1. Literature Review of Developmental and Reproductive Toxicity (DART) and Endocrine Disruption (ED) Publications

Publications suggesting glyphosate or glyphosate based formulations are developmental toxicants, reproductive toxicants or endocrine disruptors include *in vitro* studies, *in vivo* studies and epidemiological studies with weak, statistically non-significant associations. Some epidemiological studies evaluate associations with pesticides in general or classes of pesticides, with no mention of glyphosate or glyphosate based products, and thus warrant no further discussion (e.g. Benítez-Leite, 2009) other than the OECD Tier II like summary and Klimisch rating (Klimisch, 1997). Many of these published since 2000 are specifically discussed in a comprehensive glyphosate DART review publication by three internationally recognized experts (Williams et al., 2012), referenced in Doc L Table 2 and included in Doc K. Further discussions of some significant papers follow.

In addition, glyphosate was included on the US EPA Endocrine Disruptor Screening Program's (EDSP) first list of 67 compounds to Tier 1 Screening. The US EPA clearly published the criteria for inclusion on List 1 was strictly based on exposure potential, not hazard, specifically stating in the Federal Register (2009);

> "This list should not be construed as a list of known or likely endocrine disruptors".

A consortium of glyphosate registrants in North America, the Joint Glyphosate Task Force, LLC (JGTF), coordinated the conduct of the glyphosate battery of Tier 1 screening assays under the EDSP and submitted these successfully completed assays to the US EPA. The US EPA will evaluate the full battery of Tier 1 screening assays together using a weight of evidence approach, for glyphosate's potential to interact with the estrogen, androgen and thyroid endocrine pathways. The following below were submitted by the JGTF to the US EPA in early 2012 and are expected to be reviewed this year. However, the Agency gas announced they will not release their Data Evaluation Records (DERs) for individual EDSP studies until a weight of evidence review has been completed for List 1 compounds. Therefore, in an effort to disclose the findings of the glyphopsate EDSP data to the scientific community, the JGTF is considering publishing a Weight of Evidence review of glyphosate with respect to endocrine disruption.

In Vitro EDSP Glyphosate Studies submitted to the US EPA

- Androgen Receptor Binding (Rat Prostate Cytosol); OCSPP 890.1150
- Aromatase (Human Recombinant); OCSPP 890.1200
- Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC); OCSPP 890.1250
- Estrogen Receptor Transcriptional Activation (Human cell Line, HeLa-9903); OCSPP 890.1300; OECD 455
- Published OECD Validation of the Steroidogenesis Assay (Hecker et al., 2010)

In Vivo EDSP Glyphosate Studies submitted to the US EPA

- Amphibian Metamorphosis (Frog) OCSPP 890.1100; OECD 231
- *In Vivo* Hershberger Assay (Rat); OCSPP 890.1600; OECD 441
- Female Pubertal Assay; OCSPP 890.1450; OECD None
- Male Pubertal Assay; OCSPP 890.1500
- Uterotrophic Assay (Rat); OCSPP 890.1600; OECD 440
- Fish Short-Term Reproduction Assay; OCSPP 890.1350; OECD 229

The glyphosate Tier 1 screening assay study reports are owned by the JGTF. The European Glyphosate Task Force (GTF) is negotiating to procure access rights to the battery of glyphosate EDSP Tier 1 screening study reports. Results of the Hershberger and Uterotrophic *in vivo* rat studies, now in the public domain, as are the published results of the OECD validation of the Steroidogenesis assay, in which glyphosate clearly had no impact on steroidogenesis, are discussed below.

In Vitro Glyphosate DART/ED Publications

Many *in vitro* research publications have characterized pesticide formulations, including glyphosate based formulations, as toxic and endocrine disrupting products. Researchers and editorial boards have frequently overlooked the fact that surfactants (which are often components of formulated pesticide products), by their physico-chemical nature, are not suitable test substances using *in vitro* cell models. Surfactants compromise the integrity of cellular membranes, including mitochondrial membranes, and thus confound endpoint measurements considered as representative of specific toxicological modes of action or pathways. For example, Walsh et al. (2000) published research claiming that a glyphosate based formulation, but not glyphosate alone, adversely affected the steroidogenesis pathway by inhibiting progesterone production resulting in downstream reduction in mitochondrial levels of StAR protein. Subsequent research by Levine et al. (2007) demonstrated (i) no synergism between glyphosate and the surfactant since the cytotoxic effects were completely independent of glyphosate; identical dose-response curves were noted for formulated product with and without the glyphosate active ingredient; (ii) comparable cytotoxicity dose-response curves for several common household detergents or surfactants; and (iii) a variety of surfactants demonstrate cytotoxic effects that are not specific to biochemical pathways within intact cells. Levine (2007) concludes by emphasizing the importance of considering the biological plausibility of observed *in vitro* effects for in-tact animals.

Subsequent research addressing the steroidogenesis pathway confirmed glyphosate lacked endocrine disruption potential specific to this pathway. Quassinti et al. (2009) evaluated effects on gonadal steroidogenesis in frog testis and ovaries on glyphosate and another active substance, noting that glyphosate unequivocally demonstrated no effect. Forgacs et al. (2012) also tested glyphosate alone and demonstrated no effect on testosterone levels in BLTK1 murine leydig cells *in vitro*. Furthermore, the OECD multi-laboratory validation of the Steroidogenesis Assay used for Tier 1 screening of the US EPA EDSP, evaluated glyphosate and concluded no impact on steroidogenesis (Hecker et al., 2010). Consequently, the US EPA considered reference to the OECD validation report sufficient for meeting the glyphosate Steroidogenesis Assay Test Order in the EDSP Tier 1 screening of glyphosate.

