# 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 7 月 1 日～2020 年 12 月 31 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

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|---|---|
| **Data point:** | CA 5.8.3 |
| **Report author** | Ferramosca A. *et al.* |
| **Report year** | 2021 |
| **Report title** | Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency. |
| **Document No** | Reproductive Toxicology (2021), Vol. 99, pp. 48-55 |
| **Guidelines followed in study** | None |
| **Deviations from current test guideline** | Not applicable |
| **GLP/Officially recognised testing facilities** | No, not conducted under not conducted under GLP/Officially recognised testing facilities. However, all experiments were performed according to principles of good laboratory practice. |
| **Acceptability/Reliability:** | Yes (Relevant, Category A acc. EFSA GD 2092, Point 5.4.1) / Reliable with restrictions |

## 2. Full summary of the study according to OECD format

The aim of the study was to evaluate sperm mitochondria as a target of potential endocrine disrupting chemicals. In an ex vivo experimental model with human sperm cells, the oxygen consumption rate was measured under exposure to glyphosate and glufosinate. **The present summary focusses on the experiments and results obtained with glyphosate (batch # 45521; purity not reported) only.**

Using sperm cells pooled from 11 human donors, quadruplicate cell suspensions were exposed to glyphosate, solvent, negative (carbonyl cyanide 4-chlorophenyl hydrazine, CCCP) and positive controls (nicotinamide adenine dinucleotide, NADH) for one hour at 37 °C. Each substance was tested in a concentration range of 0.1 – 1000 nM. Following treatment, mitochondrial oxygen consumption was measured, based on the active ($V_3$, rate of oxygen uptake measured in the presence of respiratory substrates and ADP) and in the passive state ($V_4$, rate of oxygen uptake measured with respiratory substrates pyruvate and malate alone) of mitochondrial respiration, as well as the respiratory control ratio (RCR), an index of mitochondrial respiration efficiency.

In order to evaluate the role of physiologically relevant hormone concentrations in endocrine-disrupting chemical mitochondrial targeting, human sperm cells were co-incubated with the respective test chemical and the steroid hormones progesterone (P4), testosterone (T), di-hydroxytestosterone (DHT) and 17β-estradiol (E2). In addition, the effect of quercetin, a mitochondria-targeting flavonoid, on mitochondria oxygen consumption was assessed. Finally, sperm cells were co-incubated with glyphosate and quercetin.

In human sperm cells, glyphosate was able to reduce mitochondrial functionality, by decreasing the oxygen consumption rate in the active ($V_3$) and in the passive state ($V_4$) of mitochondrial respiration. Although the effect on $V_3$ values was more evident than $V_4$ and was already observed at 1nM, the mitochondrial respiration efficiency was negatively affected only at concentrations of ≥ 100 nM.

The positive control NADH and the negative control CCCP demonstrated the validity of the test system. NADH exposure caused a statistically significant, dose-dependent increase in the active state of mitochondrial respiration ($V_3$), resulting in a dose-dependent increase of the respiration control ratio. Conversely, CCCP treatment caused a dose-dependent increase in the resting state of respiration ($V_4$), hence resulting in a dose-dependent decrease of the respiration control ratio.

The mitochondria-targeted flavonoid quercetin had a direct effect on the passive state of mitochondrial respiration and statistically significantly induced $V_3$ and $V_4$ in a dose dependent manner. The RCR value was decreased by -43% when compared to the solvent control at the highest test concentration of 1000 nM. Upon treatment with steroid hormones, DHT was identified as the only hormone significantly reducing mitochondrial functionality at physiologically relevant concentrations. Concentrations of 10 and 100 nM caused a statistically significant decrease in the active state ($V_3$), as well as a dose-dependent, statistically significant increase in the resting state ($V_4$), thus significantly decreasing the respiration control ratio (RCR). For the remaining steroid hormones P4, T and E2, no adverse effect on

mitochondria functionality was observed.
In the presence of DHT, the negative effect of glyphosate was evident at concentrations at ≥ 0.1 nM already. $V_4$ values were increased, suggesting a stimulus of mitochondrial respiration independent of ADP phosphorylation. After co-stimulation with glyphosate and the mitochondria-targeting flavonoid quercetin, an increase in oxygen consumption rate was observed at concentrations in the range of 0.1 – 10 nM, reaching the highest levels at glyphosate and quercetin concentrations at 10 nM. Hence, it was concluded that glyphosate targets mitochondria by using a mechanism that is different from that of DHT and quercetin.

## Materials and methods

*Test material*

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| Test material: | Glyphosate* |
| Source: | Sigma Aldrich |
| Batch no.: | # 45521 |
| Purity: | Not reported |

*: Besides Glyphosate, also glufosinate was investigated in this study. The present summary focusses on the experiments and results obtained with glyphosate only.

*Control materials*

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| Solvent controls*: | Dimethylsulfoxide (DMSO), final concentration 1% |
| Negative control: | Carbonyl cyanide 4-chlorophenyl hydrazone (CCCP), 0.1 – 1000 nM |
| Positive control: | Nicotinamide adenine dinucleotide reduced (NADH), 0.1 – 1000 nM |

*: Besides DMSO, also water and ethanol were specified as solvents. It was not further specified which solvent was used for which chemical. However, solvent controls for all solvents were included in the respective experiments.

*Male recruitment and semen collection*
Semen samples were obtained from 11 healthy human volunteers. The donors were 19 – 38 years old and not on medication with sex steroids. The research on human sperm cells was approved by the Institutional Review Board of Department of biological and environmental sciences and technologies at the University of Salento.
Semen samples were collected by masturbation after 3-5 days of sexual abstinence and examined directly after liquefaction within 30 minutes. All samples underwent computer-assisted sperm analysis prior to use (CASA – SCA® 5.3.0.1: Sperm Class Analyser, LabIVF Asia Pte Ltd, Singapore).

