAKT, STAR, HSD3B, CYP11A1, CYP19A1, ERA, and ERB. (A,C) Bars represent mean +/- SEM. (B,D) Representative images of western blots for each protein with ponceau S staining represented as PS.

Conclusions

Taken together, this study determined absence of GLY-induced alterations to the reproductive endpoints measured. After both 5 and 10 weeks of GLY exposure, relative to the saline vehicle control treated mice, any impacts of GLY on endpoints measured was largely absent. This study involved sample deidentification to remove any unintentional or perceived bias, was chronic in nature and performed at a conservative dosage (relevant to human exposure). In summary, the findings of the current study do not support that chronic, oral GLY at a dose considered non-hazardous alters oestrous cyclicity, follicle number, circulating ovarian steroid hormone levels and ovarian intracellular signalling in adult female mice.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In vivo study on post natal day 42, glyphosate administered to C57BL/6 J female mice at 0 or 2 mkd (10 mice/dose). Cyclicity, follicle number, circulating ovarian steroid hormone levels and ovarian intracellular signaling parameters were tested in adult female mice during 5 or 10 weeks. Glyphosate exposure for five or ten weeks did not affect the ovarian and endocrine endpoints examined.

The article is classified as reliable with restrictions for the following reasons: only 1 dose tested (no dose relationship can be evaluated), purity of glyphosate is not clear, method of analysis for analysis test media & no validation of the analytical method was described, no GLP status stated, no OECD guideline followed. Although no HCD were available in order to compare with the equivalent concurrent controls and test groups results, the results were negative and therefore did not require HCD to interpret or provide context to any findings.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vivo* Toxicology Studies		
Publication: Ganesan S. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	Not stated
Study completely described and conducted following scientifically acceptable standards	Y	Use of control group, 10 female mice/group, 1 dose + 1 negative control, statistical analysis performed, no randomisation of animals mentioned, no HCD
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate No Purity, no storage condition stated
Only glyphosate acid or one of its salts is the tested substance	N	Glyphosate
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	C57BL/6 J mice, age, origin stated but initial BW not included
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Per os
Dose levels reported	Y	0 and 2 mg/kg/d; 5 days/week for 5 and 10 weeks
Number of animals used per dose level reported	Y	10 mice/group 5 weeks (n = 10 control; n = 10 GLY) and 10 weeks (n = 10 control; n = 10 GLY)
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	

Reliability Criteria: *In Vivo* Toxicology Studies		
Publication: Ganesan S. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	Only 1 dose tested
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	only 1 dose tested (no dose relationship can be evaluated), purity of glyphosate is not clear, method of analysis for analysis test media & no validation of the analytical method was described, no GLP status stated, no OECD guideline followed. Although no HCD were available in order to compare with the equivalent concurrent controls and test groups results, the results were negative and therefore did not require HCD to interpret or provide context to any findings.
Not reliable	N	

1. Information on the study

Data point:	CA 5.8.3
Report author	Gastiazoro M.P. *et al.*
Report year	2020
Report title	Glyphosate induces epithelial mesenchymal transition-related changes in human endometrial Ishikawa cells via estrogen receptor pathway
Document No	Molecular and cellular endocrinology, (2020), Vol. 510, Art. No. 110841
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

Ishikawa cells were exposed to glyphosate (Gly) (0.2 μM and 2 μM) or 17β-estradiol (E2: 10^{-9} M). Gly increased cell migration and invasion ability compared to vehicle, as did E2. Moreover, a down regulation of E-cadherin mRNA expression was determined in response to Gly, similar to E2-effects. These results show that Gly promotes epithelial mesenchymal transition (EMT)-related changes in Ishikawa cells. When an ER antagonist (Fulvestrant: 10^{-7} M) was co-administrated with Gly, all changes were reversed, suggesting that Gly might promote EMT-related changes via ER-dependent pathway. The results are interesting evidences of Gly effects on endometrial cancer progression via the ER-dependent pathway.

Materials and methods

Substances

All reagents and chemicals were of analytical grade. Glyphosate (CAS N° 1071-83-6) used as the PESTANAL® analytical standard (purity grade ≤ 100%) and 17β-estradiol (E2; purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Fulvestrant (ICI 182,780; purity ≥ 99%) was purchased from Tocris Bioscience (Minneapolis, Minnesota, USA).

Cell culture

The human endometrial adenocarcinoma cell line Ishikawa was provided by Masato Nishida, Department of Obstetrics and Gynecology, University of Tsukuba. These cells were obtained from an endometrial adenocarcinoma of a 39-year-old woman in 1985 by Nishida, and established as ER- and PR-positive cell line. The cells were cultured in Dulbecco's modified Eagle's medium F12 (DMEM/F12) (Biowest, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% Insulin-Transferrin-Selenium A (ITS) (Gibco-BRL, Grand Island, NY, USA) and maintained in culture 75 cm^2 flask at 5% CO2 and 37 °C. The medium was replaced every 48 h. Ishikawa cells were grown to 80% of confluence and enzymatically detached by trypsin and (0.05%) EDTA at 37 °C.

Treatment conditions

The concentrations in the experiment were chosen based on the reference dose (RfD) of 1 mg of glyphosate/kg b.w. per day established by U.S. EPA and it was in a range between 0.2 μM and 200 μM (0.2 μM, 2 μM, 20 μM and 200 μM), an interval of concentrations that include the RfD. A 1 mM Gly stock solution was prepared with ethanol and different volumes were added during the treatment to achieve the corresponding Gly concentration. In detail, to final Gly concentration 0.2 μM in a 6-well plate with 2 ml media per well, 0.4 μl of Gly stock solution was added; to final Gly concentration 2 μM was added 4 μl; to

final Gly concentration 20 μM was added 40 μl of Gly stock solution and to final Gly concentration 200 μM was added 400 μl of Gly stock solution. As a positive control estradiol (E2) at 10^{-9} M was used. ER antagonist Fulvestrant was tested at concentration of 10^{-7} M. Dimethylsulfoxide (DMSO) was used as vehicle for E2 and Fulvestrant and added in a way that the DMSO concentration in the test did not exceed 0.1%. Ethanol was used as vehicle for Gly and added in a way that ethanol concentration in the test did not exceed 2%. No difference between DMSO and ethanol was detected among assays. For that reason, vehicle is shown as a unique condition in the results. To carry out the treatments, the cells were seeded in a 6-well plate for 24 h to establish adherent monolayer after that the corresponding drug-treatment was added depending on the assay. The medium with the corresponding amount of Gly, E2, or Fulvestrant was renewed every 24 h.

Trypan blue dye exclusion assay

The authors determined the viability of Ishikawa cells after treatment with selected concentrations of Gly by trypan blue dye exclusion assay. The aim was to determine whether the assessed concentrations altered cell viability in a significant way. Four concentrations of Gly were tested, Gly 0.2: 0.2 μM, Gly 2: 2 μM, Gly 20: 20 μM and Gly 200: 200 μM. The exclusion criterium was to reject the concentrations that cause a viability ≤80% and show statistical significant difference respect to vehicle.

Scratch-wound healing assay

The Ishikawa cells were seeded in 6-well plates at density of 200,000 cells/well with DMEM/F12 - FBS 10% - ITS 1% medium and incubated at 5% CO_2 and 37 °C with vehicle, E2 or different Gly concentrations with/without addition of Fulvestrant for 24 h. Then the monolayers were scratched using a sterile 200 μl micropipette tip, and washed with PBS twice to remove the detached cells. The different treatments were renewed and the DMEM/F12 - FBS 10% - ITS 1% medium was replaced with DMEM/F12 - FBS 1% - ITS 1% medium. The cells were incubated during a 72-h period, renewed the media and treatments every 24 h. The 72-h period was selected based on the frame time of incubation on which only under optimal condition (positive control) be close to closure the scratch. In order to measure the percentage of wound closure area by migrating cells, 8–10 images (from each treatment group) of the wounded cell monolayers were taken at 0 and 72 h after scratching at 40X using an Olympus CK40 Inverted Microscope (Olympus, Japan) coupled with a camera Canon Power Shot G9 (Canon, Japan). The average area of wound covered was calculated as percentage of wound closure after 72 h = (uncovered area at 0 h – uncovered area at 72 h)/uncovered area at 0 h x 100. The uncovered areas were determined using Image J software. The results were expressed as % wound closure after 72 h relative to vehicle.

Transwell invasion assay

The invasion assay was conducted using Falcon cell culture insert 8 μm pore size placed into a 24-well plate. The inserts were uniformly coated with Matrigel® basement membrane matrix (Corning Life Science, U.S.) for 1 h at 37 °C before cells were added. Cells were treated with vehicle, E2 or different Gly concentrations with/without addition of Fulvestrant for 24 h and then, harvested by trypsinization. Cells (1 × 105 cells/well) were seeded into the upper chamber in 200 μl of DMEM/F12-FBS 1% - ITS 1% medium, while the bottom of the chamber was incubated with 750 μl DMEM/F12-ITS 1% medium containing FBS 20% as a chemoattractant. After 48 h of incubation, cells migrating from the top chamber to the lower surface of the insert, as a result of cell invasion through Matrigel®, were fixed 3 min with formaldehyde 4% and 20 min with methanol. Finally, the cells were stained 20 min with crystal violet 0.1%. To quantify the number of invasive cells, all the slides with stained cells were photographed at 40X using an Olympus CK40 Inverted Microscope (Olympus, Japan) coupled with a camera Canon Power Shot G9 (Canon, Japan). Images were analysed using the cell counting tool in Image J software. The number of invasive cells after 48 h was determined. The results were expressed as number of invasive cells after 48 h relative to vehicle.

RNA extraction, reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

The Ishikawa cells were seeded in 6-well plates with DMEM/F12 - FBS 10% - ITS 1% medium and

incubated at 5% CO_2 and 37 °C with vehicle, E2 or different Gly concentrations with/without addition of Fulvestrant for 24 h. Total RNA from cultured cells was isolated after treatment using Tri-Fast™ (Peqlab VWR, Germany) according to the manufacturer's instructions. Genomic DNA contamination was removed by enzymatic digestion (RQ1 DNase, Promega, U.S.) and checked by PCR. First-strand cDNA synthesis was performed by mixing 2 µg of digested RNA with MMLV reverse transcriptase (Promega, U.S.) and Oligo (dT) 12–18 primers (Eurofins MWG Operon, Germany). Quantitative real-time PCR was applied for cDNA amplification with SybrGreen I as the detection dye using the iCycler iQ™ Real-Time PCR Detection System (BioRad, U.S.). Product purity was confirmed by dissociation curves and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, yielding no consistent amplification. To quantify the expression relative to the vehicle-treated cells, the ΔΔCT method was used. The expression of E-cadherin and Vimentin were normalized to the housekeeping gene ribosomal protein S18 (Rps 18). All PCR reactions were conducted from three independent cell culture experiments.

Statistical analysis

Data are presented as the mean ± SEM of three independent experiments. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls comparison test using the Graph-Pad Prism software Version 5.03 (San Diego, CA, USA). P-values < 0.05 were regarded as statistically significant.

Results

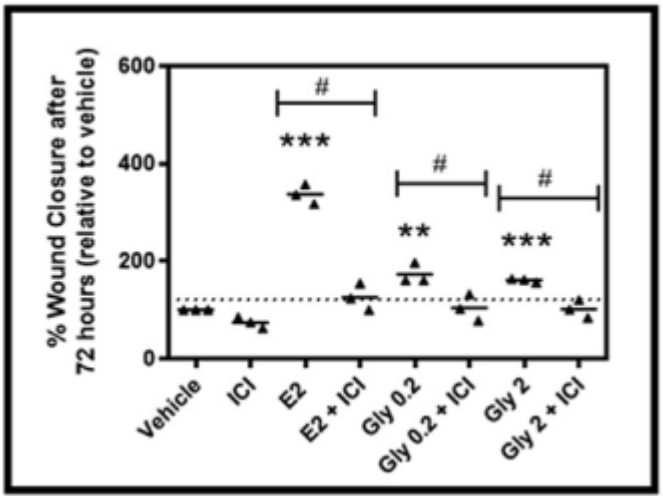
Effects of Glyphosate on cell viability of Ishikawa endometrial cancer cell

The two highest tested concentrations (20 µM and 200 µM) showed statistical significant difference respect to vehicle and the cell viability was lower than 80%. For that reason, these two concentrations were excluded. Contrary, 0.2 µM and 2 µM did not show differences respect to vehicle and the cell viability was kept above 80%. Based on these results, it was ensured that the cell survival is not altered by the two lower concentrations and it was decided to perform all the experiments with 0.2 µM and 2 µM.

Glyphosate induced Ishikawa endometrial cancer cell migration via estrogen receptor pathway

Migratory activity of Ishikawa cells was measured by scratch-wound healing assay. Fig. 1 shows the effects of Gly exposure on the percentage of wound closure after 72 h (relative to vehicle) evidencing that Gly 0.2 µM and Gly 2 µM promoted the migration of Ishikawa cells as did E2. In addition, there was no significant difference between Gly 0.2 µM and Gly 2 µM, for that reason migratory effect no dose dependency could be revealed. After Ishikawa cells were treated with E2 plus Fulvestrant or Gly plus Fulvestrant, the percentage of wound closure after 72 h did not change Fig. 1. In other words, the E2 or Gly effects were reversed by Fulvestrant.

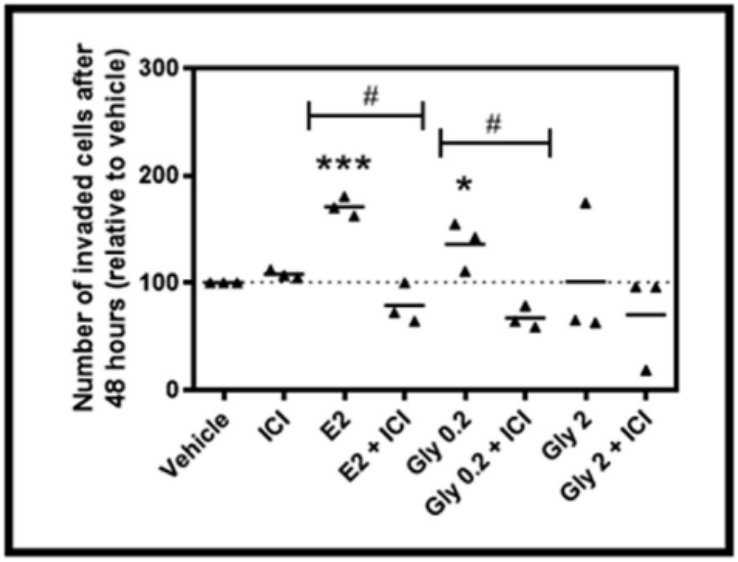
Figure 1: Effects of Gly and Fulvestrant treatment on migration ability of Ishikawa endometrial cancer cell. Results are presented as percentage of qound closure after 72 h relative to the vehicle control. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.