The Seralini laboratory at the University of Caen, France, has multiple recent publications of *in vitro* research with glyphosate and glyphosate based formulations (Richard et al, 2005; Benachour et al, 2007; Benachour and Seralini, 2009; Gasnier et al, 2009; Gasnier et al, 2010; Gasnier et al., 2011; Clair et al., 2012; Mesnage et al., 2012), with proposed extrapolations to an array of *in vivo* effects including potent endocrine disruption, aromatase inhibition, estrogen synthesis, placental toxicity, foetotoxicity, embryotoxicity and bioaccumulation. These publications are often replicates of earlier studies, using different cell lines or primary cell cultures and in some cases the same data are reported again in a subsequent publication. Firstly, the *in vitro* synergism claims are conjecture, simply because no control groups of surfactant without glyphosate were tested. Secondly, the extrapolations to *in vivo* effects are unjustifiable based on both the unsuitability of surfactants in such test systems and the supraphysiological cytotoxic concentrations at which *in vitro* effects are reported. Again often overlooked by *in vitro* researchers and editorial boards, Levine et al. (2007) presented convincing data demonstrating a lack of *in vitro* synergism for glyphosate with other formulation ingredients. Regarding Seralini's repeated claims of glyphosate induced aromatase inhibition in mircosomes (Richard et al, 2005; Benachour et al, 2007; Gasnier et al, 2009), the data are confounded and thus uninterpretable where surfactants are introduced to such *in vitro* systems. This is noted in the US EPA Aromatase Inbibition Test Guideline, OECD 890.1200, in which notes,

> "Microsomes can be denatured by detergents [surfactants]. Therefore, it is important to ensure that all glassware and other equipment used for microsome preparations be free of detergent residue."

Research from the Seralini laboratory has repeatedly gained general public and media attention, including dissemination on "you-tube" and public lecture tours in various countries, in which allegations against glyphosate based products and biotechnology in agriculture are made. The selective use of literature, with absence of contradicting research (e.g., Kojima et al. (2004) demonstrated glyphosate lacked affinity for estrogen-α, estrogen-β and androgen receptors) demonstrates consistent and undeterred bias in the authors'

publication record. Numerous authoritative reviews have discounted the relevance of the Seralini team's research to human health risk assessment; some of these are referred to in specific publication reviews below. Several more recent publications from this group investigate homeopathic plant extract remedies for effects they attribute to glyphosate exposures in formulated products *in vitro* (Gasnier et al.(2010); Gasnier et al.(2011)).

Another *in vitro* publication claiming a specific developmental toxicity pathway has gained significant public traction, media attention and widespread international public lecture tours by the lead investigator. Paganelli et al. (2010) from the Carrasco research laboratory in Argentina conducted three *in vitro* assays, (i) frog embryos exposed to glyphosate formulation; (ii) frog embryos directly injected without injection blank negative controls; and (iii) fertilized chicken embryos exposed directly to a glyphosate formulation through a hole cut in the egg shell. Key issues surrounding this research include irrelevant routes of exposure as well as excessively high and environmentally unrealistic doses.

In Vivo Glyphosate DART/ED Publications

Relatively few *in vivo* publications on glyphosate DART and ED exist in comparison with the list of *in vitro* publications. Some lack appropriate interpretation of basic toxicology; e.g. Daruich et al. (2001) and Beuret et al. (2005) (two authors are common to each paper and from the same university department) noted rats treated with a glyphosate based formulation showed reduced food intake, reduced water intake and reduced body weight gains. However, the authors did not consider attributing the effects of altered enzyme concentrations to dehydration or restricted diets. Both studies are reviewed in Williams et al. (2012).

Dallegrave et al. (2003; 2007) published results of two non-guidelines rat developmental toxicity studies, in which a glyphosate based formation containing POEA was evaluated. Numerous reporting deficiencies and inconsistencies pose difficulties in data interpretation

Romano et al. (2010) evaluated a glyphosate based formulation in a male pubertal-like assay in Wistar rats, reporting decreased preputial separation, reduced seminiferous epithelial height, increased luminal diameter of seminiferous tubules, and increased relative testicular and adrenal weights. Given the gravity of the reported findings in this publication, a very detailed review was undertaken by experts in the fields of reproductive and developmental toxicology and endocrinology; William R. Kelce, M.S., Ph.D, Fellow ATS; James C. Lamb, IV, Ph.D, DABT and Fellow ATS; John M. DeSesso, Ph.D, Fellow ATS. Their critique is referenced in Doc L and included in Appendix K. Most recently, Romano et al. (2012) reported additional findings in male rats after supposed *in utero* and *post natal* exposures which include "behavioral changes and histological and endocrine problems in reproductive parameters and these changes are reflected by a hypersecretion of androgens and increased gonadal activity, sperm production and libido". As in their first publication, Romano et al. (2012) base their hypothesis on selectively discussed literature implicating glyphosate as an endocrine disruptor, predominantly with citations to research from the Seralini laboratory.

Recently, the first publicly data available from the glyphosate Tier 1 assays under the US EPA Endocrine Disruptor Screening Program, were reported at the 2012 Society of Toxicology meeting (Saltmiras et al., 2012) for the Hershberger and Uterotrophic assays. No effects were noted for any potential for glyphosate to interact with androgenic or estrogenic pathways under these GLP studies following the US EPA 890 Series Test Guidelines.

POEA DART Studies in Williams et al. (2012)

Polyethoxylated alkylamine (POEA) surfactants are a class of non-ionic surfactant, containing a tertiary amine, an aliphatic group of variable carbon chain length and two separate sets of ethoxy (EO) chains of variable length. A dietary exposure assessment of POEAs previously submitted by Monsanto to BfR (Bleeke et al. 2010) is referenced in Doc L and included in Doc K. This exposure assessment report also refers to the US EPA Alky Amine Polyalkoxylates Human Health Risk Assessment, which includes

POEAs (http://www.regulations.gov/search/Regs/home.html#documentDetail?R=09000064809b983b). Williams et al. (2012) recently evaluated and detailed the results of DART studies with two different POEA surfactants, summarized below.