*Sperm cell suspension establishment*
Sperms from all donors were pooled, collected by centrifugation and re-suspended in isotonic salt solution (2 g/L BSA, 113 mM KCl, 12.5 mM KH2PO4, 2.5 mM K2HPO4, 3 mM MgCl2, 0.4 mM EDTA, 20 mM Tris, adjusted to pH 7.4 with HCl).

*Sperm mitochondria respiration assay*
A total of $10 \times 10^6$ sperm cells/mL were incubated with the test item, vehicle, negative or positive control at concentrations in the range of 0.1 – 1000 nM for 1 hour at 37 °C. Each experiment was performed in quadruplicates.
In order to evaluate the role of physiologically relevant hormone concentrations in endocrine-disrupting chemical mitochondrial targeting, human sperm cells were co-incubated with the test item or respective controls and steroid hormones. In the co-incubation experiments, the sperm cells were pre-incubated with the test item, solvent, negative and positive controls in the same concentration range for 20 minutes before adding 10 nM of the respective hormones (progesterone (P4), testosterone (T), di-hydroxytestosterone (DHT) and 17β-estradiol (E2) or the flavonoid quercetin (QRC).
After treatment, spermatozoa were subjected to hypotonic treatment and used for polarographic assays

of oxygen consumption as described by Ferramosca et al. (2008)[1].
Oxygen consumption was measured by using a Clark-type oxygen probe, in the presence of a solution of 10 mM pyruvate and 10 mM malate (respiratory substrates) and 0.76 µM of adenosine diphosphate (ADP).

The following parameters were evaluated:

- $V_3$, active state of mitochondrial respiration: Rate of oxygen uptake measured in the presence of respiratory substrates and ADP, reported as nmol O2 x ml- 1x min- 1 ⁄ (10 × $10^6$ cells).
- $V_4$, passive state of mitochondrial activation: Rate of oxygen uptake measured with respiratory substrates pyruvate and malate alone, reported as nmol O2 x ml- 1x min- 1 ⁄ (10 × $10^6$ cells).
- Respiratory control ratio (RCR): Index of mitochondrial respiration efficiency, calculated by dividing $V_3$ by $V_4$.

*Statistical analysis:*
Statistical analysis was carried out by the software GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as the mean of three samples with standard deviation. Student's *t*-test was performed to detect significant differences between the blank and chemicals treated spermatozoa. Differences were considered statistically significant at $p < 0.05$.

## Results

*Effects of glyphosate and the flavonoid quercetin on sperm oxygen consumption*

Upon treatment with glyphosate, there was a reduction in mitochondrial respiration efficiency. Both parameters $V_3$ and $V_4$ were statistically significantly decreased starting at 1 nM and 10 nM, respectively, reaching a maximum decrease of -31% when compared to the solvent control at the highest concentration of 1000 nM. The respiration control ratio (RCR) was statistically significantly decreased at 1000 nM.
The positive control NADH and the negative control CCCP demonstrated the validity of the test system. NADH exposure caused a statistically significant, dose-dependent increase in the active state of mitochondrial respiration ($V_3$), resulting in a dose-dependent increase of the respiration control ratio. Conversely, CCCP treatment caused a dose-dependent increase in the resting state of respiration ($V_4$), hence resulting in a dose-dependent decrease of the respiration control ratio.

The mitochondria-targeted flavonoid quercetin had a direct effect on the passive state of mitochondrial respiration. Quercetin induced a statistically significant increase of $V_3$ values from 0.1 nM constantly kept up to 100 nM, followed by a drop down to control values at the highest concentration, a statistically significant, dose-dependent increase of $V_4$ and a dose-dependent RCR decrease up to -43% when compared to control levels at the highest concentration.

[1] A. Ferramosca, R. Focarelli, P. Piomboni, L. Coppola, V. Zara, Oxygen uptake by mitochondria in demembranated human spermatozoa: a reliable tool for the evaluation of sperm respiratory efficiency, Int. J. Androl. 31 (2008) 337–345, https://doi.org/10.1111/j.1365-2605.2007.00775.x