Glyphosate induces Ishikawa endometrial cancer cell invasion via estrogen receptor pathway

To investigate the invasiveness of Ishikawa cells, transwell invasion assay was conducted. Fig. 2 shows the effects of Gly exposure on the number of invaded cells compared to vehicle, evidencing that Gly 0.2 µM promoted the invasion of Ishikawa cells as did E2. Gly 2 µM did not show statistical difference. After Ishikawa cells were treated with E2 plus Fulvestrant or Gly plus Fulvestrant, the number of invasive cells after 48 h did not change, indicating that Fulvestrant attenuated the Gly 0.2 µM and E2 effects.

Figure 2: Effects of Gly and Fulvestrant treatment on invasion ability of Ishikawa endometrial cancer cell. Invasion ability was evaluated by the Transwell Invasion Assay with Matrigel®. The results are expressed as number of invasive cells after 48 h and presented as a percentage of the vehicle. Data expressed as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

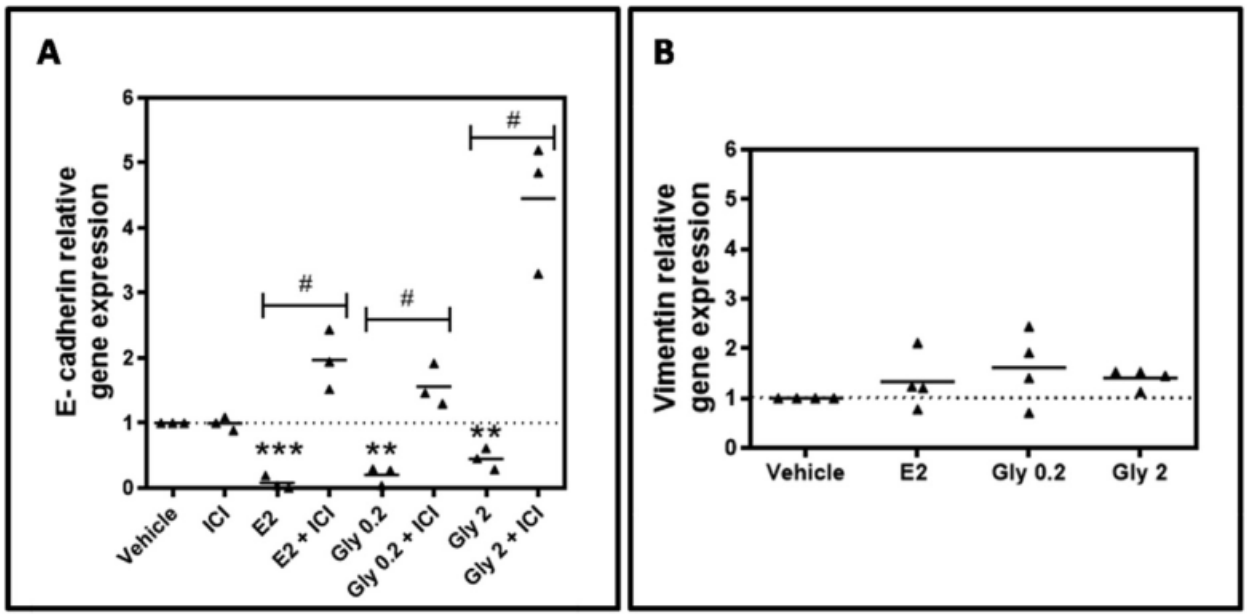


Glyphosate down-regulates E-Cadherin mRNA expression in Ishikawa endometrial cancer cells via estrogen receptor pathway

E-cadherin and vimentin mRNA expression was determined 24 h after the application of different concentrations of Gly or E2 (10^{-9} M). Fig. 3A shows that Gly down regulated E-cadherin mRNA expression

of Ishikawa cells, as did E2. Gly 0.2 μM produced a more pronounced effect on down regulation of E-cadherin mRNA expression in comparison with Gly 2 μM (Fig. 3A). Vimentin mRNA expression showed no changes with any of the treatments (Fig. 3B). After combinatorial treatment with E2 plus Fulvestrant or Gly plus Fulvestrant, E-cadherin mRNA expression was restored and even up regulated respect to the vehicle (Fig. 3A). This up regulation has higher potency to Gly 2 μM than E2 and Gly 0.2 μM.

Figure 3: Effects of Gly and Fulvestrant treatment on E-Cadherin gene expression (A) of Ishikawa endometrial cancer cell. Gly treatment effects on Vimentin gene expression (B) in Ishikawa endometrial cancer cell. Relative gene expression was quantified by RT-qPCR. The samples were normalized to the housekeeping gene RPS18. Data expressed as mean ± SEM. *p<0.05, **<0.01, ***p<0.001.



Conclusions

The results show that Gly promotes EMT process through the down regulation of E-Cadherin and increase cell migration and invasion abilities. Moreover, all changes could be prevented by the application of Fulvestrant (ER antagonist). According to our knowledge, these are the first pieces of evidence showing Gly effects on endometrial cancer cell progression via the ER-dependent pathway. The findings, in accordance with others, suggest that Gly might increase the risk of aggravating the disease for cancer patients.

Further studies are needed to shed light on the in vivo effects of Gly on the EMT process and cancer metastasis to contribute to the knowledge about carcinogenic potential of the herbicide.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Ishikawa endometrial cancer cells were treated with glyphosate at 0.2 µM and 2 µM. Glyphosate caused cell migration, invasion ability and down regulated E-cadherin mRNA expression. 17ß-estradiol, which was included as a positive control caused similar epithelial mesenchymal transition related changes, while treatment with fulvestrant (estrogen receptor antagonist) reversed the effects caused by glyphosate. The findings suggest that glyphosate has the ability to trigger the estrogen receptor-dependant pathway.

The relevance to human health risk assessment of this unvalidated *in vitro* research model in an immortal adenocarcinoma cell line containing estrogen and progesterone receptors is not clear. The results contradict a number of higher tier studies conducted across a variety of test systems,

The article is classified as reliable with restrictions for the following reason : glyphosate was tested at two different concentrations only, no test guideline was used and no historical control data were provided in order to compare with the equivalent concurrent controls and test groups results. Further, the study was not performed according to GLP.

Assessment and conclusion by RMS:

Reliability criteria for *in vitro* toxicology studies		
Publication: Gastiazoro M.P. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	Not stated
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate (purity ≤ 100%)
Only glyphosate acid or one of its salts is the tested substance	N	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Ishikawa cells
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	Not necessary
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.2 µM and 2 µM
Cytotoxicity tests reported	Y	Viability was assessed with tryptan blue dye exclusion assay
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	Y	
Dose-effect relationship reported	Y	

Reliability criteria for *in vitro* toxicology studies		
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	Glyphosate was tested at two different concentrations only, no test guideline was used and no historical control data were provided in order to compare with the equivalent concurrent controls and test groups results. Further, the study was not performed according to GLP.
Not reliable	N	

1. Information on the study

Data point:	CA 5.2.6
Report author	Lindberg T. *et al.*
Report year	2020
Report title	An integrated transcriptomic- and proteomic-based approach to evaluate the human skin sensitization potential of glyphosate and its commercial agrochemical formulations
Document No	Journal of proteomics, (2020) Vol. 217, pp. 103647
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

The objective of this study was to perform *in vitro* sensitization testing with a parallel MS-based proteomic approach to investigate the underlying cellular mechanisms induced. The experimental setup of the *in vitro* assay GARD™skin was used to stimulate cells of a human DC-like cell line with different materials, including glyphosate. As a first step, the DC maturation marker CD86 was investigated. While the positive control PPD led to a significant upregulation of CD86, glyphosate induced a significant downregulation of CD86. Furthermore, the skin sensitizing capacity of the test materials was determined. Glyphosate was predicted as a non-sensitizer.
Furthermore, the transcriptomic data were complemented with a proteomic analysis. The assembled protein groups, deduced from the peptides obtained from the MS analysis resulted in a clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate).

Materials and methods

Chemical and reagents

Dimethyl sulfoxide (DMSO), p-phenylene-diamine (PPD), HyClone™ minimum essential medium alpha modification with L-glutamine, ribo- and deoxyribonucleosides (MEM-α) and TRizol® were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was purchased from PeproTech (Rocky Hill, NJ, USA). Propidium iodide (PI), FITC-conjugated anti-human [CD86 (FUN-1), HLA-DR (L243), CD34 (581) and isotype control anti- IgG1 (MOPC-21)] and PE-conjugated anti-human [CD54 (HA58), CD80 (L307) and isotype control anti-IgG1 (MOPC-21)] antibodies were acquired from BD Biosciences (San Jose, CA, USA). FITC-conjugated anti-human CD1a (NA1/34) and PE-conjugated anti-human CD14 (TÜK4) antibodies were purchased from Dako (Santa Clara, CA, USA). Direct-zol™ RNA MiniPrep column purification kit and trypsin were acquired from Zymo Research (Irvine, CA, USA) and Promega Biotech AB (Madison, WI, USA), respectively. Hybridization buffer, reporter co-deset, capture probeset and nCounter® cartridges were purchased from NanoString® Technologies (Seattle, WA, USA). Protease inhibitor tables EDTA-free and Silica C18 UltraMicro spin columns were acquired from Roche Diagnostics GmbH (Mannheim, Germany) and The Nest group (Southborough, MA, USA), respectively. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

MUTZ-3-derived cells (DSMZ, Braunschweig, Germany) were cultured in MEM-α supplemented with 20% (v/v) fetal bovine serum (FBS) and rhGM-CSF (40 ng/mL), and maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cell viability was analyzed using LUNA™ automated cell counter (Logos Biosystems, Annandale, VA, USA), according to manufacturer's instructions. A cell viability value > 90% was considered satisfactory to carry out the experiments, which were performed using three independent batches of cells. Moreover, quality control analysis was performed for each cell batch using BD FACSCanto II flow cytometer (Biosciences, San Jose, CA, USA). For that, the following antibodies were used: FITC-conjugated anti-human CD1a, CD34, CD86 or HLA-DR, and PE-conjugated anti-human CD14, CD54 or CD80; FITC- or PE-anti-IgG1 were used as isotype controls, while staining with PI (1 μg/mL) was performed for cell viability analysis.

Cell exposure with the test materials

Cell exposures and selection of input non-cytotoxic concentration of each test material (e.g. test material concentration inducing 90% relative viability, when compared to unexposed cells, namely RV90 value) were performed as follows: test materials were diluted in appropriate vehicles (Table 1) and the cells (2 × 105 cells/mL) were exposed to different concentrations for 24 h. After that, flow cytometer analysis was conducted to evaluate the cell viability using PI staining and then to obtain the RV90 values. In the case of non-cytotoxic materials, an input concentration of 500 μM was used. After that, cells were exposed to each test material at input concentration (Table 1) to carry out the flow cytometric analysis of CD86 expression and transcriptional analysis. Cell exposures were performed in triplicate cell batch reactions, i.e. a new cell batch was used each exposure round, resulting in a total of 18 samples. Total RNA was collected by lysing 2× 105 cells/exposure in TRIzol® and stored at −20 °C until RNA purification and further processing for transcriptional analysis and GARD™ skin predictions.

GARD™skin assay

The *in vitro* assay GARD™skin (SenzaGen AB, Lund, Sweden) was used to predict the ability of chemical substances to induce skin sensitization. Total RNA was isolated from the cells lysed in TRIzol® reagent using the Direct-zol™ RNA MiniPrep column purification kit (Zymo Research), according to manufacturer' instructions. For each sample, concentration and RNA integrity was determined with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). The following gene expression analysis was performed on NanoString® GEN2 nCounter analysis system (NanoString® Technologies) using the GARD™skin assay. In brief, 5 μL RNA sample were mixed with 8 μL master mix, comprising hybridization buffer and reporter CodeSet, and 2 μL Capture ProbeSet followed by 24 h hybridization at 65 °C using a 3Prime thermal cycler (Bibby Scientific, Staffordshire, UK). Samples were then processed and transferred to a 12-well nCounter® cartridge in the GEN2 Prep station 5s set at high sensitivity followed by data collection on the Digital Analyzer 5s with an imaging resolution of 555 fields of view. The Reporter Code Count files (RCC) files were then downloaded from the Digital Analyzer and processed using SenzaGen software version 1.0 (SenzaGen AB), which conducts both NanoString® quality control and data normalization. A support vector machine (SVM) algorithm was used to assign a so-called decision value (DV) to each test material replicate. A test material is classified as skin sensitizer when the SVM median output value is > 0 of the three independent replicates, while a median DV < 0 classifies the material as a non-sensitizer.

Proteomic analysis

Protein extractions and concentration determination

For proteomic analysis, cells were also exposed to each test material at input concentration (Table 1). After 24 h, cells were harvested and processed for MS analysis by pelleting the chemically exposed cells in a maximum recovery Eppendorf tube followed by washing with phosphate buffered saline (PBS) before flash freezing of the pellet in liquid nitrogen. Cell pellets were stored at −80 °C before further MS processing. Each cell batch of chemical exposure was performed in duplicates of 2 × 106 cells/exposure, and thereafter

pooled to ensure a sufficient protein amount for MS analysis. In addition, triplicates of unexposed (water) samples were run for MS analysis. Following cellular exposure proteins were extracted from each sample by lysing the cells in 150 µL lysis buffer (8 M urea, 39 mM Tris, 5 mM MgAc, 4% CHAPS, protease inhibitor tablet) through three freeze/thaw cycles and separating supernatant and cell debris by centrifugation at 16000 ×g for 30 min. Protein extracts were stored in −80 °C until further processing. Protein concentration determination was performed using the Total Protein Micro-Lowry kit (Sigma-Aldrich) according to manufacturer's instructions. The concentration was determined by measuring the absorbance at 650 nm and 50 µg per sample were used for further processing.

Table 1: Test materials used for cell exposures.