Pregnant female rats were administered MON 0818, a POEA surfactant, at 0, 15 100 and 300 mg/kg/day. The NOAEL for maternal toxicity was 15 mg/kg/day and the NOAEL for rat developmental toxicity was the highest dose tested, 300 mg/kg/day (Holson, 2001).

A reproductive and developmental multigenerational screening study dosed MON 0818 in diets at 0, 100, 300 and 1000 ppm. The majority of endpoints evaluated were unaffected by treatment, including testis morphology, sperm parameters and testosterone and thyroid hormone levels. The mid-dose of 300 ppm (approximately 20 mg/kg/day) was considered the NOAEL for reproductive and developmental toxicity based on the following results in F0 at the high dose, 1000 ppm: increases in unaccounted for implantation sites with reduced mean number of pups and litter size in the high dose group; three high dose dams delivered litters of two-four pups each, with total litter loss by post natal day (PND) 4 in two of these litters. Upon breeding of F1 generation none of the findings noted in F0 were reproducible, and given some were not statistically significant, they were considered equivocal. However, a clear NOAEL for reproductive/developmental toxicity was considered to be the mid dose of 20 mg/kg/day (Knapp, 2007).

Another reproductive/developmental study of a different POEA surfactant, MON 8109 evaluated doses of 0, 30, 100, 300 and 2000 ppm in diet. A single dose group of MON 0818 at 1000 ppm in diet was also included to determine whether litter effects previously noted at this dose were treatment related (Knapp, 2008).

- MON 0818 dosed at 1000 ppm (76 and 86 mg/kg/day premating in males and females respectively) did not reveal the litter effects noted in the previous study at this dose. Two maternal incidents were not considered related to treatment; one female with dystocia died on PND 1 (this was also noted in one female of the control group F1 in the previous study at the same facility) and a second female was euthanized due to a ruptured uterus on gestation day 30. No test substance-related effects were noted for systemic toxicity, reproductive endpoints, pup survival or mortality. Therefore the overall DART NOAEL for MON 0818 was considered 1000 ppm, approximately 81 mg/kg/day.
- The MON 8109 systemic toxicity NOAEL in males and females was 300 ppm, based on mean body weight loss, reduced mean body weight gain and decreased food consumption at 2000 ppm. Developmental/reproductive effects at 2000 ppm included reduced mean number of implantation sites, increased number of unaccounted for implantation sites, decreased mean litter size at PND 0, reduced mean number of births, reduced survival at PND 4 and reduced mean pup weight at PND 1. The MON 0818 reproductive/developmental NOAEL was also 300 ppm (approximately 23 mg/kg/day).

Epidemiology Glyphosate DART/ED Publications

Several epidemiology studies in which glyphosate exposure was considered have evaluated the following range of reproductive outcomes; miscarriage, fecundity, pre-term delivery, gestational diabetes mellitus, birth weights, congenital malformations, neural tube defects, attention-deficit disorder / attention-deficit hyperactive disorder (ADD/ADHD). In most instances, glyphosate and reproductive outcomes lack a statistically significant positive association, as described in a recent review of glyphosate non-cancer endpoint publications by experts in the field of epidemiology, Pam Mink, Jack Mandel, Jessica Lundin and Bonnielin Sceurman (Mink et al., 2011). In evaluating ADD/ADHD a positive association with glyphosate use was reported by Garry et al (2002), but cases were parent reported with no clinical confirmation and the reported incidence rate of approximately 1% for the study population was well below the general population incidence rate of approximately 7%. Regarding *in utero* exposures, McQueen et al. (2012) report very low measured dietary exposures, from 0.005% to 2% of the current glyphosate ADI in Europe. Given the low perfusion rate of glyphosate across the placenta (Mose et al., 2008), human *in utero* exposures would be very limited.

IN VITRO DART/ED PUBLICATIONS

Author(s)	Year	Study title
Walsh, L.P. McCormick, C. Martin, C. Stocco, D.M.	2000	Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environmental Health Perspectives Volume: 108 Number: 8 Pages: 769-776

Abstract*

Recent reports demonstrate that many currently used pesticides have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the mechanisms involved in pesticide-induced infertility remain unclear. Because testicular Leydig cells play a crucial role in male reproductive function by producing testosterone, we used the mouse MA-10 Leydig tumor cell line to study the molecular events involved in pesticide-induced alterations in steroid hormone biosynthesis. We previously showed that the organochlorine insecticide lindane and the organophosphate insecticide Dimethoate directly inhibit steroidogenesis in Leydig cells by disrupting expression of the steroidogenic acute regulatory (StAR) protein. StAR protein mediates the rate-limiting and acutely regulated step in steroidogenesis, the transfer of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450 side chain cleavage (P450scc) enzyme initiates the synthesis of all steroid hormones. In the present study, we screened eight currently used pesticide formulations for their ability to inhibit steroidogenesis, concentrating on their effects on StAR expression in MA-10 cells. In addition, we determined the effects of these compounds on the levels and activities of the P450scc enzyme (which converts cholesterol to pregnenolone) and the 3 β-hydroxysteroid dehydrogenase (3 β-HSD) enzyme (which converts pregnenolone to progesterone). Of the pesticides screened, only the pesticide Roundup inhibited dibutyryl [$(Bu)_2$]cAMP-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Roundup inhibited steroidogenesis by disrupting StAR protein expression, further demonstrating the susceptibility of StAR to environmental pollutants.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Ammo, Ambush, Fusilade, Cyclone, Roundup, Banvel, Cotoran, Dual, glyphosate. Surfactants not identified or quantified in formulations.