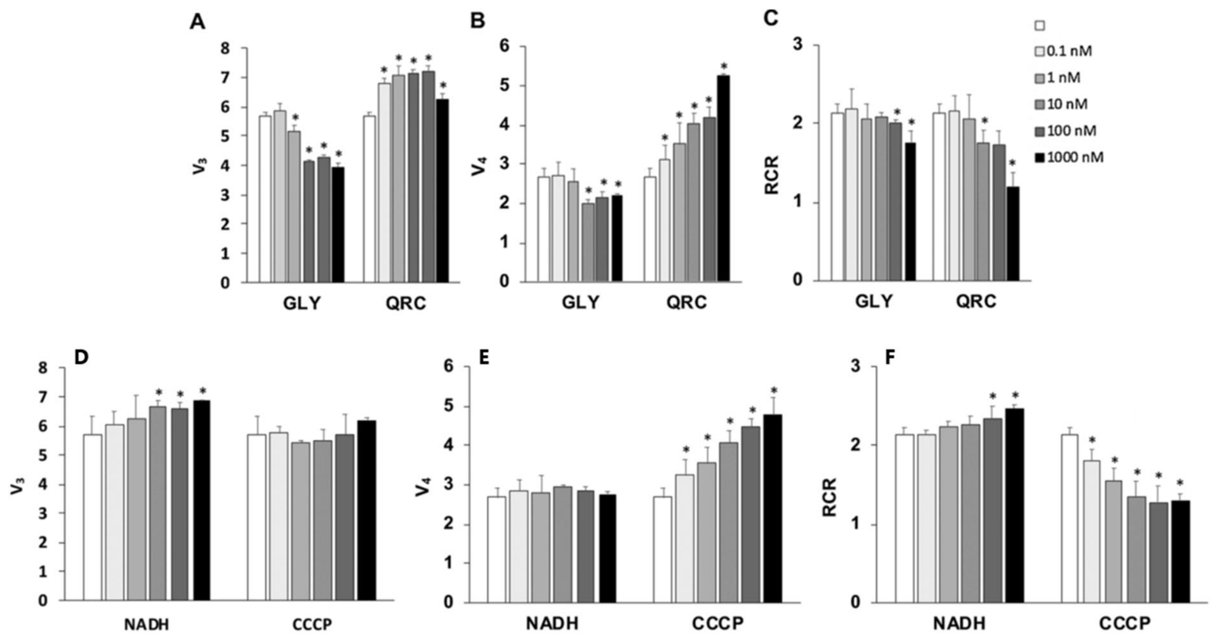


**Figure 1 (modified from the publication Ferramosca *et al.*, 2021 to report data for glyphosate only):** $V_3$, $V_4$ and RCR values upon dose-dependent treatment with glyphosate (GLY), the flavonoid quercetin (QRC), the positive control nicotinamide adenine dinucleotide (NADH) and the negative control carbonyl cyanide 4-chlorophenyl hydrazone (CCCP). **Fig. 1A**: $V_3$ of GLY and QRC; **Fig 1B**: $V_4$ of GLY and QRC; **Fig 1C**: RCR of GLY and QRC; **Fig. 1D**: $V_3$ of NADH and CCCP; **Fig 1E**: $V_4$ of NADH and CCCP; **Fig 1F**: RCR of NADH and CCCP. All data were subjected to Student's t-test ($p < 0.05$).

*Effect of steroid hormones on sperm mitochrondrial oxygen consumption*

Upon treatment with steroid hormones, di-hydroxytestosterone (DHT) was identified as the only hormone significantly reducing mitochondrial functionality at physiologically relevant concentrations. DHT at concentrations of 10 and 100 nM caused a statistically significant decrease in the active state ($V_3$), as well as a dose-dependent, statistically significant increase in the resting state ($V_4$), thus significantly decreasing the respiration control ratio (RCR).

For the remaining steroid hormones progesterone (P4), testosterone (T) and 17β-estradiol (E2) no adverse effect on mitochondria functionality was observed. Although P4 concentrations at 0.1 and 1 nM had a statistically significant increasing effect on $V_3$, mitochondrial respiration efficiency was not affected.

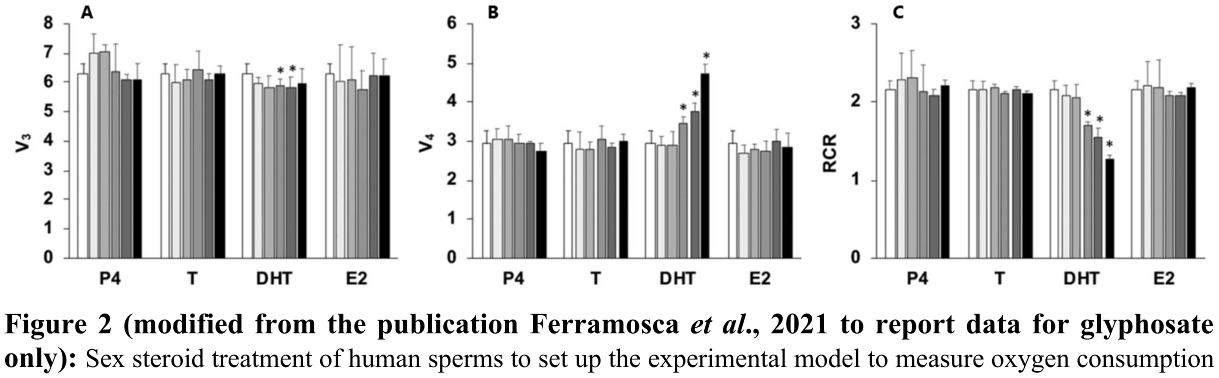


**Figure 2 (modified from the publication Ferramosca *et al.*, 2021 to report data for glyphosate only):** Sex steroid treatment of human sperms to set up the experimental model to measure oxygen consumption ($V_3$ and $V_4$) and the $V_3$:$V_4$ ratio (RCR). Human sperms were dose-dependently treated with the steroid hormones progesterone (P4), testosterone (T), di-hydroxy-testosterone (DHT) and 17β-estradiol (E2). **Fig. 2A**, $V_3$ of P4, T, DHT and E2; **Fig. 2B**, $V_4$ of P4, T, DHT and E2; **Fig. 2C**, RCR of P4, T, DHT and E2. All data were subjected to Student's *t* test (* $P < 0.05$).

*Effects of glyphosate and quercetin on sperm oxygen consumption upon co-treatment with sex steroids*

After co-incubation of sperm cells with glyphosate and steroid hormones, only treatment with di-hydroxytestosterone (DHT) was able to change the effects observed when glyphosate has been used alone. At the lowest tested concentration of 0.1 nM glyphosate, the respiration control ratio (RCR) was -31% decreased when compared to the solvent control. The decrease was attributed to an increase in the oxygen consumption rate, however, the increase in $V_4$ values much higher (55% vs. 5% at 0.1 nM; 58% vs. 114% at 1000 nM).
Also for Quercetin, a slight decrease in the RCR value was observed in the presence of DHT only.