Material	Abbreviation	CAS no.	Vehicle	Classification	Input concentration
Double-distilled water	H_2O	na	na	Unexposed	0.1% (v/v)
Dimethyl sulfoxide	DMSO	67-68-5	na	Vehicle control	0.1% (v/v)
p-Phenylenediamine	PPD	106-50-3	DMSO	Positive control	75 µM
Glyphosate	GLY	1071-83-6	H_2O	na	500 µM

na: not applicable

In gel trypsin digestion and mass spectrometry

Proteins were separated on an SDS-PAGE gel and digested into peptides using trypsin. Trypsinated peptides were then de-salted using UltraMicro spin columns (Silica C18, SUM SS18 V, Nest group) as described in Chawade et al.. Peptides were thereafter resuspended in 0.1% formic acid (FA) and loaded into an EASY-nano liquid chromatograpjy (LC) system 1200 (Thermo Fisher Scientific). Peptides were inected directly into the analytical column, a 15 cm long fused silica capillary (75 µm × 16 cm Pico Tip Emitter, New Objective, Woburn, MA, USA) packed in-house with C18 material ReproSil-Pur 1.9 µm (Dr Maisch GmbH, Germany). Peptides were separated using an 80 min gradient from 5% to 90% solvent B (80% acetonitrile, 0.1% FA, v/v) at a constant flow rate of 250 nL/min. The nLC system was coupled with a Q-Exactive™ HF-X (Hybrid Quadrupole-Orbitrap™) (Thermo Fisher Scientific) operated in a positive mode for data-dependent acquisition (DDA). The Orbitrap acquired the full MS scan with an automatic gain control (AGC) target value of 3 × 106 ions and a maximum fill time of 50 ms. The 20 most abundant peptide ions were selected from the MS for higher energy collision-induced dissociation (HCD) fragmentation (collision energy: 40 V). The instrument was scanning at a target MS1 resolution of 120,000 between 375 and 1500 m/z window with 15,000 MS/MS resolution for a target of 1 × 105 and a maximum injection time of 20 ms using an isolation window of 1.2 m/z.

Results

GARD™skin ASSAY

Following 24 h of exposure with pure glyphosate, cytotoxicity analysis was performed using PI staining to define the input concentration for each test material (Table 1). Cells were then exposed to the test materials at input concentrations for 24 h and the maturity state of the cells was assessed by measuring levels of cell surface expression of the co-stimulatory marker CD86. Only the positive control PPD induced a significant upregulation of CD86 expression ($p < 0.05$) in comparison to unexposed control. Cell exposure with pure glyphosate resulted in a modest downregulation compared to control ($p < 0.05$). No significant difference between DMSO and unexposed controls was observed.

Furthermore, skin sensitization hazard predictions were performed with the GARD™skin assay. Transcriptomic analysis of the 200 bio-markers from the GARD™skin prediction signature was carried out using the NanoString® nCounter™ System. The skin sensitizing hazard was predicted using an SVM algorithm where each test material replicate was given a DV. As expected, PPD and DMSO were correctly classified as skin sensitizer and non-sensitizer, respectively. Also, glyphosate was classified as a non-sensitizer with a mean SVM DV of −1.45.

Profiling of agrochemical-induced changes in the cellular proteome

Initially, peptide data, generated from the trypsin-digested cells exposed with test materials for 24 h were normalized and assembled to protein groups. PCA evaluation was used to identify patterns, i.e. which samples exhibit a similar protein expression profile. As a first step, unsupervised clustering of all protein groups was performed. A clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate) is observed. Additionally, by applying a multi-group comparison (FDR = 0.05) based on all the treatments, i.e. clustering of the individual treatments most alike based on their protein expression, a similar pattern was observed as when unsupervised clustering was applied.

Conclusions

The objective of this study was to perform *in vitro* sensitization testing with a parallel MS-based proteomic approach to investigate the underlying cellular mechanisms induced. The experimental setup of the *in vitro* assay GARD™skin was used to stimulate cells of a human DC-like cell line with different materials, including glyphosate. As a first step, the DC maturation marker CD86 was investigated. While the positive control PPD led to a significant upregulation of CD86, glyphosate induced a significant downregulation of CD86. Furthermore, the skin sensitizing capacity of the test materials was determined. Glyphosate was predicted as a non-sensitizer.
Furthermore, the transcriptomic data were complemented with a proteomic analysis. The assembled protein groups, deduced from the peptides obtained from the MS analysis resulted in a clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate).

3. Assessment and conclusion

Assessment and conclusion by applicant:
Investigation of molecular mechanisms in the skin sensitization process specifically focusing on DC activation using an integrated transcriptomic- and proteomic approach.
First, Mutz-3-derived cells were exposed to PPD, DMSO, unexposed sample and glyphosate. No cytotoxicity was observed for glyphosate and glyphosate was classified as non-sensitising.
Second, PPD, DMSO, unexposed sample and glyphosate were assembled to protein groups. A clear separation between sensitizers (PPD) and non-sensitizers (unexposed, DMSO, glyphosate) was observed. Data on glyphosate are consistent with other available validated assay results.

The article is classified as reliable with restrictions for the following reason: This is a non-validated test system. The purity and origin of glyphosate is unclear. only 1 dose tested (no dose relationship can be evaluated), no HCD were available in order to compare with the equivalent concurrent controls and test groups results.

Assessment and conclusion by RMS:

Reliability Criteria: *In Vitro* Toxicology Studies		
Publication: Lindberg T. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Not applicable because there is no OECD guideline for this type of test
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	The only information is that glyphosate's input concentration was 500 μM. The purity, origin etc. is unclear.
Only glyphosate acid or one of its salts is the tested substance	N	Glyphosate alone and EU non-representative formulations were tested (summary above is provided only for glyphosate)
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	n.a.	Not applicable as no metabolic activation system used
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	N	Not applicable as only 1 concentration was used
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	This is a non-validated test system. The purity and origin of glyphosate is unclear. Only 1 dose tested (no dose relationship can be evaluated). No HCD were available in order to compare with the equivalent concurrent controls and test groups results.
Not reliable	N	

1. Information on the study

Data point:	CA 5.5/027
Report author	Portier, C.J.
Report year	2020
Report title	A comprehensive analysis of the animal carcinogenicity data for glyphosate from chronic exposure rodent carcinogenicity studies
Document No	Environ Health (2020) Vol. 19, 18. https://doi.org/10.1186/s12940-020-00574-1
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	-/Reliable with restrictions

2. Full summary of the study according to OECD format

Abstract

Since the introduction of glyphosate-tolerant genetically-modified plants, the global use of glyphosate has increased dramatically making it the most widely used pesticide on the planet. There is considerable controversy concerning the carcinogenicity of glyphosate with scientists and regulatory authorities involved in the review of glyphosate having markedly different opinions. One key aspect of these opinions is the degree to which glyphosate causes cancer in laboratory animals after lifetime exposure. In this review, twenty-one chronic exposure animal carcinogenicity studies of glyphosate are identified from regulatory documents and reviews; 13 studies are of sufficient quality and detail to be reanalyzed in this review using trend tests, historical control tests and pooled analyses. The analyses identify 37 significant tumour findings in these studies and demonstrate consistency across studies in the same sex/species/strain for many of these tumours. Considering analyses of the individual studies, the consistency of the data across studies, the pooled analyses, the historical control data, non-neoplastic lesions, mechanistic evidence and the associated scientific literature, the tumour increases seen in this review are categorized as to the strength of the evidence that glyphosate causes these cancers. The strongest evidence shows that glyphosate causes hemangiosarcomas, kidney tumours and malignant lymphomas in male CD-1 mice, hemangiomas and malignant lymphomas in female CD-1 mice, hemangiomas in female Swiss albino mice, kidney adenomas, liver adenomas, skin keratoacanthomas and skin basal cell tumours in male Sprague-Dawley rats, adrenal cortical carcinomas in female Sprague-Dawley rats and hepatocellular adenomas and skin keratoacanthomas in male Wistar rats.

Materials and methods

Bioassay data - The animal carcinogenicity data analyzed in this publication were derived from the published literature, the EPA review, the addendum to the EFSA review prepared by the German Institute for Risk Analysis, the JMPR review, the review of the carcinogenicity of glyphosate by a panel of scientists on behalf of industry, and full laboratory reports. The 13 cancer bioassays considered acceptable for this evaluation are presented in Table 1.

Table 1: Characteristics of the cancer bioassays of glyphosate analyzed in this study

Study Reference	Duration (months)	Strain		Dietary exposure dose levels (mg/kg/day)	Animals per Group	Purity (%)	Comments on survival and weight
		Mouse	Rat				
A: Knezevich and Hogan (1983) [11]	24	CD-1		M: 0, 157, 814, 4841 F: 0, 190, 955, 5874	50	99.8	No survival differences, slight weight reduction in high dose (M)
B: Atkinson et al. (1993) [12]	24	CD-1		M: 0, 98, 297, 988 F: 0, 102, 298, 1000	50	> 97.0	No survival differences, no weight differences
C: Sugimoto (1997) [13]	18	CD-1		M: 0, 165, 838.1, 4348 F: 0, 153.2, 786.8, 4116	50	94.6–95.7	No survival differences, slight weight reduction in mid (F) & high dose (M + F)
D: Wood et al. (2009) [14]	18	CD-1		M: 0, 71.4, 234.2, 810 F: 0, 97.9, 299.5, 1081.2	51	95.7	No survival differences, no weight differences
E: Takahashi (1999a) [15]	18	CD-1		M: 0, 167.6, 685, 7470 F: 0, 93.2, 909, 8690	50	97.5	Reduced survival high dose (M), slight weight reduction in mid (M) & high dose (M + F). This study was only mentioned by JMPR [7] and provides limited tumor data.
F: Kumar (2001) [16]	18	S-A[a]		M: 0, 85.5, 285.2, 1077.4 F: 0, 104.5, 348.6, 1381.9	50	> 95.0	No survival differences, no weight differences
G: Lankas (1981) [17]	26		SD[b]	M: 0, 3.05, 10.3, 31.49 F: 0, 3.37, 11.22, 34.02	50	98.7	No survival differences, no weight differences
H: Stout and Ruecker (1990) [18]	24		SD[b]	M: 89, 362, 940 F: 0, 113, 457, 1183	50	98.7	No survival differences, slight weight reduction in high dose (F)
I: Atkinson (1993) [19]	24		SD[b]	M: 0, 11, 112, 320, 1147 F: 0, 12, 109, 347, 1134	50	98.9	No survival differences, slight weight reduction in high dose (M + F)
J: Enemoto (1997) [20]	24		SD[b]	M: 0, 104, 354, 1127 F: 0, 115, 393, 1247	50	95.7	Reduced survival high dose (M), slight weight reduction in high dose (M + F)
K: Suresh (1996) [21]	24		W[c]	M: 0, 6.3, 59.4, 595.2 F: 0, 8.6, 88.5, 886	50	96.8	No survival differences, no weight differences
L: Brammer (2001) [22]	24		W[c]	M: 0, 121, 361, 1214 F: 0, 145, 437, 1498	53	97.6	High-dose survived longer (M), reduced weight highest dose (M + F)
M: Wood et al. (2009) [23]	24		W[c]	M: 0, 165, 838.1, 4348 F: 0, 153.2, 786.8, 4116	51	94.7–97.6	No survival differences, no weight differences

[a]Swiss Albino mouse; [b]Sprague-Dawley rat; [c]Wistar rat

For twelve of these studies full study reports were available and most of them were conducted in accordance with the appropriate regulatory guidelines. A full study report was not available for the Takahashi (1999) study but the data on kidney tumours in males and malignant lymphomas in females could be obtained from a JMPR review.

Statistical tests - Individual tumour counts for the individual studies were re-analyzed using the exact Cochran-Armitage one-sided linear trend test in proportions. Re-analyses were conducted on all primary tumours where there were at least 3 tumours in all of the animals in a sex/species/strain combination. In addition, any tumour where a significant positive trend ($p \leq 0.05$) was seen in at least one study was also evaluated in all studies of the same sex/species/strain, regardless of the number of animals with the tumour. When adenomas and carcinomas were observed in the same tissue, a combined analysis of adenomas and carcinomas was also conducted. Pairwise comparisons between individual exposed groups and the control group are conducted using Fisher's exact test. To evaluate the consistency of a tumour finding across multiple studies using the same sex-species-strain combinations, logistic regression with individual background responses and dose trends are fit to the pooled data using maximum likelihood estimation. A common positive trend is seen in the pooled analysis when the null hypothesis that the slope is 0 is rejected (statistical p-value ≤ 0.05 using a likelihood-ratio test) in favor of the alternative that the slope is greater than 0. Heterogeneity is seen in the pooled analysis when the null hypothesis that the slopes are equal is rejected (statistical p-value ≤ 0.05 using a likelihood-ratio test) in favor of the alternative that at least one of the slopes is different. For CD-1 mice analyses were conducted separately for 18 month and 24 month studies and then a combined analysis was performed. Similar grouped analyses were conducted for SD rats with one study of 26 months and 3 studies of 24 months. Only the combined analysis over all study durations is provided. The same methods of analysis were used to evaluate the incidence of non-neoplastic toxicity in tissues where tumours were observed. In cases of rare tumours where the increase in incidence didn't reach statistical significance the test proposed by Tarone (1982) was applied using an appropriate historical control group. All analyses were done using MATLAB, version R2017b.

Results

The purpose of this analysis is to understand the potential of glyphosate to produce tumours across all studies and not one study at a time. Thus, rather than presenting the results of each study separately, this review focuses on the tumours that are seen as positive in any one study and compares the findings across all studies of the same tumour in the same sex/species/strain combination.

Re-analysis of the data from CD-1 mice - From Table 2 it can be derived that a significant ($p \leq 0.05$) positive trend was found in males for kidney adenomas and kidney adenomas/carcinomas in the Takahashi study, malignant lymphomas in the Sugimoto and the Wood studies, hemangiosarcomas in the Atkinson study, and alveolar-bronchiolar carcinomas in the Wood study. When Tarone's test was applied using historical control data then a significant increase was found for kidney adenomas in the Sugimoto study, kidney carcinomas in the Knezevich and Hogan study, kidney adenomas/carcinomas in the Knezevich and Hogan and the Sugimoto study, and hemagiosarcomas in the Sugimoto study. In females a significant ($p \leq 0.05$) positive trend was found for hemangiomas, harderian gland adenomas and adenomas/carcinomas in the Sugimoto study, alveolar-bronchiolar adenomas/carcinomas in the Atkinson study, and malignant lymphomas in the Takahashi study. A significant common trend was found for kidney adenomas, carcinomas and adenomas/carcinomas, hemangiosarcomas in males and hemangiomas and malignant lymphomas in females.

Table 2: P-values for the exact Cochran-Armitage trend test and pooled logistic regression analysis for tumours with at least one significant trend test ($p \leq 0.05$) or Fisher's exact test ($p \leq 0.05$) in male and female CD-1 mice.