Active substance(s):
- Ammo: **cypermethrin**: (*R,S*)-α-cyano-3-phenoxybenzyl(1*R,S*)-*cis*,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Ambush: **permethrin**: 3-phenoxybenzyl(1*R,S*)-*c i s* ,*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate
- Fusilade: **fluazifop-*p*-butyl**: (*R*)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionic acid
- Cyclone: **paraquat**: 1,1´-dimethyl-4,4´-bipyridinium
- Roundup: **glyphosate**: *N*-(phosphonomethyl) glycine
- Banvel: **dicamba**: 3,6-dichloro-*o*anisic acid
- Cotoran: **fluometuron**: 1,1-dimethyl-3-(α,α,α-trifluoro-*m*-tolyl) urea

Purity:	• Dual: **metolachlor**: 2-chloro-6´ethyl-*N*-(2-methoxy-1-methylethyl)aceto-toluidine. • Ammo (300 g/L cypermethrin) • Ambush (240 g/L permethrin) • Fusilade (120 g/L fluazifop-*p*-butyl) • Cyclone (240 g/L paraquat) • Roundup (180 g/L glyphosate) • Banvel (480 g/L dicamba) • Cotoran (480 g/L fluometuron) • Dual (958 g/L metolachlor)
Source:	Glyphosate – Sigma Other pesticides – unknown source

2. Vehicle and/or positive control:

Vehicle control:	Yes (DMSO, ethanol < 0.4 %)
Positive control:	No data

3. Test system / cells / animals:

Cell culture:	Mouse MA-10 Leydig tumor cell line
Species:	Mouse
Source:	M. Ascoli, University of Iowa College of Medicine (Iowa City, IA)
Maintenance conditions:	Waymouth's MB 752/1 medium + 15% horse serum Temperature: 37° C, Atmosphere: 5% CO_2
Plate cultures #1:	75,000 cells/well in a 96-well plate. For dose–response, time–course, steroidogenic enzyme activity, reversibility, and mixture studies.
Plate cultures #2:	50 x 10^6 cells onto 25 x 25 cm tissue culture dishes. For nuclear run-on analysis.
Plate cultures #3:	1.5 x 10^6 cells into 100-mm culture dishes, Grown until 80% confluence. For the remaining studies.

4. Test methods:

Study type:	Inhibition of steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	2 or 4 h
Dose/concentration levels:	Ambush, Ammo: 5, 10, 50 µg/mL Banvel, Cotoran, Dual, Fusilade: 1, 5, 10 µg/mL Cyclone: 0.5, 1, 5 µg/mL Roundup: 12.5, 25, 50, 100 µg/mL
Treatment:	MA-10 cells were stimulated using a maximal stimulatory dose of $(Bu)_2$cAMP (1 mM). In some tests (P450scc and 3β-HSD

enzyme activity), steroidogenic substrates (22R-HC, 25 µM or pregnenolone, 10 µM) were provided.

All treatments were performed in serum-free media.

Final concentrations of the solvents DMSO and ethanol were < 0.4 %.

5. Observations/analyses:

Dose–response and time-course studies:

Measurement: Steroid levels and total protein synthesis.

Calculation: IC_{50} values (concentration that leads to an inhibition of 50%) were calculated as the slope of the linear regression line obtained from Eadie/Hofstee plots of steroidogenesis dose–response data.

Analysis: For steroid determination in Roundup-treated cells, each data point was the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate.

For progesterone production in cells treated with other pesticides, each data point is the mean ± SE of four replicates in a single experiment that was repeated once.

Progesterone production and total cellular protein synthesis

Radioimmunoassay (RIA).

Measurement: Quantification of progesterone

Preparation of samples: Standard curves were prepared in serum-free Waymouth's medium.

Analysis: Analysis of RIA data was performed using a computer program specifically designed for this purpose (not further specified).

Data are expressed as ng/mL media.

Determination of total cellular protein synthesis:

Measurement: Total protein content was determined using a modification of the Bradford method (no treatment with Expre^{35}S^{35}S).

Preparation of samples: After treatment, cells were solubilized in 0.25 M NaOH at 37°C. Protein was precipitated overnight at 4°C using cold 20% trichloroacetic acid (TCA). TCA-precipitable material was transferred onto glass fiber filters, rinsed with 5% TCA, dried, and counted in a liquid scintillation counter.

Analysis: Results were reported as counts per minute per mg protein (2 or 4 h).

Each data point is the mean ± SE of four replicates in a single experiment, which was performed three times.

Determination of P450scc and 3β-HSD activity and reversibility:

Measurement: <u>P450scc enzyme activity:</u> Pregnenolone in medium
<u>3β-HSD enzyme activity:</u> Progesterone in medium

Preparation: <u>Evaluation of P450scc enzyme activity</u>:
22*R*-HC was provided as substrate to MA-10 cells in the presence and absence of the xenobiotic as well as cyanoketone

	and SU 10603 (inhibitors of 3β-HSD and P450c17, respectively). Evaluation of 3β-HSD enzyme activity: pregnenolone was provided as substrate, and MA-10 cells were treated in the presence and absence of the xenobiotic
Analysis:	Each data point represents the average ± SE of the means from at least three separate experiments in which treatments were performed in quadruplicate.
Effects on enzyme and StAR expression:	Protein levels, mRNA levels, gene transcription
Isolation of mitochondria and Western blot analysis:	
Measurement:	Protein levels of P450scc, β-HSD, StAR
Preparation:	Western blot analysis of mitochondrial protein was performed. Mitochondria were isolated by homogenization of the cells followed by differential centrifugation. After detection of StAR, membranes were stripped and then successively probed with P450scc or 3β-HSD antisera.
Analysis:	The bands of interest were quantitated using a BioImage Visage 2000 imaging system. Values obtained were expressed as integrated optical density units. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.
Isolation of RNA and Northern blot analysis:	
Measurement:	mRNA levels of of P450scc, β-HSD, StAR
Preparation:	Total RNA was isolated using Trizol Reagent and quantitated. For Northern blot analysis 20 µg total RNA was loaded into each well. Labeling of cDNA probes for mouse StAR, P450scc, 3β-HSD, and 18S rRNA was achieved by random priming (Prime-It II; Stratagene, La Jolla, CA) using [α-32 P] dCTP (SA 3,000 Ci/mmol; New England Nuclear) according to the manufacturer's protocol. After Northern blot analysis with StAR cDNA, blots were stripped and then successively probed with P450scc, 3β-HSD, and 18S rRNA cDNA.
Analysis:	The bands of interest (RNA) were quantified. Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.
Gene expression:	
Measurement:	StAR, P450scc
Isolation of nuclei:	
Preparation:	After treatment, cells were harvested with a rubber policeman and centrifuged. The cell pellet was resuspended and homogenized. The homogenate was layered and centrifuged. The supernatant was discarded and the pellet containing nuclei