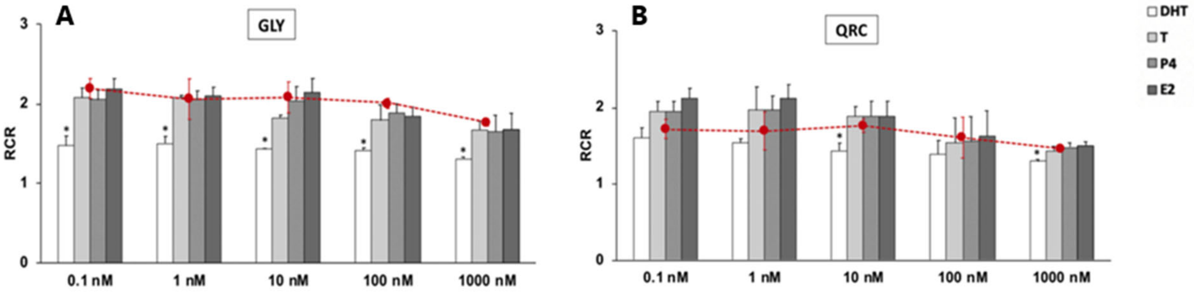


**Figure 3 (modified from the publication Ferramosca *et al*., 2021)**: RCR values upon dose-dependent treatments with GLY and QRC in the presence of 10 nM DHT, or 10 nM testosterone (T), or 10 nM progesterone (P4), or 10 nM 17β-estradiol (E2). **Fig. 3A**, RCR of GLY (0.1- 1000 nM) in presence of 10 nM DHT, T, P4 or E2; **Fig. 3B**, RCR of QRC (0.1- 1000 nM) in presence of 10 nM DHT, T, P4 or E2. The red dotted line shows RCR values obtained when GLY or QRC were tested individually. All data were subjected to Student's *t*-test (* $P < 0.05$).

**Table 1:** Mitochondrial oxygen uptake in the active ($V_3$) and in the resting ($V_4$) state after treatment with glyphosate alone (GLY) and glyphosate in combination with di-hydroxytestosterone (DHT) published by Ferramosca *et al*., 2021

| Concentrations (nM) | $V_3$ (nmol $O_2$ x $ml^{-1}$x $min^{-1}$/(10 x $10^6$ cells)) | | $V_4$ (nmol $O_2$ x $ml^{-1}$x $min^{-1}$/(10 x $10^6$ cells)) | |
|---|---|---|---|---|
| | **GLY** | **GLY + DHT** | **GLY** | **GLY + DHT** |
| 0.1 | 5.85 ± 0.15 | 6.17 ± 0.76 | 2.70 ± 0.50 | 4.20 ± 0.20 |
| 1 | 5.17 ± 0.29 | 6.07 ± 0.40 | 2.53 ± 0.35 | 4.03 ± 0.25 |
| 10 | 4.12 ± 0.12 | 6.30 ± 0.66 | 1.98 ± 0.12 | 4.40 ± 0.40 |
| 100 | 4.30 ± 0.20 | 6.67 ± 0.21 | 2.15 ± 0.15 | 4.70 ± 0.10 |
| 1000 | 3.93 ± 0.28 | 6.23 + 0.25 | 2.23 ± 0.02 | 4.77 ± 0.23 |

*Effects of glyphosate on sperm oxygen consumption upon co-treatment with quercetin*

In the presence of 10 nM quercetin, glyphosate slightly reduced $V_3$ and $V_4$ in a dose-dependent manner (statistically not significant). RCR values were dose-dependently decreased, reaching statistical significance at the highest concentrations tested. When compared to individual glyphosate treatments, the co-incubation with quercetin caused an increased consumption rate mainly at concentrations in the range of 0.1 – 10 nM, reaching the highest values when glyphosate and quercetin concentrations were in the same range. The findings suggest a stimulus of mitochondrial respiration independent of ADP phosphorylation.

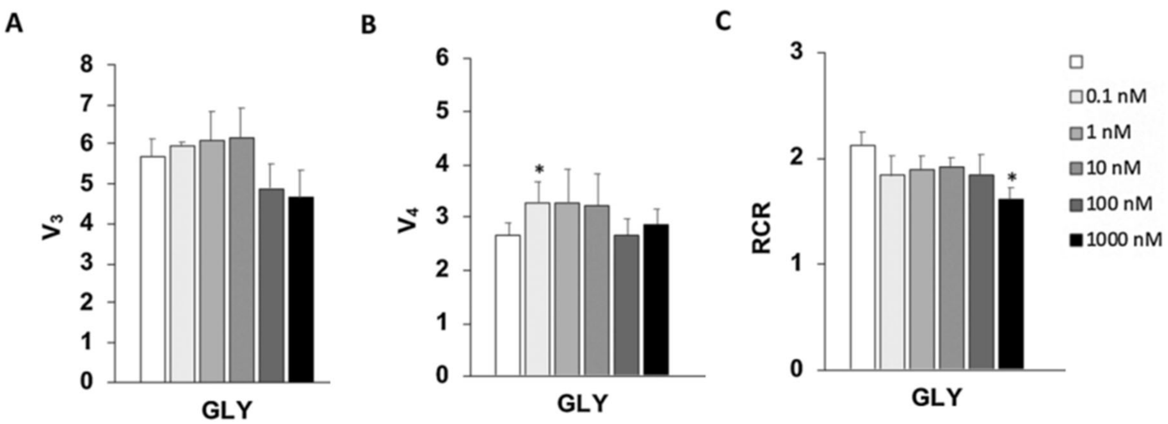


**Figure 4 (modified from the publication Ferramosca *et al.*, 2021):** $V_3$, $V_4$ and RCR values upon dose-dependent treatments with glyphosate (GLY) in the presence of the flavonoid quercetin (QRC). **Fig. 4A,** $V_3$ of GLY in presence of 10 nM QRC; **Fig. 4B**, $V_4$ of GLY in presence of 10 nM QRC; **Fig. 4C**, RCR of GLY in presence of 10 nM QRC. All data were subjected to Student's *t*-test (* $P < 0.05$).

The effects of di-hydroxytestosterone, glyphosate and quercetin on sperm mitochondria are summarised in the figure below.