Tumor	Individual study *p*-values for trend[a]					Common Trend	Heterogeneity Test
Males	A	B	C	D	E		
Kidney Adenomas	0.442 (0.138)[d]	0.938	0.062 (**0.009**)[d]	---[b]	**0.019**	**0.006**	0.268
Kidney Carcinomas	0.063 (**< 0.001**)[d]	0.938	---[b]	---[b]	0.250	**0.031**	0.546
Kidney Adenomas and Carcinomas	0.065 (**0.008**)[d]	0.981	0.062 (**0.009**)[d]	---[b]	**0.005**	**< 0.001**	0.106
Malignant Lymphomas	0.754	0.087	**0.016**	**0.007**	ND[c]	0.093	0.007
Hemangiosarcomas	0.505	**0.004**	0.062 (**0.005**)[d]	---[b]	ND[c]	**0.033**	0.007
Alveolar-Bronchiolar Adenomas	0.294	0.231	0.513	0.924	ND[c]	0.384	0.409
Alveolar-Bronchiolar Carcinomas	0.918	0.456	0.148	**0.028**	ND[c]	0.407	0.083
Alveolar-Bronchiolar Adenomas and Carcinomas	0.576	0.231	0.294	0.336	ND[c]	0.346	0.826
Females	A	B	C	D	E		
Hemangiomas	0.631	---[b]	**0.002**	0.438	ND[c]	**0.031**	0.155
Harderian Gland Adenomas	0.877	ND[c]	**0.040**	0.155	ND[c]	0.155	0.052
Harderian Gland Carcinomas	---[b]	ND[c]	---[b]	1.000	ND[c]	0.500	1.00
Harderian Gland Adenomas and Carcinomas	0.877	ND[c]	**0.040**	0.372	ND[c]	0.184	0.110
Alveolar-Bronchiolar Adenomas	0.999	0.144	0.800	0.656	ND[c]	0.996	0.211
Alveolar-Bronchiolar Carcinomas	0.183	0.110	0.623	0.601	ND[c]	0.268	0.544
Alveolar-Bronchiolar Adenomas and Carcinomas	0.985	**0.048**	0.842	0.688	ND[c]	0.982	0.241
Malignant Lymphomas	0.070[e]	0.484	0.294	0.353	**0.050**	**0.012**	0.995

[a] – Study A is Knezevich and Hogan [11] (Additional file 2: Table S1), Study B is Atkinson et al. [12] (Additional file 2: Table S2), Study C is Sugimoto [13] (Additional file 2: Table S3), Study D is Wood [14] (Additional file 2: Table S4), Study E is Takahashi [15] (Additional file 2: Table S5); [b] – three dashes "---" indicates all tumor counts are zero; [c] – ND indicates there is no data available for this tumor in this study; [d] – using historical control data (see text for details) and Tarone's test; [e] – Spleen composite lymphosarcomas (malignant lymphomas) are also significantly increased in female mice in this study (see Additional file 2: Table S1)

Re-analysis of the data from Swiss albino mice - The single study with Swiss albino mice (Kumar, 2001) shows a significant increase in hemangiomas in female mice ($p = 0.004$).

Re-analysis of the data from SD rats - From Table 3 it can be derived that a significant ($p \leq 0.05$) positive trend was found in males for testicular interstitial cell tumours in the Lankas study, hepatocellular adenomas and adenomas/carcinomas in the Stout and Ruecker study, kidney adenomas in the Enemoto study, skin keratoacanthomas in the Stout and Ruecker, Atkinson and Enemoto studies, and skin basal cell tumours in the Enomoto study. In females a significant ($p \leq 0.05$) positive trend was found for thyroid C-cell adenomas in the Stout and Ruecker study, C-cell carcinomas in the Lankas study, and adrenal cortical carcinoma in the Stout and Ruecker study. When Tarone's test was applied using historical control data then a significant increase was found for pancreatic islet cell adenomas in males in the Stout and Ruecker study, and thyroid C-cell adenomas/carcinomas in females in the Lankas study. A significant common trend was found for hepatocellular adenomas, kidney adenomas, skin keratoacanthomas and skin basal cell tumours in males. In females the common trend was statistically significant for adrenal cortical carcinoma.

Table 3: P-values for the exact Cochran-Armitage trend test and pooled logistic regression analysis for tumours with at least one significant trend test or Fisher's exact test ($p \leq 0.05$) in male and female Sprague-Dawley rats

Tumor	Individual study *p*-values for trend[a]				Common Trend	Heterogeneity Test
Males	G	H	I	J		
Testicular Interstitial Cell Tumors	**0.009**	0.296	0.580	0.594	0.461	0.105
Pancreas Islet Cell Adenomas	0.512	0.147 (**0.007**)[c]	0.974	0.859	0.849	0.143
Pancreas Islet Cell Carcinomas	0.251	1.000	–	0.500	0.731	0.166
Pancreas Islet Cell Adenomas or Carcinomas	0.316	0.206	0.974	0.844	0.875	0.185
Thyroid C-cell Adenomas	0.743	0.089	0.278	0.631	0.210	0.532
Thyroid C-cell Carcinomas	0.505	0.442	0.495	0.565	0.322	0.898
Thyroid C-cell Adenomas and Carcinomas	0.748	0.097	0.197	0.642	0.175	0.526
Thyroid Follicular-cell Adenomas	0.122	0.408	0.067	0.966	0.464	0.055
Thyroid Follicular-cell Carcinomas	---[b]	0.255	0.443	1.000	0.448	0.137
Thyroid Follicular-cell Adenoma and Carcinoma	0.122	0.232	0.099	0.986	0.446	0.031
Hepatocellular Adenomas	0.471	**0.015**	0.325	0.500	**0.029**	0.664
Hepatocellular Carcinomas	0.062	0.637	0.760	0.642	0.803	0.269
Hepatocellular Adenomas and Carcinomas	0.173	**0.050**	0.480	0.690	0.144	0.428
Kidney Adenomas	0.938	0.813	1.000	**0.004**	**0.039**	0.002
Skin Keratoacanthomas	---[b]	**0.042**	**0.047**	**0.029**	**< 0.001**	0.998
Skin Basal Cell Tumors	0.251	0.249	1.000	**0.004**	**< 0.001**	0.009
Females	G	H	I	J		
Thyroid C-cell Adenomas	0.679	**0.049**	0.207	0.912	0.287	0.150
Thyroid C-cell Carcinomas	**0.003 (< 0.001)**[c]	0.500	---[b]	---[b]	0.385	0.041
Thyroid C-cell Adenomas and Carcinomas	0.072 (**0.037**)[c]	0.052	0.207	0.912	0.275	0.071
Adrenal Cortical Adenoma	0.851	0.603	---[b]	0.626	0.713	0.750
Adrenal Cortical Carcinoma	0.386	**0.015**	0.493	---[b]	**0.031**	0.199
Adrenal Cortical Adenoma and Carcinoma	0.801	0.090	0.493	0.626	0.195	0.520

[a] – Study G is Lankas [17] (Additional file 2: Table S7), Study H is Stout and Ruecker [18] (Additional file 2: Table S8), Study I is Atkinson et al. [12] (Additional file 2: Table S9) and Study J is Enemoto [20] (Additional file 2: Table S10); [b] – three dashes "---" indicates all tumor counts are zero; [c] – using historical control data (see text for details) and Tarone's test

Re-analysis of the data from Wistar rats - From Table 4 it can be derived that a significant ($p \leq 0.05$) positive trend was found in males for hepatocellular adenomas and adenomas/carcinomas in the Brammer study, pituitary adenomas in the Wood study, skin keratoacanthomas in the Wood study, and adrenal pheochromocytomas in the Suresh study. In females a significant ($p \leq 0.05$) positive trend was found for mammary gland adenocarcinomas and adenomas/adenocarcinomas, pituitary adenomas and adenomas/carcinomas all in the Wood study. A significant common trend was found for hepatocellular adenomas and adenomas/carcinomas and skin keratoacanthomas in males. No statistically significant common trend was found for the tumours in females.

Table 4: P-values for the exact Cochran-Armitage trend test and pooled logistic regression analysis for tumours with at least one significant trend test or Fisher's exact test ($p \leq 0.05$) in male and female Wistar rats

Tumor	Individual study *p*-values for trend[a]			Common Trend	Homogeneity Test
Males	K	L	M		
Hepatocellular Adenomas	0.391	**0.008**	0.418	**0.048**	0.156
Hepatocellular Carcinomas	0.418	---[b]	1.000	0.492	0.242
Hepatocellular Adenomas and Carcinomas	0.286	**0.008**	0.610	**0.029**	0.194
Pituitary Adenomas	0.376	0.277	**0.045**	0.057	0.664
Pituitary Carcinomas	0.692	---[b]	1.000	0.771	0.956
Pituitary Adenomas and Carcinomas	0.454	0.277	0.059	0.073	0.700
Skin Keratoacanthomas	---[b]	0.387	**0.030**	**0.032**	0.823
Adrenal Pheochromocytomas	**0.048**	0.721	0.306	0.273	0.210
Females	K	L	M		
Mammary Gland Adenomas	0.539	0.941	0.062	0.448	0.015
Mammary Gland Adenocarcinomas	1.000	0.271	**0.042**	0.071	0.008
Mammary Gland Adenomas and Adenocarcinomas	0.729	0.590	**0.007**	0.113	0.064
Pituitary Adenomas	0.967	0.261	**0.014**	0.105	0.023
Pituitary Carcinomas	1.000	–	0.750	0.748	0.491
Pituitary Adenomas and Carcinomas	0.976	0.261	**0.017**	0.129	0.019

[a] – Study J is Suresh [21] (Additional file 2: Table S11), Study K is Brammer [22] (Additional file 2: Table S12), and Study L is Wood et al. [14] (Additional file 2: Table S13); [b] – three dashes "---" indicates all tumor counts are zero

False positive errors - The evaluation of any one animal cancer study involves a large number of statistical tests that could lead to false positives. To evaluate this issue, the probability that all of the results in any sex/species/strain could be due to false positive results is calculated. Overall, a total of 496 evaluations were done for these 13 studies including the few evaluations done against historical controls. There are 41 evaluations at 37 tumour/site combinations with a trend test $p \leq 0.05$. The probability that all of these are due to false positives is 0.001. Similarly, looking at the evaluations resulting in $p \leq 0.01$, the probability that all of the findings are due to false positives is < 0.001. The strongest evidence was found for male CD-1 mice where the probability for 11 positive findings to occur at $p \leq 0.05$ and 8 positive findings at $p \leq 0.01$ are both below 0.001.

Conclusions

Oral exposure of rats and mice to glyphosate *via* the diet in 13 separate carcinogenicity studies demonstrates that glyphosate causes a variety of tumours that differ by sex, species, strain and length of exposure. To summarize the results of the strength-of-evidence analysis, each tumour is placed in any of the following categories: Clear evidence (CE) when there is a causal link between glyphosate exposure and the tumour; Some evidence (SE) when there is a causal link between glyphosate exposure and the tumour but chance, although unlikely, cannot be ruled out; Equivocal evidence (EE) when there is a causal link between glyphosate exposure and the tumour but chance is as likely an explanation for the association as is exposure to glyphosate; No evidence (NE) indicates that any causal link between glyphosate exposure and the tumour is almost certainly due to chance. The factors used to place tumours into these categories include the analyses of the individual studies, the consistency of the data across studies (pooled analyses), the analyses using historical control data, the analyses of non-neoplastic lesions, mechanistic evidence and the associated scientific literature.

The weight-of-evidence analysis conducted in this study indicates that there is clear evidence (CE) that oral exposure to glyphosate *via* the diet produces adrenal cortical carcinoma in the female SD rat, hemangioma in the female mouse (CD-1 and Swiss albino), hemangiosarcoma in the male CD-1 mouse, kidney tumours in the male CD-1 mouse and SD rat, liver adenoma in male rats (SD and Wistar), malignant lymphoma in the male and female CD-1 mouse, skin basal cell tumour in the male SD rat and skin keratoacanthoma in male rats (SD and Wistar). Some evidence (SE) for a causal relationship was found for kidney tumours in male Swiss albino mice, mammary tumours in female Wistar rats, malignant lymphoma in male and female Swiss albino mice, pituitary adenoma in the male and the female Wistar rat, and testicular interstitial cell tumours in the male SD rat. The results of the analyses conducted in this study are supportive of the conclusions of IARC that there is sufficient evidence to consider glyphosate as a rodent carcinogen.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Thirteen glyphosate cancer bioassays considered acceptable for this re-analysis were selected from the published literature, the EPA review, the review from the German Institute for Risk Analysis, the JMPR review, and full laboratory reports. For twelve of them full study reports were available. Individual tumour counts for the individual studies were re-analyzed using the exact Cochran-Armitage one-sided linear trend test. Re-analyses were conducted on all primary tumours where there were at least 3 tumours in all of the animals in a sex/species/strain combination. In addition, any tumour where a significant positive trend ($p \leq 0.05$) was found in at least one study was also evaluated in all the other studies of the same sex/species/strain combination, regardless of the number of animals with the tumour. Pairwise comparisons between individual exposed groups and the control group were conducted using Fisher's exact test. To evaluate the consistency of a tumour finding across multiple studies using the same sex-species-strain combinations, logistic regression with individual background responses and dose trends are fit to the pooled data using maximum likelihood estimation. The same methods of analysis were used to evaluate the incidence of non-neoplastic lesions in tissues where tumours were observed. In cases of rare tumours where the increase in incidence didn't reach statistical significance the test proposed by Tarone (1982) was applied using an appropriate historical control group. To summarize the results of the strength-of-evidence analysis, each tumour is placed in any of the following categories: Clear evidence (CE), some evidence (SE), equivocal evidence (EE), and no evidence (NE). The factors used to place tumours into these categories include the analyses of the individual studies, the consistency of the data across studies (pooled analyses), the analyses using historical control data, the analyses of non-neoplastic lesions, and mechanistic evidence with the associated scientific literature. The author's weight-of-evidence analysis indicates that there is clear evidence (CE) that oral exposure to glyphosate *via* the diet produces adrenal cortical carcinoma in the female SD rat, hemangioma in the female mouse (CD-1 and Swiss albino), hemangiosarcoma in the male CD-1 mouse, kidney tumours in the male CD-1 mouse and SD rat, liver adenoma in the male rat (SD and Wistar), malignant lymphoma in the male and female CD-1 mouse, skin basal cell tumour in the male SD rat and skin keratoacanthoma in male rats (SD and Wistar). Some evidence (SE) for a causal relationship was put forth for kidney tumours in the male Swiss albino mouse, mammary tumours in the female Wistar rat, malignant lymphoma in the male and female Swiss albino mouse, pituitary adenoma in the male and the female Wistar rat, and testicular interstitial cell tumours in the male SD rat.