was resuspended, frozen on dry ice, and stored in liquid nitrogen.

Nuclear run-on analysis:

Measurement: Radioactivity was detected using a Phosphorimager 445 SI.

Analysis: Signals were quantitated using ImageQuant version 4.1 software in volume mode, which integrates the intensity of each pixel within the defined area.

Values were obtained as arbitrary units. Each data point represents the average ± SE of five separate experiments.

Protein kinase A (PKA) activity determination:

Measurement: PKA activity was measured with the SignaTECT cAMP-dependent protein kinase assay system.

Analysis: Three separate experiments were performed in which treatments were performed in triplicate.

Mixture studies:

Measurement: Progesterone was measured.

Analysis: Each data point represents the average ± SE of the means from three separate experiments in which treatments were performed in triplicate.

Statistics: Statistically significant differences were determined by one-way analysis of variance and Fisher–protected least-square difference multiple comparison using the software program Statview SE + Graphics.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions – Not reliable for Roundup**

Comment: Non-standard test systems, but publication meets basic scientific principles. However, surfactant blend in Roundup confounds results.

2. Relevance of study: **Relevant with restrictions:** Different effects of glyphosate alone and glyphosate formulations were observed. No conclusion can be drawn that the observed effects are result of glyphosate exposure. Roundup data unreliable for endpoints measured, due to mitochondrial membrane damage.

3. Klimisch code: **2 for glyphosate data, 3 for Roundup data**

Response - GTF

- Glyphosate did not affect steroidogenesis in the test system.
- Roundup formulation data was confounded by mitochondrial membrane damage, attributable to the surfactant in the tested formulation.
- Roundup results were comprehensively addressed in Levine et al. (2007).
    - Roundup formulation containing glyphosate and Roundup formulation blank without the active ingredient was shown to have “indistinguishable” dose response curves for reductions in progesterone production in hCG stimulated MA-10 Leydig cells. Therefore

the effect on progesterone levels shown by Walsh (2000) were independent of glyphosate and attributable to the surfactant component of the formulation.
  - Comparable rates of progesterone inhibition for several different surfactants suggest a common mode of action for surfactants.
  - Roundup formulation containing glyphosate and Roundup formulation blank without the active ingredient was shown to have almost identical concentration-dependent decreases in MTT activity in MA-10 cells, suggesting the surfactant alone was responsible for the observed cytotoxicity and effect on mitochondrial function.
  - The JC-1 assay demonstrated the decreased progesterone production in MA-10 Leydig cells was accompanied by loss of mitochondrial membrane potential. These results confirm StAR protein function and steroidogenesis require intact mitrchondrial membrane potential.
  - StAR protein expression were not affected by treatments, indicating that perturbed mitochondrial membrane, not StAR protein inhibition, was responsible for the effects noted by Walsh et al. (2000).

- Given the significant differences in physico-chemical properties between glyphosate and formulation surfactants, environmental fate and transport of these compounds are likely to be different. Likewise, absorption, distribution, metabolism and excretion (ADME) differences between glyphosate and formulation surfactants at low concentration exposures in the field, environment or food residues will very likely result in insignificant concomitant physiological exposures.

Author(s)	Year	Study title
Paganelli, A. Gnazzo, V. Acosta H. Lopez, S.L. Carrasco, A.E.	2010	Glyphosate-Based Herbicides Produce Teratogenic Effects on Vertebrates by Impairing Retinoic Acid Signalling Chemical Research in Toxicology Volume: 23 Pages: 1586-1595

Abstract*

The broad spectrum herbicide glyphosate is widely used in agriculture worldwide. There has been ongoing controversy regarding the possible adverse effects of glyphosate on the environment and on human health. Reports of neural defects and craniofacial malformations from regions where glyphosatebased herbicides (GBH) are used led us to undertake an embryological approach to explore the effects of low doses of glyphosate in development. *Xenopus lae*V*is* embryos were incubated with 1/5000 dilutions of a commercial GBH. The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the anterior-posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Moreover, GBH produced similar effects in chicken embryos, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly. This suggests that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in *Xenopus* embryos and cotreatment with a RA antagonist rescued the teratogenic effects of the GBH. Therefore, we conclude that the phenotypes produced by GBH are mainly a consequence of the increase of endogenous retinoid activity. This is consistent with the decrease of Sonic hedgehog (Shh) signaling from the embryonic dorsal midline, with the inhibition of *otx2* expression and with the disruption of cephalic neural crest development. The direct effect of glyphosate on early mechanisms of morphogenesis in vertebrate embryos opens concerns about the clinical findings from human offspring in populations exposed to GBH in agricultural fields.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Classic ®; Glyphosate
Active substance(s):	Glyphosate
Source:	Roundup Classic ®: Monsanto Glyphosate: Sigma Aldrich
Purity:	Roundup Classic ®: 48% (w/v) glyphosate salt Glyphosate: not reported