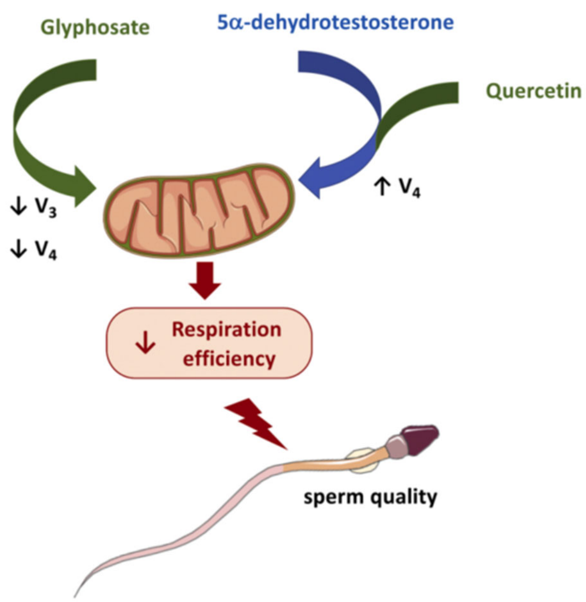


**Figure 5:** Effects overview of di-hydroxytestosterone, glyphosate and quercetin on sperm mitochondria published by Ferramosca *et al.*, 2021

**Conclusion**

In human sperm cells, glyphosate was able decrease oxygen consumption rate in the active ($V_3$) and in the passive state ($V_4$) of mitochondrial respiration, which is assumed to reflect a reduction in mitochondrial function. The mitochondrial respiration efficiency (respiratory concentration ratio, RCR) was negatively affected only at concentrations of ≥ 100 nM.

In the presence of the steroid hormone di-hydroxytestosterone (DHT), the negative effect of glyphosate was evident at concentrations at ≥ 0.1 nM already. $V_4$ values were increased, suggesting a stimulus of mitochondrial respiration independent of ADP phosphorylation.

After co-stimulation with glyphosate and the mitochondria-targeting flavonoid quercetin, an increase in oxygen consumption rate was observed at concentrations in the range of 0.1 – 10 nM, reaching the highest levels at glyphosate and quercetin concentrations at 10 nM. The authors concluded that glyphosate targets mitochondria by using a mechanism that is different from that of DHT and quercetin.

## 3. Assessment and conclusion

**<u>Assessment and conclusion by applicant:</u>**

Glyphosate was reported to reduce mitochondrial functionality, by decreasing the oxygen consumption rate in the active and in the passive state of mitochondrial respiration. The mitochondrial respiration efficiency was negatively affected only at concentrations of ≥ 100 nM. In the presence of the sex steroid hormone di-hydroxytestosterone (DHT), the negative effect on mitochondria functionality caused by glyphosate was observed from ≥ 0.1 nM. The passive state of mitochondrial respiration was found to be increased, suggesting a stimulus of mitochondrial respiration independent of ADP phosphorylation. In the presence of the mitochondria-targeting flavonoid quercetin, an increase in oxygen consumption rate was observed at concentrations in the range of 0.1 – 10 nM, reaching the highest levels at glyphosate and quercetin concentrations at 10 nM. Glyphosate was concluded to target mitochondria by using a mechanism that is different from that of DHT and quercetin but not described.

The study did not follow any OECD guideline and was not performed under GLP. No information on the test item with regard to purity was given, however, the supplier and batch number were reported. Cytotoxicity tests were not included, but a broad concentration range from 0.1 - 1000 nM was tested to cover the sexual hormones physiologically relevant concentrations (10 nM), triggering endogenously hormone-dependent signalling pathways, and the estimated (nM range) QRC dietary intake. However, it is not clear how these concentrations may be relevant in term of exposure to glyphosate. The authors stated that these concentration are below the NOAEL and acceptable daily intake (ADI) for the glyphosate (50 and 0.5 mg/kg bw per day, respectively). But no calculations have been presented to show whether spermatozoa could be exposed under the normal condition of glyphosate use. Given the novel study type and underlaying assumptions, evaluation of other comparator molecules to which humans are regularly systemically exposed (e.g. in the diet) would provide context to the relevance of these results and credibility to the assay’s predictive capacity for effects in humans.

It was not clear from the publication which solvent has been used for which chemical. As solvent controls were included for all solvents used, the weakness was considered to be of minor degree. The criteria for a biological response were not provided.

Overall, the study is sufficiently documented to generally accepted scientific principles. It is considered to be reliable with restriction, but the information provided are not robust enough to impact the risk assessment.

**<u>Assessment and conclusion by RMS:</u>**

**Reliability criteria for *in vitro* toxicology studies**

| **Publication:** Ferramosca, 2021: Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency. | **Criteria met? Y/N/Uncertain** | **Comments** |
|---|---|---|
| **Guideline-specific** | | |
| Study is in accordance to valid internationally accepted testing guidelines. | N | |
| Study is performed according to GLP. | N | |
| Study is completely described and conducted following scientifically acceptable standards. | Y | |
| **Test substance** | | |
| Test material (glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions) | Y | Purity for glyphosate not reported but batch No. given (#45521, Sigma Aldrich) |
| Only glyphosate acid or one of its salts is the tested substance. | N | Glyphosate alone and in combination with steroid hormones. |