After thorough analysis and considering all factors that are important in the interpretation of cancer studies none of the tumours identified by the author as indicating clear evidence (CE) or some evidence (SE) of carcinogenicity were found relevant for reconsideration under the necessary due diligence of the European AIR5 review of glyphosate. Most of the tumours selected by the author were previously dismissed by the EU experts as not relevant even before the last review of glyphosate in 2017, and the applicant believes that there is no solid toxicological evidence for glyphosate exposure related carcinogenicity in the mouse and the rat that warrants any science-based concerns for human health. The discussion of each of the suspect tumours is given below.

Clear evidence (CE) for carcinogenicity:

Adrenal cortical carcinoma in the female SD rat (Stout and Ruecker study):
The tumour incidences were 0/60, 0/60, 0/60, 3/60 at 0, 113, 457, and 1183 mg/kg bw/day, respectively. This tumour has not been considered treatment related by the authors of the study. There is no dose-related increase in adrenal cortical adenoma (1/60, 3/60, 2/60, 1/60), no dose-related increase in pre-neoplastic lesions and this tumour was not found in the males of the same study or in other rat studies. Therefore, this tumour has been considered not relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

Hemangioma in female CD-1 mice (Sugimoto study):
In the Sugimoto study hemangiomas were observed in different tissues:
- In liver with an incidence of 0/50, 0/50, 1/50, 1/50;
- In the ovary with an incidence of 0/50, 0/50, 0/50, 1/50;
- In the uterus with an incidence of 0/50, 0/50, 1/50, 2/50;
- In the spleen with an incidence of 0/50, 0/50, 1/50, 0/50;
- In the abdominal cavity with an incidence of 0/8, 0/9, 0/9, 1/9;

At 0, 153.2, 786.8, and 4116 mg/kg bw/day, respectively. Taken together as systemic tumours a significant positive trend is obtained. However, hemangiomas have also been observed in males (liver and testes) but without any dose-response relationship and the highest incidence found was in the control group (1/50). These tumours have not been confirmed in the other carcinogenicity studies in the CD-1 mouse. Moreover, the dose level (4116 mg/kg bw/day) at which the incidence was statistically significantly increased when compared against the control, is more than 4-fold the limit dose for the testing of carcinogens in rodent species. If that dose is ignored there is no significative positive trend. Therefore, this tumour has been considered not relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

Hemangioma in female Swiss albino mice (Kumar study):
In the re-analysis of the tumour data of the Kumar study by K. Weber (report submitted in 2017) no statistically significant trend was found for systemic neoplasms using the Peto test. When analyzed using the Fischer's exact test no statistically significant increase in incidence was found in pair-wise comparisons of all dose groups with the control group. It is important to emphasize that this study was compromised by non-identified ecto-and endoparasites in a large number of animals. Therefore, this tumour has been considered not relevant for the assessment of glyphosate.

Hemangiosarcoma in the male CD-1 mouse (Atkinson study):
The tumour incidences were 0/50, 0/50, 0/50, 4/50 (3 in liver and 1 in prostate) at 0, 98, 297, and 988 mg/kg bw/day, respectively. The incidence at the highest dose (8 %) was still within the historical control range of the test laboratory (0-8 %, 300 male mice in 6 studies up to 1993). This tumour was not confirmed in other mouse studies of which one (Knezevich and Hogan study) with a dose level nearly 5-fold that of the Atkinson study (4841 mg/kg bw/day). Therefore, this tumour has been considered not relevant for the assessment of glyphosate.

Kidney tumours in the male CD-1 mouse (Takahashi study, as reported by JMPR, 2016):
Renal cell adenoma (3/50) and renal cell carcinoma (1/50) were observed in males at 7470 mg/kg bw/day, but, according to the authors, there was no statistically significant difference with the control group. It is of note that the high dose considered in this study for males is extraordinarily high, more than 7-fold the limit dose for the testing of carcinogens in rodent species. If this dose is ignored there is no significant positive trend. These tumours were re-examined by the original study pathologist in 2012 because the Pesticide Expert Panel of the Food Safety Commission of Japan requested more information on historical control data and association with the non-neoplastic renal findings. After re-examination, the incidences for renal cell adenoma were 1/50, 1/50, and 1/50 at 167.6, 685, and 7470 mg/kg bw/day, showing no dose-response relationship. The incidence for renal cell carcinoma was confirmed to be 1/50 at 7470 mg/kg bw/day. The historical control data for the Takahashi study were not available, but the historical control values described in the re-examination document for renal cell carcinoma were 1/725 (0.13 %) in males and 0/725 (0 %) in females and for renal cell adenoma were 3/564 (0.53 %) in males and 0/564 (0 %) in females. The re-examination report also

provides reference data of 0-1.8 % in males and 0 % for all doses in females for renal cell carcinoma, and 0-1.8 % in males and females for renal cell adenoma. The results of the re-examination revealed also that the tubular epithelial cell hypertrophy was localized with an incidence in each treatment group that did not significantly differ from that in the control group. There was no association between the tubular epithelial cell hypertrophy and the development of renal tumours. The renal cell tumours observed in this study are thus not relevant for the human risk assessment of glyphosate because (1) the incidence of renal tumours in males at 7470 mg/kg bw/day did not significantly differ from that in the control group upon re-evaluation; (2) none of the females had neoplastic or non-neoplastic lesions; and (3) the high dose considered in this study for males is more than 7-fold the limit dose for the testing of carcinogens in rodent species. Therefore, this tumour has been considered not relevant for the assessment of glyphosate.

Kidney tumours in the male SD rat (Enemoto study):
The incidences of kidney adenoma were 0/76, 0/75, 0/80, 4/78 at 0, 104, 354, and 1127 mg/kg bw/day. An increasing trend in the incidence of adenomas in the kidney was observed in males of the high dose group (animal 193: killed in extremis at week 92, animal 167: found dead at week 94, animal 159: found dead at week 101, and animal 169: killed by design after 104 weeks of treatment) and this incidence was greater than the historical control range referred to in the study report (0-2.9 %). However, according to the authors of this study, the increase observed was not statistically significant. No kidney tumours were found in the females and nearly all male rats at all dose levels suffered from chronic nephropathy (62/76, 63/75, 56/80, 67/78). This tumour in this study was not considered relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

Hepatocellular adenomas in the male SD rat (Stout and Ruecker study):
The tumour incidences for adenomas were 3/60, 2/60, 3/60, 8/60 and of carcinomas were 3/60, 2/60, 1/60, 2/60 at 0, 89, 362, and 940 mg/kg bw/day, respectively. The incidence of adenomas at the high dose (13.3 %) is still within the historical control range of the test laboratory (1.4-18.3 %). Foci of cellular alteration were observed at all dose levels without any dose-response relationship and there were no signs of hepatocellular hypertrophy, a prerequisite for hepatocellular carcinogenesis. Beside the Brammer study no increase in hepatocellular adenomas was noted in the other rat studies. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Hepatocellular adenomas in the male Wistar rat (Brammer study):
The tumour incidences were 0/64, 2/64, 0/64, 5/64 at 0, 121, 361, 1214 mg/kg bw/day, respectively. The positive trend is significant and the incidence at the high dose is significantly different from the control. However, the incidence at the high dose (7.8 %) is still within the historical control range of the test laboratory (0-11.5 %, 26 studies in 1984-2003). There were no histopathological signs of liver enzyme induction or pre-neoplastic lesions. The high dose animals in this study survived longer when compared to the other groups. This may also influence the spontaneous tumour rate. Beside the Stout and Ruecker study no increase in hepatocellular adenomas was noted in the other rat studies. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Malignant lymphoma in the male CD-1 mouse (Sugimoto study):
The tumour incidences were 2/50, 2/50, 0/50, 6/50 at 0, 165, 838, 4348 mg/kg bw/day, respectively. The positive trend is significant but the incidence at the high dose is not significantly different from the control. Moreover, the incidence at the high dose (12 %) is still within the historical control range of the test laboratory (3.6-19.2 %, 458 male mice in 12 studies in 1993-1998). The trend has been found significantly positive because of the elevated incidence at a dose level that is over 4-fold the limit dose for carcinogenicity studies in rodents. If this dose is ignored the trend is not positive. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Malignant lymphoma in the female CD-1 mouse (Takahashi study):
The tumour incidences were 3/50, 1/50, 4/50, 6/50 at 0, 93.2, 909, and 8690 mg/kg bw/day, respectively. The increased incidence of lymphoma at the high dose was statistically significant in the trend test but not in a pairwise comparison. The trend has been found significantly positive

because of the elevated incidence at an extraordinarily high dose level, more than 8-fold the limit dose for carcinogenicity studies in rodents. If this dose is ignored the trend is no longer significant. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Skin basal cell tumour in the male SD rat (Enemoto study):
The tumour incidences were 0/78, 0/75, 0/80, 3/78 for adenoma and 0/78, 0/75, 0/80, 1/78 for carcinoma at 0, 104, 354, and 1127 mg/kg bw/day. No increased incidence of this tumour was observed in the females or other rat studies and may be associated with other skin lesions (follicular hyperkeratosis and/or folliculitis/follicular abscess) observed in this study. Although there is a significant positive trend for the adenomas, the increase in incidence at the high dose level was not considered relevant for the risk assessment of glyphosate by the authors of this study. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male SD rat (Stout and Ruecker study):
The tumour incidences were 1/60, 3/60, 4/60, 5/60 at 0, 89, 362, and 940 mg/kg bw/day. Although there is a significant positive trend the incidence at the high dose was not statistically significantly different from the control and considered not related to treatment. Skin keratoacanthoma is one of the most common spontaneous benign neoplasms in male Sprague Dawley rats. Therefore, this tumour was not considered relevant for the risk assessment of glyphosate by the authors of this study. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male SD rat (Atkinson study):
The combined incidences of intracutaneous cornifying epithelioma (keratoacanthoma) were 1/50, 2/25, 0/19, 0/21, 5/50 at 0, 11, 112, 320, and 1147 mg/kg bw/day. Although the trend was significant, the incidence at the high dose was not statistically significantly different from the control and considered not related to treatment by the authors of this study. Skin keratoacanthoma is one of the most common spontaneous benign neoplasms in male Sprague Dawley rats. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male SD rat (Enemoto study):
The incidences of the tumour were 4/76, 3/75, 0/80, 7/78 at 0, 104, 354, and 1127 mg/kg bw/day. The increased incidence of this skin tumour at the high dose may be associated with other skin lesions (follicular hyperkeratosis and/or folliculitis/follicular abscess) observed in this study. Although there is a significant positive trend for this tumour, the increase in incidence at the high dose level was not statistically significantly different from the control. Skin keratoacanthoma is one of the most common spontaneous benign neoplasms in male Sprague Dawley rats and considered by the authors of this study not relevant for the risk assessment of glyphosate. This tumour was not discussed further in the previous EU review of glyphosate.

Skin keratoacanthoma in the male Wistar rat (Wood study):
There were no treatment-related conditions seen in the skin or in subcutaneous tissues, but several spontaneous lesions were observed. Epidermal ulceration and scab formation, inflammatory lesions, abscess formation, focal acanthosis, focal mineralisation, focal dermal thickening, and focal necrosis were seen, occasionally or rarely and without significance. This tumour was not discussed further in the previous EU review of glyphosate.

Some evidence for carcinogenicity (SE)
Kidney tumours in the male Swiss albino mouse (Kumar study):
In the re-analysis of the tumour data of the Kumar study by K. Weber (submitted in 2017) no statistically significant trend was found for systemic neoplasms in the Peto test. When analyzed using the Fischer's exact test no statistically significant increase in incidence was found in pair-wise comparisons of all dose groups with the control group. It is important to emphasize that this study was compromised by non-identified ecto-and endoparasites in a large number of animals. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Mammary tumour in the female Wistar rat (Wood study):

At interim and terminal sacrifice combined mammary neoplasia was seen in 6 female mice. There were no mammary neoplasms in the control group but carcinomas were seen with incidences of 2/51, 3/51, and 1/51 at 153.2, 786.8, and 4116 mg/kg bw/day, respectively. All neoplasms were adenocarcinomas with the exception of one adenosquamous carcinoma seen in a low dose group animal. No increase in the incidence of these tumours was reported in the females of other rat studies. The authors concluded that there was no effect of treatment upon the incidence of mammary neoplasia in this study. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Malignant lymphoma in the male Swiss albino mouse (Kumar study):
In the re-analysis of the tumour data of the Kumar study by K. Weber (2017) no statistically significant trend was found for systemic neoplasms in the Peto test. When analyzed using the Fischer's exact test no statistically significant increase in incidence was found in pair-wise comparisons of all dose groups with the control group. It is important to emphasize that this study was compromised by non-identified ecto-and endoparasites in a large number of animals. Therefore, this tumour was not considered as relevant for the assessment of glyphosate.

Pituitary adenomas in the male and the female Wistar rat (Wood study):
Pituitary adenomas were only seen in female mice with incidences of 0/51, 1/51, 0/51, 2/52 at 0, 104.5, 348.6, and 1381.9 mg/kg bw/day. The group distribution was unrelated to treatment. Therefore, this tumour was not considered relevant for the assessment of glyphosate.

Testicular interstitial cell tumour in the male SD rat (Lankas study):
The incidences of this tumour were 0/50, 3/50, 1/50, 6/50 at 0, 3.05, 10.30, and 31.49 mg/kg bw/day, respectively. The positive trend is statistically significant and the incidence at the high dose level (12 %) is statistically significantly different from the control and greater that the historical control rate of the test laboratory (3.4-6.6 %). However, there was no dose-response relationship for interstitial cell hyperplasia (1/50, 1/50, 1/50, 0/50). Since the dose range considered in this study (0-31.5 mg/kg bw/day) is approximately at least 30-fold lower than that of all the other studies in rats where no increase of such tumours was found this finding should be considered as spontaneous in nature. Therefore, this tumour was not considered relevant for the risk assessment of glyphosate and was not discussed further in the previous EU review of glyphosate.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because some of the statistical methods employed were not described in sufficient detail. Besides, the results of this study are not in agreement with the findings of Crump *et al*. 2020 in relation to the estimation of false positives and the overall evaluation of the significance of the tumours by the EU regulatory authorities. All the tumours that were identified by the author as providing clear evidence for the carcinogenicity of glyphosate have been previously dismissed in the EU regulatory process.