2. Positive control: Specified under the respective test

3. Test organisms and systems:

Species:	*Xenopus laevis*
Embryo culture:	*Xenopus laevis* embryos obtained by in vitro fertilisation
Source:	Not specified
Culture conditions:	Embyos were incubated in 0.1 x modified Barth´s saline (MBS)
Species:	Chicken

Strain:	White Leghorn
Source:	Not specified
Stage:	Egg (fertilized)
Guideline:	Non-guideline tests
GLP:	No
Guideline deviations:	Not applicable
***Xenopus* embryo Culture and Treatments:**	
Stage of embryos:	2 cell
Dose levels:	1/3000, 1/4000, and 1/5000-dilutions of Roundup Classic® prepared in 0.1× MBS (modified Barth's saline)
Treatment:	Treatments were performed from the 2-cell stage.
Rescue experiments:	0.5 or 1 µM Ro-415253 was added at the 9-cell stage
Culture conditions:	Embryos were incubated in 0.1 x MBS. Cyclopamine was used at 100 µM concentration in 0.1 x MBS and was applied from the 2-cell stage until fixation. Embryos were fixed in MEMFA when sibling controls reached the desired stage.
Negative control:	Not adequately described
Positive control:	None
Xenopus Embryo Injections, Whole Mount in Situ Hybridization and Cartilage Staining:	
Dose levels:	360 or 500 pg of glyphosate (N-(phosphonomethyl) glycine (Sigma 337757).
Exposure route:	injection
Stage of embryos:	2 cell
Treatment:	Embryos were injected with 360 or 500 pg of glyphosate (N-(phosphonomethyl) glycine (Sigma 337757) per cell into one or both cells at the 2-cell stage. Glyphosate was coinjected with 10 ng of Dextran Oregon Green (DOG, Molecular Probes) to identify the injected side.
Culture condition:	Embyos were incubated in 0.1 x MBS. And fixed in MEMFA when sibling controls reached the desired stage.
In situ hybridisation:	Wholemount in situ hybridisation (WMISH) was performed with digoxigenin-labeled antisense RNA probes, but without the proteinase K step. Embryos were fixed in MEMFA at stages 45-47, washed with PBS, stained overnight in 0.04 %Alcian Blue, 20% acetic acid, and 80 % ethanol. Afterwards embryos were washed.
Detection of RA Activity:	
Dose levels:	1/3000, 1/4000, and 1/5000 Roundup Classic® dilutions
Exposure route:	injection
Stage of embryos:	1-2 cell
Treatment:	Embryos were injected with 320 pg of the plasmid RAREhplacZ (RAREZ) per cell into one cell at the 2-cell stage and placed immediately in the test substance dilutions
Negative control:	Negative control was not evaluated with vehicle injection.

	Therefore effects of decreased pH or vehicle coformulant (Dextran Orange Green) were not assessed.
Positive control:	Xenopus embryos were injected with the RAREZ plasmid and incubated at late blastula stage with 0.5 or 5 μM all-transretinoic acid (RA, Sigma R2625).
Rescue experiment:	Embryos injected with the reporter plasmid were incubated in a 1/4000 test substance dilution from the 2-cell stage, and when they reached the blastula stage, 1 μM of Ro 41-5253 was added.

Treatments of Chicken Embryos:

Stage:	Egg
Dose levels:	20 μL of 1/3500 or 1/4500 dilutions of Roundup Classic®.
Treatment:	Injection after opening a small window in the shell of fertilized chicken eggs, above the air chamber in the inner membrane. After injection the window was sealed with transparent adhesive tape
Negative control:	Injected with 20 μL of H2O without pH or osmolality adjustment
Positive Control	None
Pre-incubation conditions:	Placement: eggs were placed with their blunt end up; Temperature: room temperature; Duration: 30 minutes.
Incubation conditions:	Light: Darkness; Temperature: 38 C; Humidity: 56-58% Rotation: regular

Whole-Mount Inmunofluorescence and WMISH of Chicken Embryos:

Treatment:	Embryos were fixed 2-4 hin freshly prepared 4% paraformaldehyde, rinsed and processed for analysis.
	Wholemount in situ hybridization (WMISH) was performed as described for Xenopus embryos, using a c-shh probe.

4. Measurements/analyses:

Measurements:	Basal luminiscence was detected in uninjected and untreated embryos.
	The endogenous RA activity was measured in embryos injected with RAREZ (plasmid RAREhplacZ).
	When sibling controls reached the neurula stages, all embryos were processed for chemiluminiscent quantitation of the reporter activity by using the β-gal reporter gene assay (Roche).
	Luminiscence was measured on duplicate samples in FlexStation 3 equipment (Molecular Devices), and values were normalized by protein content.
Statistics:	A two-tailed t-test was employed to analyze the significance in the difference of the means. The experiment was repeated three times.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline study that is not sufficiently described for assessment. Inadequate positive and negative control experiments.

2. Relevance of study: **Not relevant:** Irrelevant routes of exposure and inappropriately high doses. Test system not adequate for human risk assessment.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- No pH adjustment for doses and thus effects may be in response to the acidic nature of glyphosate technical acid.
- Inappropriate and irrelevant routes of exposure.
- Data requires further substantiation before consideration in risk assessment.

Response 2 – Saltmiras et al. (2012) letter to the Editor

- Multiple high quality toxicological studies and expert review panels consistently agree glyphosate is not a teratogen or reproductive toxicant.
- The authors' justification for this research is flawed, providing no valid basis, other than an opinion, of an increase in the rate of birth defects in Argentina.
- Direct injection of frog embryos and through chicken shells do not reflect real world exposure scenarios to either environmental species or humans.
- Doses were excessively high and irrelevant for risk assessment purposes. Frog embryos were also bathed in glyphosate formulation at doses 9-15 times greater than the acute LC50 same species of frog. Calculating equivalent oral doses based on pharmacokinetics studies, such doses are 150000000 times greater than worst case human exposure monitoring data.
- "…. the results from this research cannot be used in isolation to reach the conclusions expressed in the publication. Instead, the type of data in this research paper must be interpreted relative to all other available data on the specific materials under study and with balanced consideration for higher tier apical studies."