| **Publication:** Ferramosca, 2021: Herbicides glyphosate and glufosinate ammonium negatively affect human sperm mitochondria respiration efficiency. | **Criteria met? Y/N/Uncertain** | **Comments** |
|---|---|---|
| AMPA or other glyphosate metabolites is the tested substance. | N | |
| **Study** | | |
| Test system is clearly and completely described. | Y | |
| Test conditions are clearly and completely described. | Y | |
| Metabolic activation system is clearly and completely described. | N | |
| Test concentrations is in physiologically acceptable range (< 1 mM). | Y | 0.1-1000 nM |
| Cytotoxicity tests are reported. | N | Concentration range covers both the sexual hormones physiologically relevant concentrations (10 nM), triggering endogenously hormone-dependent signaling pathways, and the estimated (nM range) QRC dietary intake. |
| Positive and negative controls. | Y | |
| Complete reporting of effects observed. | Y | |
| Statistical methods described. | Y | |
| Historical negative and positive control data reported. | N | Criteria for a biological relevant response not provided. |
| Dose-effect relationship reported. | Y | |
| **Overall assessment** | | |
| Reliable without restrictions | N | |
| Reliable with restrictions | Y | No information on the test item with regard to purity was given, however, the supplier and batch number were reported. Cytotoxicity tests were not included, but a broad concentration range from 0.1 - 1000 nM was tested. Historical control data were not reported. Results contradict higher tier in vivo multigenerational studies dosed at several orders of magnitude higher, which do not report any adverse outcomes in fecundity or reproductive outcome.<br>No information on whether the tested concentration may reflect physiological exposure to human spermatozoa in vivo following exposure to the accepted regulatory dose levels following glyphosate use as herbicide. |
| Not reliable | N | |

## 1. Information on the study

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|---|---|
| **Data point:** | CA 5.9.4 |
| **Report author** | Shrestha S. *et al.* |
| **Report year** | 2020 |
| **Report title** | Pesticide use and incident Parkinson's disease in a cohort of farmers and their spouses. |
| **Document No** | Environmental Research (2020), Vol. 191, Article No. 110186<br>https://doi.org/10.1016/j.envres.2020.110186 |
| **Guidelines followed in study** | None |
| **Deviations from current test guideline** | Not applicable |
| **GLP/Officially recognised testing facilities** | Not applicable |
| **Acceptability/Reliability:** | Yes (Relevance Category A)/Reliable without restrictions |

## 2. Full summary of the study according to OECD format

The authors studied the use of pesticides and possible associations with incident Parkinson's disease (PD) for 38,274 pesticide applicators and 27,836 of their spouses in the Agricultural Health Study (AHS) cohort with follow-up of more than 20 years. **Methods:** Self-reported AHS enrollment questionnaire information regarding use of 50 specific pesticides and follow-up questionnaire information from applicators and spouses was employed in the analysis. There were two exposure metrics: ever use of specific pesticides and intensity-weighted lifetime days of use (IWLD). The statistical analysis involved Cox regression, adjusted for covariates, to estimate hazard ratios (HRs) and 95% confidence intervals (95% CIs) for incident PD for each of the 50 pesticides. PD risk was also evaluated based on head injury and the use or non-use of chemical resistant gloves. **Results:** A total of 373 applicators and 118 spouses self-reported incident doctor-diagnosed PD after enrollment. Ever-use of the insecticide terbufos (HR 1.31, 95% CI 1.02, 1.68) and the herbicides trifluralin (HR 1.29, 95% CI 0.99, 1.70) and 2,4,5-T (HR 1.57, 95% CI 1.21, 2.04) was associated with elevated PD risk. On the other hand, diazinon (HR 0.73, 95% CI 0.58, 0.94) and 2,4,5-TP (HR 0.39, 95% CI 0.25, 0.62) were associated with reduced risk. The authors observed higher risk for certain pesticides among those who reported a history of head injury or who did not use chemical resistant gloves. PD risk was also elevated for applicators in the highest category of IWLD for dichlorvos, permethrin (animal use), and benomyl. **Conclusions:** The authors found evidence of increased PD risk for some pesticides and concluded that there was higher susceptibility for pesticide-associated PD among individuals with a prior head injury as well as protection from PD with use of chemical resistant gloves.

### Materials and methods

The AHS cohort enrolled 52,394 applicators and 32,345 spouses. Excluded for the purposes of this study were those who did not participate in any AHS follow-up: 13,596 applicators and 4,107 spouses. Also excluded were those who: reported PD at enrollment (96 applicators and 34 spouses), did not respond to follow-up PD questions (249 applicators and 306 spouses), had PD reported on a death certificate but no other information available (84 applicators and 25 spouses), reported PD that could not be confirmed (88 applicators and 36 spouses), or gave inconsistent responses across surveys (7 applicators, 1 spouse). Thus, the analysis cohort for this study included 38,274 applicators and 27,836 spouses.

Two pesticide exposure metrics were used in the analysis. First, the authors evaluated PD risk according to ever use of 50 pesticides. Second, PD risk was evaluated according to exposure intensity-weighted lifetime days of use (IWLD) calculated based on information on days of use for specific pesticides, mixing practices, application methods, equipment repair, and personal protective equipment use. Cox proportional hazards regression was used to estimate HRs and 95% CIs for specific pesticides and PD. In the IWLD analyses, a test for trend was conducted using the median IWLD value as an ordinal variable in regression models. Multiple imputation was used to fill in missing covariate data.

**Results**

In a combined analysis of applicators and spouses, the authors found positive associations for the organophosphate insecticide terbufos (HR 1.30, 95% CI 1.02, 1.68) and the herbicides trifluralin (HR 1.29, 95% CI 0.99, 1.70) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) (HR 1.57, 95% CI 1.21, 2.04), and inverse (viz., decreased risk) associations for ever-use of the organophosphate insecticide diazinon (HR 0.73, 95% CI 0.58, 0.94), the fumigant ethylene dibromide (HR 0.35, 95% CI 0.14, 0.84), and the herbicide 2,4,5-TP [2,4,5-T,P, 2-(2,4,5-trichlorophenoxy) propionic acid] (HR 0.39, 95% CI 0.25, 0.62). The corresponding analysis for glyphosate showed no association with PD (HR 1.10, 95% CI 0.87, 1.39).