Reliability criteria for *in vivo* toxicology studies

Publication: Portier, 2020.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N.A.	
Study performed according to GLP	N. A.	Most of the study reports analysed were GLP compliant.
Study completely described and conducted following scientifically acceptable standards	Y	Re-analysis of the tumour data of 13 selected glyphosate cancer bioassays.
Test substance		
Test material (Glyphosate) is sufficiently documented and	Y	Purity of glyphosate used in

reported (i.e. purity, source, content, storage conditions)		every cancer bioassay mentioned.
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	N.A.	Described in the test reports of 12 of the selected studies. The data from one study were derived from a JMPR review.
Route and mode of administration described	Y	Oral *via* the diet.
Dose levels reported	Y	Dose range from 71.4 to 8690 mg/kg bw in the mouse and from 3.05 to 4348 mg/kg bw in the rat.
Number of animals used per dose level reported	Y	About 50 per dose group.
Method of analysis described for analysis test media	N.A.	Described in the original test reports.
Validation of the analytical method	N.A.	
Analytical verifications of test media	N.A.	
Complete reporting of effects observed	Y	
Statistical methods described	Y	All statistical methods used in the re-analysis of the tumour data were reported, however sometimes not in sufficient detail.
Historical control data of the laboratory reported	N.A.	
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 some of the statistical methods employed were not described in sufficient detail. Besides, the results of this study are not in agreement with the findings of Crump *et al*. 2020 in relation to the estimation of false positives and the overall evaluation of the significance of the tumours by the EU regulatory authorities. All the tumours that were identified by the author as providing clear evidence for the carcinogenicity of glyphosate have been dismissed in the EU regulatory process.		

1. Information on the study

Data point:	CA 5.8.3
Report author	Xia Y. *et al*.
Report year	2020
Report title	The endoplasmic reticulum stress and related signal pathway mediated the glyphosate-induced testosterone synthesis inhibition in TM3 cells
Document No	Environmental Pollution, (2020) 260, 113949 DOI: 10.1016/j.envpol.2020.113949
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes/Reliable with restrictions

2. Full summary of the study according to OECD format

The authors investigated the effects of glyphosate on testosterone secretion and the role of endoplasmic reticulum (ER) stress in the process in TM3 cells. The effects of glyphosate at different concentrations on the viability of TM3 cells were detected by the CCK8 method. The effect of glyphosate exposure on testosterone secretion was determined by enzyme-linked immunosorbent assay (ELISA). The expression levels of testosterone synthases and ER stress-related proteins were detected by Western blot and Immunofluorescence stain. Results showed that exposure to glyphosate at concentrations below 200 mg/L had no effect on cell viability, while the glyphosate above 0.5 mg/L could inhibit the testosterone secretion in TM3 cells. Treatment TM3 cells with glyphosate at 5 mg/L not only reduced the protein levels of testosterone synthase StAR and CYP17A1, inhibited testosterone secretion, but also increased the protein level of ER stress molecule Bip and promoted the phosphorylation of PERK and eIF2α. Pre-treatment cells with PBA, an inhibitor of ER stress, alleviated glyphosate-induced increase in Bip, p-PERK, and p-eIF2α protein levels, meanwhile rescuing glyphosate-induced testosterone synthesis disorders. When pre-treatment with GSK2606414, a PERK inhibitor, the glyphosate-induced phosphorylation of PERK and eIF2α was blocked, and the glyphosate-inhibited testosterone synthesis and secretion were also restored. Overall, the authors suggest that glyphosate can interfere with the expression of StAR and CYP17A1 and inhibit testosterone synthesis and secretion via ER stress-mediated the activation of PERK/eIF2α signalling pathway in Leydig cells.

Materials and methods

Chemicals and antibodies

The following chemicals and antibodies were used in this study: Cell Counting Kit (CCK)-8 (Dojindo Molecular Technologies, Shanghai, China), Testosterone ELISA Kit (Mlbio, Shanghai, China), GSK2606414 (Absin, Shanghai, China), 4-phenylbutyric acid (PBA), goat anti-rabbit IgG-TRITC antibody and mouse anti-3β-HSD antibody (Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-Bip antibody, rabbit anti-PERK antibody, rabbit anti-PERK (phosphor T980) antibody, rabbit anti-eIF2α antibody, rabbit anti-eIF2α (phosphor S51) antibody, rabbit anti-StAR antibody, rabbit anti-CYP11A1 antibody, rabbit anti-CYP17A1 antibody, rabbit antiGAPDH antibody, rabbit anti-β-actin antibody, goat anti-mouse IgG, HRP-linked antibody and goat anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Beverly, MA, USA), glyphosate, poly-L-lysine solution (sigma-aldrich, Santa Louis, MO, USA), goat serum blocking solution (ZSJB-BIO, Beijing, China), 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China). PBA was dissolved as a stock solution (0.1 M) in PBS and the final concentration was 10 μM. GSK2606414 was dissolved as a stock solution (2 mM) in DMSO and the final concentration was 0.5 μM.

Cell cultures and treatment

TM3 cells (purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Shanghai, China), a mouse Leydig cell line, were cultured in DMEM/F12 complete medium containing 5 % horse serum and 2.5 % FBS (Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Shanghai, China) at 37 °C in a humidified atmosphere with 5 % CO_2. For cell viability test, cells were seeded at a density of 5 x 10^3 cells/well in 96-well plates. For ELISA, cells were seeded in 24-well plates (4 x 10^4 cells/well) and the concentration of testosterone in cell culture medium was detected by ELISA. For western blotting analysis, cells were seeded at a density of 4 x 10^5 cells/dish in Ø 60 mm Petri dishes. Cells were cultured for 24 h before experimental treatment.

Cell viability test

Cell viability was tested using CCK-8 Kit according to the manufacturer's protocol. Briefly, TM3 cells were seeded in 96-well plates for 24 h before the treatment. After exposed to glyphosate at different concentrations from 0.01 to 2000 mg/L for 24 h, cells in each well were added with 10 μL reagent from the Cell Counting Kit and then incubated at 37 °C for 80 min. Finally, OD value of each well at the wavelength of 450 nm was read by a multimode plate reader (Thermo Scientific, NYC, NY, USA). The experiment was repeated independently four times, and six duplicate wells were set in each group.

Testosterone determination by ELISA

TM3 cells were cultured in a 24-well plate and exposed to glyphosate at different concentrations for 24 h with or without PBA or GSK2606414 pre-treatment. Then, the medium was collected and centrifuged at 12,000 rpm for 5 min at 4 °C. The testosterone in supernatant was assayed using ELISA kit according to the manufacturer's protocol. Briefly, each well of ELISA plate was added with 40 μL sample dilution, 10 μL sample and 100 μL enzyme labelling reagent respectively, then the plate was covered and incubated at 37 °C for 60 min. After thoroughly aspirating solution from wells, the plate was washed with washing solution for five times. Then each well was added with 50 μL developer A and 50 μL developer B, and incubated at 37 °C for 15 min in the dark. Finally, 50 μL stop solution was added to terminate the reaction. The absorbance of each well was measured at a wavelength of 450 nm. The experiment was repeated independently four times, and three duplicate wells were set in each group.

Western blotting analysis

Cells were seeded in Ø 60 mm Petri dishes. After exposed to glyphosate for 24 h with or without PBA or GSK2606414 pre-treatment, cells were extracted using the RIPA lysis buffer (Beyotime, Shanghai, China) containing 1 % phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail. Protein concentrations were detected by BCA protein assay kit (Beyotime, Shanghai, China). Equal amount of protein was prepared and separated by 10 % SDS-PAGE. Then, protein was transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 5 % bovine serum albumin in Tris buffered saline (TBS) for 2 h, the membranes were incubated with primary antibodies overnight at 4 °C. Next day, the membranes were washed 3 times with TBS containing 0.1 % tween-20 (TBST) for 10 min/time and incubated with secondary antibody for 1 h at room temperature. After washed with TBST, the bolts were detected with BeyoECL Star (Beyotime, Shanghai, China) and the grey scales of protein bands were measured by a Chemiluminescence Imager (BIO-RAD, Hercules, CA, USA). Experiments were repeated independently for eight times.

Immunofluorescence analysis

For immunofluorescence analysis, glass slides were coated with 0.01 % (w/v) poly-L-lysine solution for 5 min. The excess solution was removed and the slides were dried at room temperature. Then the slides were put into Ø 35 mm Petri dishes and cells were seeded in them. After exposed to glyphosate for 24 h with or without PBA or GSK2606414 pre-treatment, cells were fixed with 4 % paraformaldehyde for 20 min and permeabilized with 0.5 % Triton X-100 for 15 min at 4 °C. Then, cells were treated with goat serum blocking solution for 2 h at room temperature and incubated with primary antibodies overnight at 4 °C. Subsequently, cells were incubated with TRITC-conjugated secondary antibody for 1 h and DAPI for 15 min at room temperature. Finally, the stained slides were photographed with laser scanning confocal microscope (Olympus, Tokyo, Japan) and the fluorescence intensity was evaluated using ImageJ software. Each experiment was repeated four times and two background groups (without primary antibody or secondary antibody) were set.

Statistical analysis
Data were presented as mean ± SD and analysed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test using SPSS 22 statistical software. A difference at $p < 0.05$ was considered statistically significant.

Results

Glyphosate at low concentration inhibited testosterone secretion but not to affect cell viability in TM3 cells
After treated with glyphosate at different concentrations from 0.01 to 2000 mg/L for 24 h, cell viability was measured using CCK8 and the concentration of testosterone in the culture medium was detected using ELISA kit. The results showed that glyphosate above 500 mg/L could decrease TM3 cell viability ($p < 0.05$), whereas glyphosate at concentrations below 200 mg/L had no significant effect on TM3 cell viability (Figure 1). In contrast to cell viability, as shown in Figure 2, exposure to glyphosate at a concentration above 0.5 mg/L could significantly decreased the concentrations of testosterone in culture medium ($p < 0.05$). These findings suggested that glyphosate at low concentrations which had no effect on cell viability could inhibit testosterone secretion in TM3 cells.

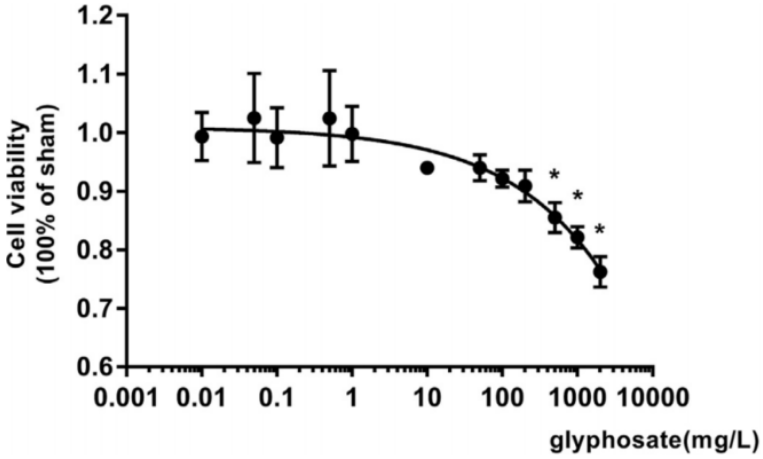
Glyphosate inhibited the expression of testosterone synthases StAR and CYP17A1
In the experiment, the effect of glyphosate on expression of testosterone key synthases was investigated. TM3 cells were treated with 5 mg/L glyphosate for different durations (1, 3, 6, 12 or 24 h), and the protein level of testosterone synthases was detected by western blotting. The results showed that glyphosate exposure significantly reduced the protein levels of StAR and CYP17A1 ($p < 0.05$) (Figure 3A and B). However, the protein levels of CYP11A1 and 3β-HSD were not affected by glyphosate (Figure 3C and D). It suggested that glyphosate decreased testosterone secretion through selectively inhibiting expression levels of key synthesis enzymes of StAR and CYP17A1 in TM3 cells.

Glyphosate induced ER stress and activated the PERK/eIF2α signaling pathway in TM3 cells
To investigate whether glyphosate exposure causes ER stress and activates the PERK/eIF2α signaling pathway in TM3 cells, the expression levels of related proteins were detected by western blotting. Results found that exposure to glyphosate at 5 mg/L for 1 h significantly increased all of the protein levels of Bip, p-PERK, PERK and p-eIF2α, which peaked at around 12 h ($p < 0.05$) (Figure 4A-D), but the protein level of eIF2α was not affected by glyphosate (Figure 4E). When TM3 cells were pretreated with 10 mM PBA, an inhibitor of ER stress for 2 h, all of the glyphosate-induced increases in protein levels of Bip, p-PERK, PERK and p-eIF2α were completely inhibited (Figure 5A-D) ($p < 0.05$). These results indicated that glyphosate could induce ER stress and the activation of PERK/eIF2α signaling pathway in TM3 cells.

Glyphosate-induced testosterone synthesis disorders depended on ER stress
In the present experiment, the role of ER stress in glyphosate-induced testosterone synthesis disorders was investigated. The results showed that PBA, the inhibitor of ER stress not only significantly rescued glyphosate-inhibited expressions both of StAR and CYP17A1 ($p < 0.05$), but also effectively restored the testosterone secretion inhibited by glyphosate ($p < 0.05$). It indicated that glyphosate-induced testosterone synthesis disorders were mediated by the ER stress.

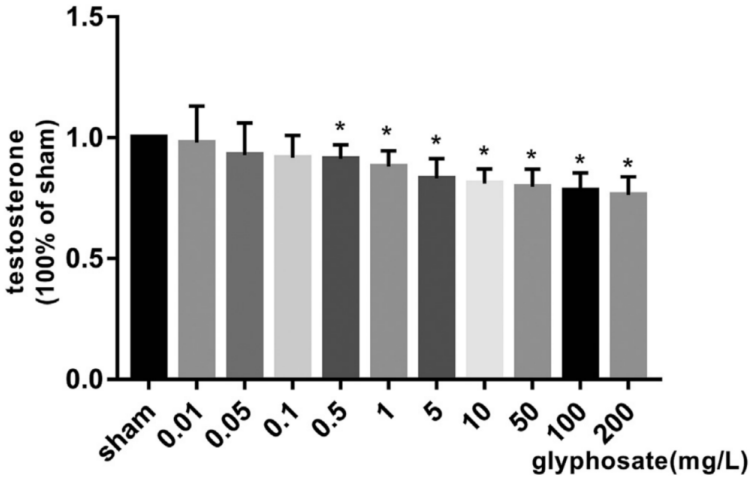
PERK/eIF2α signaling pathway mediated glyphosate-induced testosterone synthesis disorders
To investigate the role of PERK/eIF2α pathway in the glyphosate-induced testosterone synthesis disorders, TM3 cells were pretreated with 0.5 μM GSK2606414, an inhibitor of PERK activation for 2 h, and then incubated with 5 mg/L glyphosate for 24 h. The results showed that GSK2606414 pre-treatment significantly reduced glyphosate-induced phosphorylation of PERK and eIF2α ($p < 0.05$). In addition, the decrease both of StAR and CYP17A1 protein expression induced by glyphosate was completely blocked ($p < 0.05$), and the glyphosate-inhibited testosterone secretion also was effectively rescued ($p < 0.05$). These results suggested glyphosate-induced testosterone synthesis disorders depended on StAR and CYP17A1 which mediated by PERK/eIF2α signaling pathway.