Response 3 – Mulet (2012) letter to the Editor

- Notes the premise for this research is falsely based on an incorrectly cited local pediatric bulletin from Paraguay.
- "…. this article refers to a study in a single hospital in Paraguay showing a correlation between pesticide use (not herbicides as mentioned by Paganelli et al.) and birth malformations. In the cited study (Benitez et al.), the authors state that the results are preliminary and must be confirmed. Is important to remark that the Benitez et al. study does not include any mention to glyphosate, so does not account for what the authors are stating in the Introduction…..This journal is also wrongly cited in the Discussion referring to increased malformations due to herbicides, which is not the result of the study."

Response 4 – comments from BVL (2010)

- Highly artificial experimental conditions.
- Inappropriate models to replace validated mammalian reproductive and developmental toxicity testing methods for use in human health risk assessment.
- Inappropriate routes of exposure.
- Lack of corroborative evidence in humans.
- "In spite of long-lasting use of glyphosate-based herbicides worldwide, no evidence of teratogenicity in humans has been obtained so far."

Response 5– comments from European Commission Standing Committee on the Food Chain and Animal Health (2011)

- The EU commission supports the German Authorities position, "that that there is a comprehensive and reliable toxicological database for glyphosate and the effects observed have not been revealed in mammalian studies, nor evidenced epidemiologically in humans."
- "…. the Commission does not consider there is currently a solid basis to ban or impose specific restrictions on the use of glyphosate in the EU."

Summaries of the follow up published letters to the Editor by Mulet, Palmer follow

Author(s)	Year	Study title
Mulet, J.M.	2011	Letter to the Editor Regarding the Article by Paganelli et al. (2010) Chemical Research in Toxicology Volume: 24 Number: 5 Pages: 609

Abstract

No abstract.

[The author of the letter states that the study of Paganelli et al., 2010, about teratogenic effect of glyphosate when injected invertebrate embryos, is based on misused citations or non-peer reviewed data]

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Classic
Active substance(s):	Glyphosate
Description:	Not reported
Source of test medium:	Not reported
Lot/Batch #:	Not reported
Concentration:	480 g/glyphosate IPA salt/L

2. Studies addressed:

Paganelli et al. (Chem. Res. Toxicol. (2010), 23, 1586-1595)

In vitro teratology studies:

Xenopus embryo culture and treatments with glyphosate

Xenopus embryo treatment with glyphosate and whole-mount in *situ* hybridization and cartilage staining

Detection of RA (retinoic acid) activity

Treatment of chicken embryos with glyphosate and whole-mount immunofluorescence

KLIMISCH EVALUATION

1. Reliability of study: **Not applicable**

Comment: In this publication the author expresses some major concern about the article by Paganelli et al. (Chem. Res. Toxicol. (2010), 23, 1586-1595) in terms of over interpretation of results

2. Relevance of study: **Relevant** (no original publication but letter to the editor regarding the article by Paganelli et al., 2010)

3. Klimisch code: **Not applicable**

Author(s)	Year	Study title
Palma, G.	2011	Letter to the Editor Regarding the Article by Paganelli et al. (2010) Chemical Research in Toxicology Volume: 24 Number: 6 Pages: 775-776

Abstract

No abstract.

[The author of the letter claims that the study by Paganelli et al., 2010, described effects of glyphosate only at unrealistic high concentrations or via unrealistic routes of exposure. The data are thought to be inconsistent with the literature, and therefore not suitable or relevant for the risk assessment for humans and wildlife. Furthermore the author asserts that findings do not support the extrapolation to human health as stated in the publication]

MATERIALS AND METHODS

1. Test material:

Test item: Roundup Classic

Active substance(s): Glyphosate (isopropylamine salt)

Description: Not reported

Source of test medium: Not reported

Lot/Batch #: Not reported

Concentration: 480 g/glyphosate IPA salt/L

2. Studies addressed: Paganelli et al.(Chem. Res. Toxicol. (2010), 23, 1586-1595)

In vitro teratology studies: *Xenopus* embryo culture and treatments with glyphosate

Xenopus embryo treatment with glyphosate and whole-mount in *situ* hybridization and cartilage staining

Detection of RA (retinoic acid) Activity

Treatment of chicken embryos with glyphosate and whole-

mount immunofluorescence

KLIMISCH EVALUATION

1. Reliability of study: **Not applicable**

Comment: In this publication the article by Paganelli et al. (Chem. Res. Toxicol. (2010), 23, 1586-1595) is discussed in detail. The author of the letter claims that the study by Paganelli et al. contains major deficiencies and errors in terms of experimental design, descriptions of the methods used, and the interpretation of results

2. Relevance of study: **Relevant** (No original publication but letter to the editor regarding the article by Paganelli et al., 2010)

3. Klimisch code: **Not applicable**

Author(s)	Year	Study title
Richard, S. Moslemi, S. Sipahutar, H. Benachour, N. Seralini, G.E.	2005	Differential effects of glyphosate and roundup on human placental cells and aromatase. Environmental Health Perspectives Volume: 113 Pages: 716-720

Abstract*

Roundup is a glyphosate-based herbicide used worldwide, including on most genetically modified plants that have been designed to tolerate it. Its residues may thus enter the food chain, and glyphosate is found as a contaminant in rivers. Some agricultural workers using glyphosate have pregnancy problems, but its mechanism of action in mammals is questioned. Here we show that glyphosate is toxic to human placental JEG3 cells within 18 hr with concentrations lower than those found with agricultural use, and this effect increases with concentration and time or in the presence of Roundup adjuvants. Surprisingly, Roundup is always more toxic than its active ingredient. We tested the effects of glyphosate and Roundup at lower nontoxic concentrations on aromatase, the enzyme responsible for estrogen synthesis. The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation in microsomes or in cell culture. We conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. We suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate
Active substance(s):	Glyphosate
Source of test item:	Glyphosate: Sigma-Aldrich, Saint Quentin Fallavier, France
Lot / Batch #:	Not specified
Purity:	not reported
Test item:	Roundup ®
Active substance(s):	Glyphosate
Source of test item:	Roundup®, (produced by Monsanto, obtained from a commercial source)
Lot / Batch #:	Not specified
Purity:	Roundup ®: 360 g/L acid