In analyses stratified by presence or absence of head injury, the authors found increased risk for those with a history of head injury, versus no increased risk for those without head injury, for chlordane, dichlorodiphenyltrichloroethane (DDT), toxaphene, diazinon, phorate, permethrin, methyl bromide, paraquat and pendimethalin. Results for glyphosate were not presented for those with/without a history of head injury.

In analyses stratified by use or non-use of chemical resistant gloves, the authors found 5 herbicides (dicamba, imazethapyr, metolachlor, trifluralin, and metribuzin) that were associated with increased PD risk among those who did not wear chemical resistant gloves when there was no increased risk for these herbicides among users of chemical resistant gloves. Results for glyphosate were not presented stratified by use of non-use of chemical resistant gloves.

In IWLD analyses, none of the 50 pesticides were found to have a significant trend of increasing PD HRs with increasing IWLDs, though there were elevated HRs for individuals in the highest category of IWLD for the insecticides dichlorvos (HR 1.46, 95% CI: 0.98, 2.19, p-trend 0.06) and permethrin (animal use) (HR 1.44, 95% CI: 0.85, 2.44, p-trend 0.21), and the fungicides benomyl (HR 1.34, 95% CI 0.64, 2.80, p-trend 0.31), captan (HR 1.27, 95% CI 0.74, 2.20, p-trend 0.36), and chlorothalonil (HR 1.29, 95% CI 0.66, 2.56, p-trend 0.41) as compared to those who never used those pesticides. Glyphosate did not show a positive trend with increasing IWLDs and the HR in the highest IWLD category was 0.85 (95% CI 0.62, 1.17).

**Conclusions**

The authors concluded that they found evidence of increased PD risk for terbufos, trifluralin, and 2,4,5-T and decreased PD risk for diazinon and 2,4,5-TP. They also concluded that some pesticide-PD associations were more apparent in those with a past head injury and that using chemical resistant gloves reduced some pesticide-PD associations. The authors did not reach a conclusion specific for glyphosate.

## 3. Assessment and conclusion

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| **<u>Assessment and conclusion by applicant:</u>**<br><br>Glyphosate was not associated with PD in analyses based on ever use or in analyses based on IWLDs of use. Given that there is no plausible mechanism for glyphosate causing PD and that glyphosate systemic dose has been found to be minimal for applicators and spouses (Acquavella et al. 2004), those results are considered to be a valid.<br><br>**References**<br><br>Acquavella JF, Alexander BH, Mandel JS, et al. Glyphosate biomonitoring for farmers and their families: Results from the farm family exposure study. Environ. Health Perspect. 2004; 112:321-326. |

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| **<u>Assessment and conclusion by RMS:</u>** |

**Reliability criteria for epidemiology studies**

| **Publication:** Shrestha S. et al., 2020, Pesticide use and incident Parkinson's disease in a cohort of farmers and their spouses. | **Criteria met? Y/N/?** | **Comments** |
|---|---|---|
| **Guideline-specific** | | |
| Study is in accordance to valid internationally accepted testing guidelines/practices. | n/a | Not applicable |
| Study is completely described and conducted following scientifically acceptable standards. | Yes | |
| **Test substance** | | |
| Exposure to formulations with only glyphosate as a.i. | Yes | |
| Exposure to formulations with glyphosate combined with other a.i. | No | |
| Exposure to various formulations of pesticides. | Yes | 50 pesticides total |
| **Study** | | |
| Study design – epidemiological method followed. | Yes | |
| Description of population is investigated. | Yes | |
| Description of exposure circumstances. | Yes | |
| Description of results. | Yes | |
| Have confounding factors been considered. | Yes | |
| Statistical analysis. | Yes | |
| **Overall assessment** | | |
| Reliable without restrictions | Yes | The finding of no association between glyphosate and Parkinson's disease risk in this study is considered to be valid. The results fit with what is known about glyphosate toxicology and exposure potential. |
| Reliable with restrictions | No | |
| Reliability not assignable | No | |
| Not reliable | No | |

## 1. Information on the study

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| **Data point:** | CA 5.9.4 |
| **Report author** | Werder E. J. *et al.* |
| **Report year** | 2020 |
| **Report title** | Herbicide, fumigant, and fungicide use and breast cancer risk among farmers' wives. |
| **Document No** | Environmental Epidemiology (2020), Vol. 4, No. 3, Art. No. e097 |
| **Guidelines followed in study** | None |
| **Deviations from current test guideline** | Not applicable |
| **GLP/Officially recognised testing facilities** | Not applicable |
| **Acceptability/Reliability:** | Yes (Relevance Category A)/Reliable without restrictions |

## 2. Full summary of the study according to OECD format

**Methods:** The authors examined exposure to herbicides, fumigants, and fungicides in relation to breast cancer risk among farmers' wives with no prior history of breast cancer in the Agricultural Health Study (AHS). Women provided information on pesticide use, demographics, and reproductive history at enrollment (1993–1997) and at a 5-year follow-up interview. Cox proportional hazards regression was used to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) between the women's and their husbands' self-reported use of individual pesticides and incident breast cancer risk. **Results:** Out of 30,594 women, 38% reported using herbicides, fumigants, or fungicides and 1,081 were diagnosed with breast cancer during a median 15.3 years of follow-up. There were elevated HRs in relation to women's ever use of the fungicide benomyl (HR = 1.6; 95% CI = 0.9, 2.7) and the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (HR = 1.6; 95% CI = 0.8, 3.1) and for their husbands' use of the herbicide 2-(2,4,5-trichlorophenoxy) propionic acid (2,4,5-TP) (HR = 1.5; 95% CI = 0.9, 2.7). Few other chemical associations were observed and there was little evidence of differential risk by tumor estrogen receptor status or linear exposure-response relationships. **Conclusions:** The authors did not find a general pattern of increased breast cancer risk with use of specific pesticides, though they did find some evidence of increased risk with women's use of benomyl and 2,4,5-T and husbands' use of 2,4,5-TP.