Figure 1: **Effects of glyphosate on TM3 cell viability**



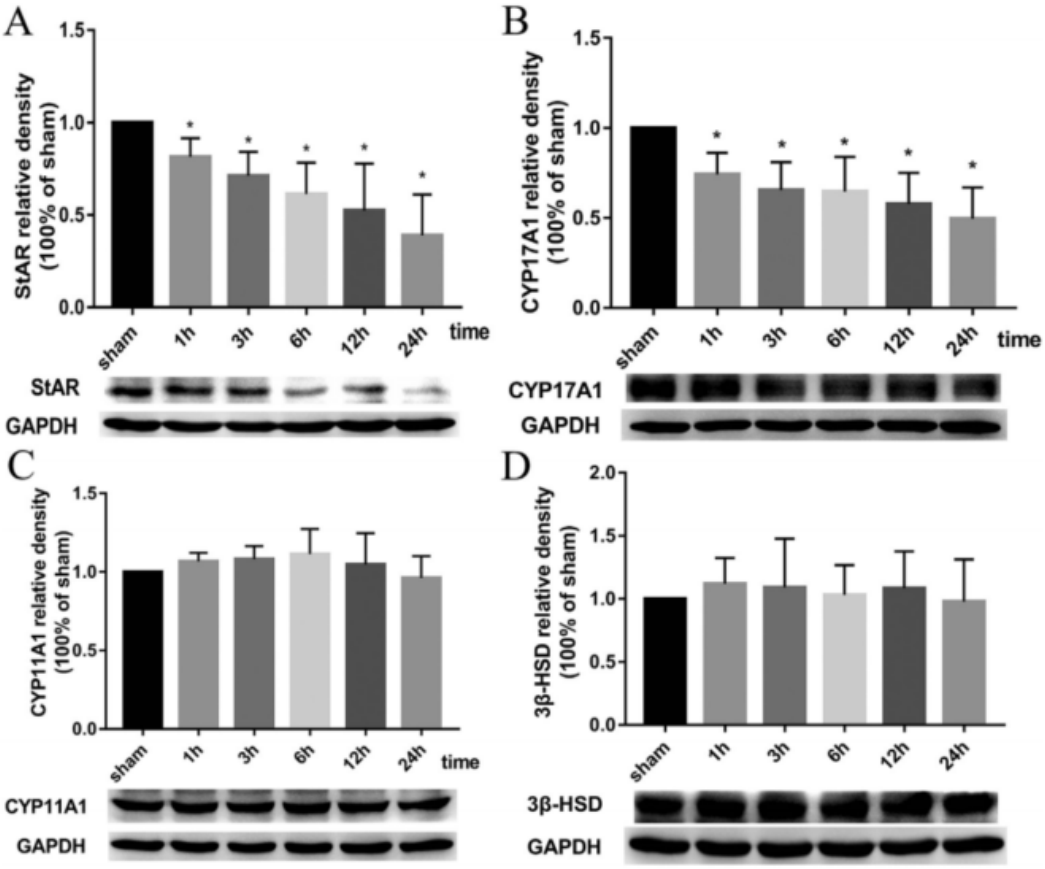
TM3 cells were exposed to different doses of glyphosate in 96-wells plate for 24 h and cell viability was measured using CCK8. Data were presented as the mean ± SD for n = 4 independent experiments.
*p <0.05, compared with the sham group.

Figure 2: **Glyphosate inhibited testosterone secretion of TM3 cells**



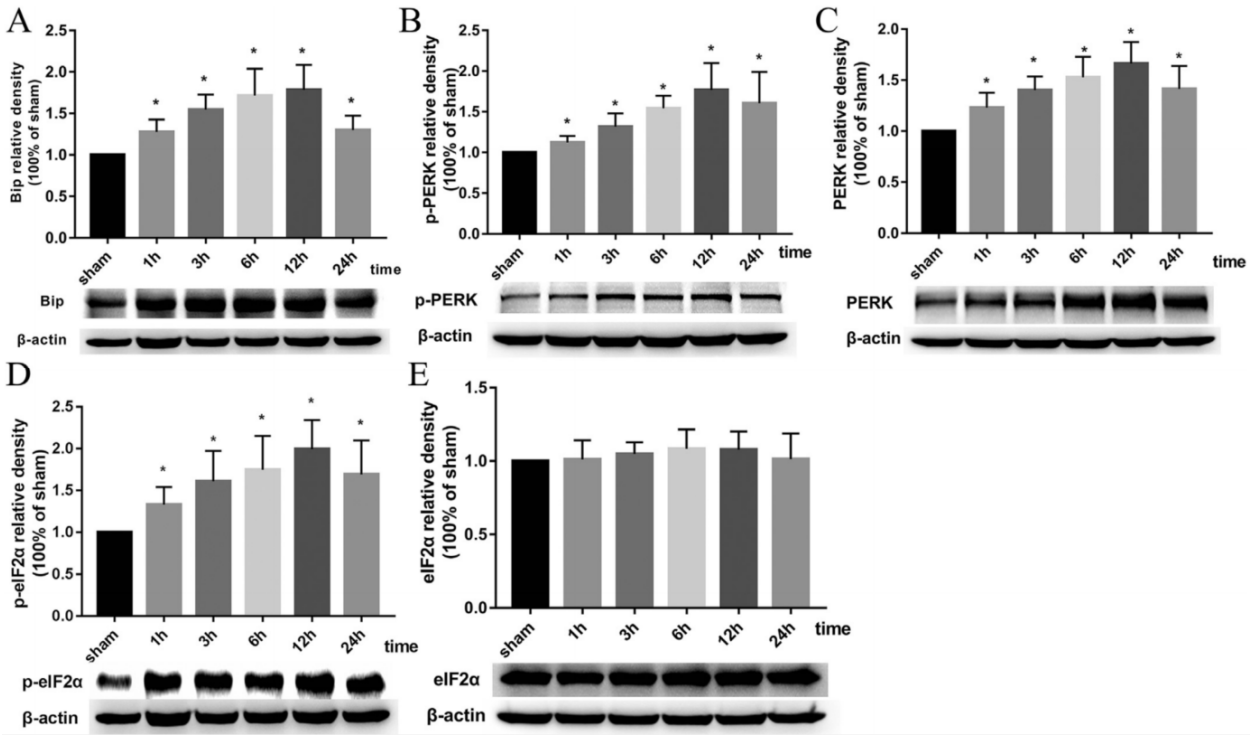
TM3 cells were exposed to different doses of glyphosate for 24 h. The cell supernatants were collected and the testosterone was assayed using ELISA kit. Data were presented as the mean ± SD for n = 4 independent experiments.
*p <0.05, compared with the sham group.

Figure 3: **Glyphosate inhibited the protein expression levels of testosterone synthesis enzymes in TM3 cells**



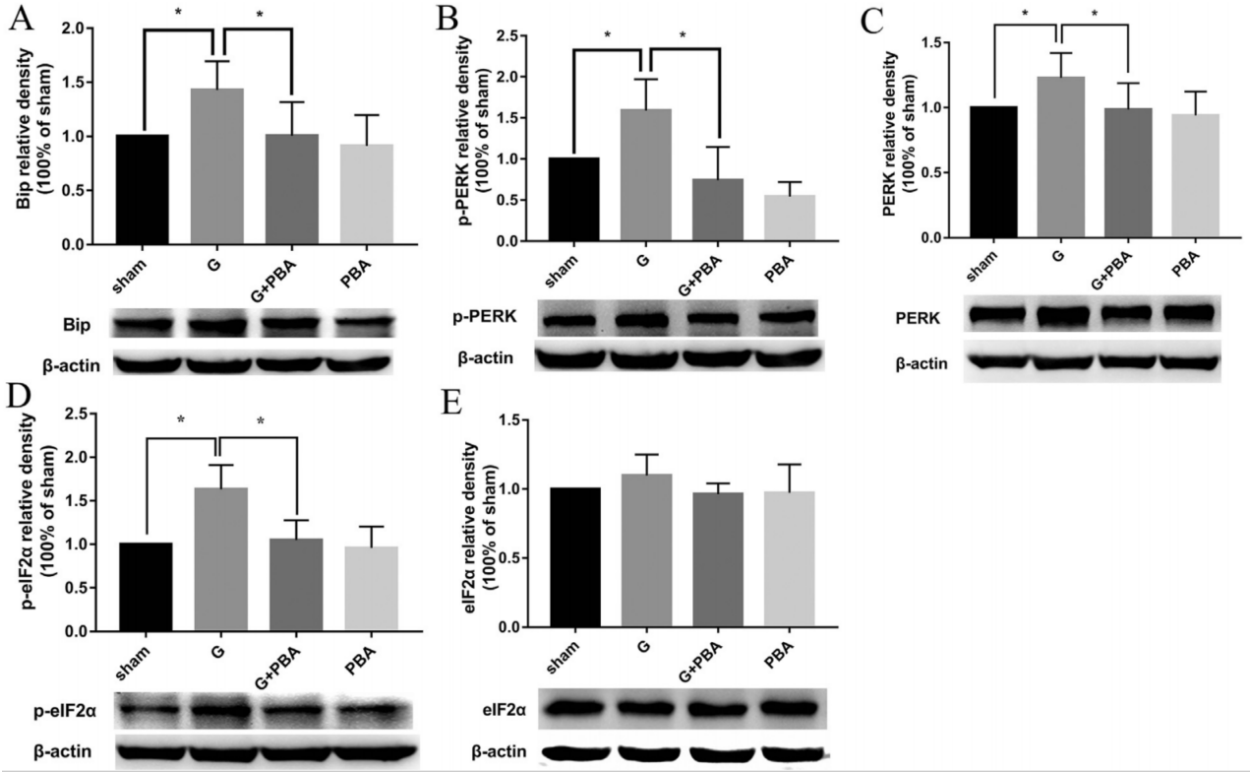
TM3 cells were exposed to 5 mg/L glyphosate for 1-24 h. The level of StAR, CYP17A1, CYP11A1 and 3β-HSD were detected with western blotting. Data were presented as the mean ± SD for n = 8 independent experiments.
*p <0.05, compared with the sham group.

Figure 4: **Glyphosate induced ER stress and activated the PERK/eIF2α signalling pathway in TM3 cells**



TM3 cells were exposed to 5 mg/L glyphosate for 1-24 h. The level of Bip, p-PERK, PERK, p-eIF2α and eIF2α were detected with western blotting. Data were presented as the mean ± SD for n = 8 independent experiments.
*p <0.05, compared with the sham group.

Figure 5: **PBA alleviated ER stress induced by glyphosate in TM3 cells**



TM3 cells were pre-treated with 10 μM PBA for 2 h, and then incubated with 5 mg/L glyphosate for 24 h. The level of Bip, p-PERK, PERK, p-eIF2α and eIF2α were detected with western blotting. Data were presented as the mean ± SD for n = 8 independent experiments.
*p <0.05. Panel labels are Sham for negative control, G for glyphosate exposure only, G + PBA for PBA treatment before glyphosate exposure, and PBA for PBA treatment only. G: glyphosate; PBA: phenylbutyric acid.

Conclusions

The authors conclude that glyphosate could inhibit testosterone synthesis via decreasing the expression of testosterone synthase StAR and CYP17A1, which depends on the ER stress and activation of PERK/eIF2α signalling pathway in Leydig cells.

3. Assessment and conclusion

Assessment and conclusion by applicant:

In vitro study on the effects of glyphosate on testosterone secretion and the role of endoplasmic reticulum stress in the process were investigated in TM3 cells. Results showed that exposure to glyphosate at concentrations below 200 mg/L had no effect on cell viability, while glyphosate at concentrations above 0.5 mg/L could inhibit the testosterone secretion in TM3 cells. Treatment of TM3 cells with glyphosate at 5 mg/L not only reduced the protein levels of testosterone synthase StAR and CYP17A1 but also inhibited testosterone secretion.

The article is classified as reliable with restrictions for the following reason: not enough information on the tested material (purity) was provided, no positive controls were used, and no statistical methods were described. Furthermore, no OECD guidelines were followed, no GLP status was stated, and no historical control data (HCD) were provided to compare the relevance of data. In addition, key literature in disagreement with the authors' findings appear to have been disregarded, suggesting bias within the research and the following publications: Hecker (2011), OECD validation of the H295R steroidogenesis assay with glyphosate; Levine (2007), demonstrating a lack of effect of glyphosate on the StAR protein; US EPA (2015), glyphosate EDSP weight of evidence evaluation; and EFSA (2017), peer review of glyphosate potential endocrine-disrupting properties.

Hecker M et al (2011), The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study, Environmental Science and Pollution Research 18(3):503-15

Levine S. L. Et al. (2007), Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis, Cell Biol Toxicol (2007) 23:385-400

US EPA (2015), EDSP Weight of Evidence Conclusions on the Tier 1 Screening Assays for the List 1 Chemicals - EDSP: WEIGHT OF EVIDENCE ANALYSIS OF POTENTIAL INTERACTION WITH THE ESTROGEN, ANDROGEN OR THYROID PATHWAYS - CHEMICAL: GLYPHOSATE

EFSA (2017): Peer review of the pesticide risk assessment of the potential endocrine disrupting properties of glyphosate, Question number: EFSA-Q-2016-00663

Assessment and conclusion by RMS:

Reliability Criteria: *In Vitro* Toxicology Studies		
Publication: Xia Y. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	Not stated
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate Purity not stated

Reliability Criteria: *In Vitro* Toxicology Studies		
Publication: Xia Y. *et al.* 2020	**Criteria met? Y/N/?**	**Comments**
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	N	TM3 cells (no purity stated)
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	Not necessary
Test concentrations in physiologically acceptable range (<1 mM)	N	0.01 to 200 mg/L
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive controls
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions	N	
Reliable with restrictions	Y	Not enough information on the tested material (purity), no positive controls were used, no statistical methods were described. Furthermore, no OECD guideline followed, no GLP status was stated. In addtion, no HCD were available in order to compare the relevance of the data. In addition, key literature in disagreement with the authors' findings appear to have been disregarded, suggesting bias within the research and publication: Hecker (2011) OECD validation of the H295R steroidogenesis assay with glyphosate; Levine (2007) demonstrating a lack of effect of glyphosate on the StAR protein; US EPA (2015) glyphosate EDSP weight of evidence evaluation; EFSA (2017) peer review of glyphosate potential endocrine-disrupting properties.
Not reliable	N	

1. Information on the study

Data point:	CA 5.8.2
Report author	Yahfoufi Z. A. *et al.*
Report year	2020
Report title	Glyphosate Induces Metaphase II Oocyte Deterioration and Embryo Damageby Zinc Depletion and Overproduction of Reactive Oxygen Species
Document No	Toxicology, (2020) Vol. 439, Art. No. 152466
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	Yes / Reliable with restrictions

2. Full summary of the study according to OECD format

In this study effects of glyphosate (0 – 300 μM) on metaphase II mouse oocyte quality and embryo damage was investigated to obtain insight on its mechanisms of cellular action and the tolerance of oocytes and embryos towards this chemical. The work shows that glyphosate exposure impairs metaphase II mouse oocyte quality via two mechanisms: 1) disruption of the microtubule organizing center and chromosomes such as anomalous pericentrin formation, spindle fiber destruction and disappearance, and defective chromosomal alignment and 2) substantial depletion of intracellular zinc bioavailability and enhancement of reactive oxygen species accumulation. Similar effects were found in embryos.

Materials and methods

Materials

Metaphase II noncumulus oocytes and embryos from a B6C3F1 mouse crossed with a B6D2F1 mouse were obtained commercially from Embryotech Inc. in cryopreserved straws using the ethylene glycol-based slow freeze cryopreservation protocol. Human tubular fluid (HTF) medium with gentamicin was obtained from Irvine Scientific Inc. Anti-α tubulin antibody, fluorescein isothiocyanate (FITC) conjugate anti-goat antibody, 4′,6′-diamino-2-phenylindole (DAPI), and 0.1% Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The anti-fade agent, formaldehyde, SYTO 64 Red Fluorescent Nucleic Acid Stain, Alexa Fluor® 633 goat anti-mouse IgG, and dimethylsulfoxide (DMSO) were obtained from Thermo Fisher Scientific Inc. The Purified Mouse Anti-Mouse Pericentrin was purchased from BD Biosciences and Fab Fragment Affinity Purified Antibody was purchased from Jackson ImmunoResearch Inc (West Grove, PA, USA). The cellular ROS detection assay kit and zinc detection kit (Zinquin ethyl ester) were obtained from Abcam (Cambridge, UK). Glyphosate ($C_3H_8NO_5P$, powder form) was purchased from Research Products International (Mt. Prospect, IL, USA; catalog G36060, CAS 1071-83-6).

Methods

Frozen mouse metaphase II oocytes and embryos were obtained from Embryotech (MA, USA). The concentrations of glyphosate treatment used here ranged from 0 to 300 μM.

Study the effect of glyphosate on MT and CH alignment of metaphase II noncumulus oocytes (total number of oocytes ≈ 180)

Oocytes were thawed and transferred from straws to phosphate buffer saline (Dulbecco PBS) and washed for 3 min to remove cryo-protectant. Oocytes were then transferred to HTF media and incubated at 37 °C and 5% carbon dioxide (CO_2) for 60 min to allow spindle re-polymerization and attainment of normal oocyte architecture. The oocytes were then screened for the presence of the polar body confirming their Metaphase II stage. Immature oocytes or those that displayed disrupted zona pellucida were discarded.

In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average; total ∼180 oocytes used across replicates) were exposed to 0, 25, 50, 75, 100, 200, and 300 μM glyphosate for a 2 h incubation period (37 °C, 5% CO_2) to observe the effects on oocyte MT and CH alignment. Only some concentrations were selected for presentation to improve focus. Untreated oocytes served as controls.

Immunostaining of the oocytes and confocal microscopy examination

Oocytes were subsequently fixed in a solution prepared from 2% formaldehyde and 0.2% Triton X-100 for 30 min at 25 °C, then washed with PBS for 3 min. Subsequently, the oocytes were subjected to indirect immunostaining in mouse primary anti-α-tubulin antibody (1:300) against the MT overnight at 25 °C, then washed and placed in secondary FITC conjugated anti-goat antibody (1:50) for 45 min at 25 °C. After washing in PBS, the chromosomes were stained using DAPI (1:100) for 15 min at 25 °C. For immunostaining of pericentrin, oocytes were treated with primary antibody, Purified Mouse Anti-Mouse Pericentrin (1:100), overnight at 4 °C prior to staining with secondary antibody, Alexa Fluor® 633 goat anti-mouse IgG (1:200) for 2 h at 25 °C. Oocytes were then washed 3 times before immunoglobulin blocking with Fab Fragment Affinity Purified Antibody (20 μg/ml) for 30 min at 25 °C, prior to washing and staining for MT and chromosomes.
Stained oocytes were loaded into anti-fade agent on slides with two etched rings and cover slips placed using transparent nail varnish. Slides were stored at −20 °C and protected from light until they were evaluated for more details by confocal microscopy. To obtain confocal images, slides were examined using a Zeiss LSM 510 META NLO microscope using FITC (green), DAPI (blue), and Alexa Fluor® 633 (bright red) fluorescent filters with excitation and emission wavelengths of 495 and 519 nm, 358 and 461 nm, and 632 and 647 nm, respectively. Oocytes were localized using a 10x magnification lens and spindle alterations were assessed using 40x oil immersion lens. The MT was stained fluorescent green, which was distinct from the fluorescent blue staining of chromosomes.

Assessment of MT AND CH Alignment (Scoring System)

Each treated and control oocyte from every experiment set was closely examined for spindle and chromosome configurations. Three independent observers who were blinded to treatment group assignment and used comprehensive evaluation of the individual optical sections performed the categorization of oocytes based on MT and CH configurations.
Three observers scored the oocytes and compared the alterations in the MT and CH alignment to controls. Scores of 1-4 were assigned for both MT and CH alterations, with good outcomes associated with scores 1 or 2 and poor outcomes associated with scores 3 or 4. Good spindle configurations (score 1 or 2) generally exhibit barrel-shaped microtubules, whereas poor or abnormal configurations (score 3 or 4) display shortened spindle length, disorganization, or completely missing spindles. Good chromosomal configurations (score 1 or 2) generally exhibit chromosomes arranged at the equator of the spindle, while poor chromosomes (score 3 or 4) appear dispersed, irregular, or less condensed.

Detection of intracellular zinc in oocytes and embryos after glyphosate exposure (total number of oocytes ≈ 180; total number of embryos ≈ 180)

In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average) were thawed, prepared, and pretreated with increasing concentration of glyphosate for 2 h (0, 25, 50, 75, 100, 200, and 300 μM). Subsequently, oocytes were stained with 25 μM Zinquin ethyl ester in combination with 1 μM SYTO64 and incubated for 1 h at 37 °C, 5% CO_2. A single dilution of Zinquin ethyl ester was used in each dish to ensure that all dishes received the same concentration of dye. This zinc-selective fluorescent probe permeates the cell and labels the intracellular zinc ions to emit a quantifiable blue fluorescent signal. Cells were then washed in PBS for 3 min to remove any excess dye before fixation with 2% formaldehyde and 0.2 % Triton X-100 for 15 min at 25 °C. After washing, cells were loaded into anti-fade agent on slides for subsequent evaluation by confocal microscopy. A similar procedure was used for embryos (n = 7 embryos per group on average).

Detection of ROS generation in oocytes after glyphosate exposure (total number of oocytes ≈ 180; total number of embryos ≈ 180)

In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average) were thawed, prepared,

and exposed to 0, 25, 50, 75, 100, 200, and 300 μM glyphosate as outlined above in detail (under "oocyte preparation"). Oocytes were incubated at 37 °C, 5% CO_2 in glyphosate treatment for 2 h and in 100 μM pyocyanin for 45 min. Oocytes treated with pyocyanin served as positive control, while untreated oocytes served as negative control.
After incubating with the treatment, oocytes were stained with 500 μL volume of ROS deep red working solution (a mixture of 250 μL HTF media, 249 μL ROS buffer, and 1 μL ROS Deep Red dye) and incubated for 1 h at 37 °C, 5% CO_2 before subsequent washing in PBS. After fixation by 2% formaldehyde and permeabilization with 0.2% Triton X- 100 for 30 min, cells were washed and nuclei were lastly stained with DAPI (1:100) for 15 min at 25 °C. Cells were loaded into anti-fade agent on slides for subsequent evaluation by confocal microscopy. A similar procedure was used for embryos (n = 7 embryos per group on average).

Results

Glyphosate altered MT and CH alignment of metaphase II mouse oocytes

The untreated oocytes displayed the same normal spindle morphology throughout the 180 min incubation time, however, the treated oocytes displayed maximum MT and CH alterations (80-85%) at 120 min and remained unchanged beyond this point.
Untreated oocytes had a well-organized barrel shaped spindle structure (green) with chromosomes tightly aligned at the equatorial plate of the spindles (blue). Oocytes exposed to low glyphosate concentration had altered spindle configuration indicated by an enlarged "balloon" shape and some disturbance in the chromosome configuration, while oocytes treated with high glyphosate concentration (> 100 μM) exhibited a scattered pattern of chromosomes and missing microtubules. The damaging effects of glyphosate on both MT and CH quality were exhibited by the significant increase in the quantity of oocytes with poor scores when exposed to higher concentrations of glyphosate compared to control ($p < 0.001$). The control group had approximately 8.69% and 21.7% poor scores for both MT and CH scoring, respectively. The remaining percentage of oocytes with poor MT scores were 22.2% ($p < 0.05$), 72.2%, 77.7%, and 80% ($p < 0.001$) and poor CH scores were 50% ($p < 0.05$), 77.7%, 72.2%, and 80% ($p < 0.001$) for 50, 100, 200, and 300 μM glyphosate, respectively.
Overall, the results demonstrate that increasing glyphosate concentration induces damage to MT and CH alignment as well as substantial oocyte deterioration exhibited beyond 100 μM.

Glyphosate mediates pericentrin disappearance

Control oocytes had normal pericentrin (bright red) localized at each spindle pole, maintaining the spindle force balance with a well-organized MT structure (green) and chromosomes (blue) tightly aligned at the equatorial plate. Low treatment groups (50 μM) displayed pericentrin proteins moving closer together from each spindle pole, while remaining within the polar axis. However, at higher glyphosate concentrations (> 75 μM), pericentrin disappears disrupting the spindle force balance and causing chromosomal dispersion and spindle loss. Pericentrin was diminished significantly at 50 μM ($p < 0.01$), and the effect increases dramatically at 100 μM and higher ($p < 0.001$).
Overall, these findings demonstrate that increasing glyphosate concentration can cause substantial pericentrin damage, thus influencing MT and CH alignment and morphology.

Glyphosate reduced intracellular zinc content

Increasing glyphosate concentration correlated with zinc depletion as indicated by a decrease in blue fluorescent intensity compared to untreated control, which is likely attributed to glyphosate's chelating ability. Normalized CTCF was calculated for the groups tested. The difference between the CTCF of each treatment group compared to the untreated control was found to be statistically significant ($p < 0.001$). Oocytes treated with 300 μM had the lowest zinc-mediated fluorescence, while oocytes treated with 50 μM had the highest fluorescence with an average normalized CTCF of 3.8 ± 1.6 and 19.4 ± 3.4, respectively (mean ± SEM).

Effect of glyphosate exposure on intracellular zinc content in embryos: Similar to oocytes, intracellular zinc declined with glyphosate treatment. 50 μM glyphosate treatments reduced in tracellular zinc by 37%, and increasing the concentration of glyphosate maintained this effect ($p < 0.01$ for comparisons of treatment groups with the control).

Glyphosate increased ROS generation

Increasing glyphosate concentration correlated with an increase in ROS formation as signified by an increase in deep red fluorescence compared to negative control. Normalized CTCF was calculated for the groups tested. The difference between the CTCF of each treatment group compared to the negative control was found to be statistically significant ($p < 0.001$). Oocytes treated with 50 μM had the lowest ROS fluorescence, while oocytes treated with 300 μM had the highest ROS fluorescence with an average normalized CTCF of 28.3 ± 1.1 and 73.8 ± 2.6, respectively (mean ± SEM).
Similarly, changes in ROS production resulting from glyphosate exposure were also examined in embryos Glyphosate exerted a comparable effect in embryos, which showed increase in ROS production as the embryos were exposed to glyphosate. The experiment showed a significant increase in ROS production in the treatment groups when compared to control ($p < 0.01$ for comparisons of all treatment groups with the control).

Conclusion

The work demonstrates for the first time that glyphosate exposure causes metaphase II oocyte quality deterioration in dual mechanisms involving i) pericentrin abnormalities, spindle fiber destruction and disappearance, and disturbance in chromosomal alignment and ii) significant interference with intracellular zinc bioavailability and ROS accumulation. The work also links ROS enhancement and zinc deficiency mediated by glyphosate exposure to possible embryo damage.

3. Assessment and conclusion

Assessment and conclusion by applicant:

The quality of metaphase II noncumulus oocytes and embryos from mice were investigated following glyphosate exposure at different concentrations (max 300 μM). The concentrations were in the range of those found in human blood following accidental acute exposure or suicidal attempts.

Results indicate that glyphosate provokes disruption of the microtubule organizing center and chromosomal disorganization at the mid-position of the spindle due to spindle disappearance, and defective chromosomal alignment as well as depletion of intracellular zinc bioavailability and enhancement of reactive oxygen species (ROS) accumulation in the mouse oocytes. In the embryos (not specified the source and the embryonal stage) zinc depletion and accumulation of ROS was also observed in a dose-related manner.

The article is classified as *reliable with restrictions* for the following reason: Not performed according to GLP or an OECD test guideline. No purity of the test substance stated. No information of the source and the embryonal stage of the embryos were provided. There were no concurrent positive control or substances known to deteriorates oocyte quality through disassembly of microtubule organizing centers (like peroxynitrite) or ROS accumulation (like hydrogen peroxide, and hypochlorous acid) or dimercapto-1-propanesulfonic acid (DMPS) for zinc depletion.

Assessment and conclusion by RMS:

Reliability criteria for *in vitro* toxicology studies		
Publication: Yahfoufi Z. A. et al. 2020	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	No GLP or OECD test guideline followed
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	No purity, content, storage conditions
Only glyphosate acid or one of its salts is the tested substance	Unknown	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	No metabolic activation system present
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Max. concentration 300 µM
Cytotoxicity tests reported	N	
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	N	
Reliable with restrictions	Y	Not performed according to GLP or an OECD test guideline. No purity of the test substance stated. No appropriate information on the tested embryos was provided. Tested dose correspond to level of glyphosate that may be found in human blood following human accidental acute exposure intoxication. No reference compound were tested in parallel to confirm the appropriateness of the testing procedures and the relevance of the results.
Not reliable	N	