### Materials and methods

The underlying biological hypothesis for this study was that some pesticides are endocrine disruptors or exhibit estrogenic activity, so research is warranted into whether direct pesticide use by spouses or possible indirect pesticide exposure through the applicator's use of specific pesticides may relate to higher breast cancer risk.

The study population was restricted to female wives of AHS pesticide applicators. Of the 32,126 AHS wives who enrolled in the AHS cohort, 19,578 (61%) completed the enrollment spouse questionnaire about farm exposures and the female health questionnaire providing a reproductive health history. In addition, 23,676 of the wives (74%) completed a 5-year follow-up telephone interview. Of the 32,126 AHS wives, 478 were excluded for having prevalent breast cancer at enrollment, 113 were excluded for living outside of Iowa or North Carolina at enrollment (residence was necessary for detection of breast cancers in the statewide cancer registries), and 948 were excluded for having provided no data on pesticide use. This left a study population of 30,594 women [Note, the numbers actually add up to 30, 587.]

Cox proportional hazards models were used to estimate HRs and 95% CIs adjusted for time-varying menopausal status, race, state, parity, age at first birth, and for all other pesticides found to be associated with breast cancer. Missing covariate data were imputed. Results were similar with/without the imputed covariate data. Estrogen and progesterone receptor status, known for 87% of cases, was considered via joint proportional hazards models.

**Results**

Risk of breast cancer was not associated with spouses ever using any herbicide (HR 0.9; 95% CI 0.8, 1.0), any fungicide (HR 0.9; 95% CI 0.6, 1.2), or any fumigant (HR 0.9; 95% CI 0.5, 1.4). For specific pesticides, breast cancer HRs were elevated non-significantly for any use of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (HR 1.6; 95% CI 0.8, 3.1) and the fungicide benomyl (HR 1.6; 95% CI 0.9, 2.7) and the HR was reduced for any use of butylate (HR 0.4; 95% CI 0.2, 1.0). For glyphosate, the HR of 0.9 (95% CI 0.8, 1.0) indicated no association with breast cancer risk with any personal use.

The authors considered possible indirect exposure based on the husbands reported use of pesticides for women who did not apply pesticides themselves (n = 13,500). Ever use of 2-(2,4,5-trichlorophenoxy) propionic acid (2,4,5-TP) was associated with a non-significantly elevated breast cancer risk (HR 1.5; 95%CI 0.9, 2.7) as was husbands' use of 2,4,5-T below the cumulative days of use median (HR 1.5; 95% CI 0.7, 2.9) and above the median (HR = 1.3; 95% CI = 0.8, 2.0), with limited evidence of a monotonic exposure-response trend (p trend = 0.08). Husbands' ever use of trifluralin was associated with a significant reduction in breast cancer risk (HR 0.7; 95% CI 0.5, 0.9) with a significant, but nonmonotonic, exposure-response trend across quartiles of exposure (p trend 0.01). There was no association between husband's extent of use of glyphosate and breast cancer risk. The HR was 1.0 (95% CI 0.7, 1.3) for ever use and HRs varied from 0.8 to 1.1 over cumulative days of use quartiles.

**Conclusions**

The authors concluded that they did not observe any clear associations between use of pesticides by female spouses and breast cancer or from possible indirect exposure based on pesticides that were used by the husbands. However, they did note elevated HRs associated with women's use of benomyl and 2,4,5-T and husbands' use of 2,4,5-TP.

**3. Assessment and conclusion**

**Assessment and conclusion by applicant:**

This study was undertaken based on the assumption that the properties of pesticides – on the endocrine disruption and estrogenic activity scales – are such that an increase in breast cancer risk is possible from direct use of specific pesticides by female AHS spouses or from presumed indirect exposure related to their husbands' use of specific pesticides. The presumed biologic properties of pesticides underlying the study's hypotheses do not apply to glyphosate, at systemic doses from direct or indirect exposure ($10^{-4}$ mg/kg direct, $10^{-5}$ mg/kg indirect – see Acquavella et al. 2004). The results of the study did not find clear associations between pesticide use and breast cancer risk and results for glyphosate were consistent across the various analyses in indicating no association with breast cancer.

We conclude that this study provides evidence that glyphosate is not related to breast cancer risk.

**References**

Acquavella JF, Alexander BH, Mandel JS, et al. Glyphosate biomonitoring for farmers and their families: Results from the farm family exposure study. Environ. Health Perspect. 2004; 112:321-326.

| Assessment and conclusion by RMS: |
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**Reliability criteria for epidemiology studies**

| **Publication:** Werder E. J. et al., 2020, Herbicide, fumigant, and fungicide use and breast cancer risk among farmers' wives. | **Criteria met? Y/N/?** | **Comments** |
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| **Guideline-specific** | | |
| Study is in accordance to valid internationally accepted testing guidelines/practices. | n/a | Not applicable |
| Study is completely described and conducted following scientifically acceptable standards. | Yes | |
| **Test substance** | | |
| Exposure to formulations with only glyphosate as a.i. | Yes | |
| Exposure to formulations with glyphosate combined with other a.i. | Uncertain | |
| Exposure to various formulations of pesticides. | Yes | 26 pesticides |
| **Study** | | |
| Study design – epidemiological method followed. | Yes | |
| Description of population is investigated. | Yes | |
| Description of exposure circumstances. | Uncertain | No description of how farm spouses applied pesticides. |
| Description of results. | Yes | |
| Have confounding factors been considered. | Yes | |
| Statistical analysis. | Yes | Good. |
| **Overall assessment** | | |
| Reliable without restrictions | Yes | This study did not show a relationship between glyphosate and breast cancer. That result is consistent with glyphosate's exposure and toxicological properties. |
| Reliable with restrictions | No | |
| Reliability not assignable | No | |
| Not reliable | No | |