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells / animals:

Cell line:	Human choriocarcinoma derived placental cell line (ref JEG3, ECACC 92120308)
Species:	Human
Source:	CERDIC (Sophia-Antipolis, France)

Maintenance medium:	Phenol red–free EMEM containing 2 mM glutamine, 1% nonessential amino acids, 100 U/mL antibiotics (mix of penicillin, streptomycin, and fungizone), 1 mM sodium pyruvate, and 10% fetal calf serum
Cells:	Human placental microsomes
	Equine testicular microsomes
Source:	Human:
	Full-term placentas of young healthy and non-smoking women (Centre Hospitalier Régional de Caen, France) and equine testis by differential centrifugations.
	Equus:
	Equine testis
Microsome preparation:	Microsomal fractions (endoplasmatic reticulum) were obtained using differential centrifugations.
	Tissues were washed with 0.5 M KCl, homogenised in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM DTT, and centrifuged at 20,000 g. The supernant was ultracentrifuged at 100,000 g, and the pellet was washed twice, disoolved in the same buffer containing 20% glyceol and stored at -70°C until use. All preparations steps were carried out at 4°C.

4. Test methods:

GLP:	No (for all tests)
MTT assay:	Assessment of cell viability
	Cleavage of MTT into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase, to evaluate JEG3 cell viability exposed to Roundup or glyphosate during various times.
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Test substance preparations:	2% solution of Roundup and an equivalent solution of glyphosate were prepared in Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France), and the pH of glyphosate solution was adjusted to the pH of the 2% Roundup solution (~ pH 5.8). Successive dilutions were then obtained with serum-free EMEM.
Dose concentrations:	In serum-containing medium (18, 24, 48 h): Roundup: 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 % Glyphosate: 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0 % In serum-free medium: Roundup (1 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 % Glyphosate (1 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 % Glyphosate + Roundup 0.02% (18 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 % Glyphosate + Roundup 0.1% (18 h): 0.02, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 %
Treatment:	Fifty thousand cells per well in 24-well plates were grown to 80% confluence, washed with serum-free EMEM and exposed to various concentrations of Roundup or equivalent glyphosate concentrations

Incubation conditions:	Cells were washed with serum-free EMEM and incubated with 250 µL MTT per well for 3 h at 37°C. 250 µL of 0.04 N-hydrochloric acid–containing isopropanol solution was added to each well.
Positive control:	None
Negative control:	None
Replicates per dose level:	3 x 3
Radioimmunoassay (RIA):	Measurement of aromatase activity *in vitro*
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	In serum free medium: Roundup (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2 % Glyphosate (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 % Roundup (18 h): 0.01, 0.02, 0.04, 0.08 % Glyphosate (18 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6 %
Positive control:	None
Negative control:	None
Incubation conditions:	Duration: 90 min Temperature: 37 C Atmosphere: 5% CO_2 200 nM androstenedione
Replicates per dose level:	3 x 3
RT-PCR:	Quantification of cytochrom P450 aromatase mRNA levels in JEG3 cells
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	In serum free medium: Roundup (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2 % Glyphosate (1 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 % Roundup (18 h): 0.01, 0.02, 0.04, 0.08 % Glyphosate (18 h): 0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, 0.6 %
Positive control:	None
Negative control:	None
Incubation conditions:	Duration: 90 min Temperature: 37 C Atmosphere: 5% CO_2 200 nM androstenedione
Sample preparation:	Total RNA was isolated from JEG3 cells using the guanidium/phenol/chloroform method. RNA samples were treated with DNase I at 37 C for 30 min to remove genomic DNA. Then DNase I was inactivated at 65°C for 10 min.
Tritiated water release assay:	Assessment of aromatase activity in human placental microsomes *in vitro*
Guideline:	Non-guideline assays

Guideline deviations:	Not applicable
Dose concentrations:	Roundup: 0.01, 0.06, 0.1, 0.5, 0.7, 1.0 , 3.0, 6.0 % Glyphosate: 0.01, 0.06, 0.1, 0.7, 1.0 , 3.0 %
Positive control:	None
Negative control:	None
Treatment of human microsomal fractions:	50 µg of human placental microsomes were incubated with radiolabeled androstenedione (100 pmol/tube) at 37°C for 15 min in the presence or absence of various concentrations of Roundup or glyphosate in 1 mL total volume of 50 mM Tris-maleate buffer (pH 7.4).The reaction was started by adding 100 µL of 0.6 mM H±NADPH and stopped with 1.5 mL chloroform and then centrifuged at 2,700 g at 4°C for 5 min. After adding 0.5 mL 7% charcoal/1.5% dextran T-70 solution into the preparation, the centrifugation was repeated fro 10 min.
Treatment of equine microsomal fractions:	2 µg of equine testicular microsomes were incubated for 3 min at 25°C with various concentrations of radiolabeled androstenedione (in the presence or absence of various concentrations of Roundup in 0.5 mL of H^+-NADPH containing Tris-maleate buffer (pH 7.4).
Spectral studies:	Assessment of reductase and aromatase activities
Guideline:	Non-guideline assays
Guideline deviations:	Not applicable
Dose concentrations:	Roundup: 0.1 % Glyphosate: 0.0046 %
Positive control:	None
Negative control:	None
Purification of reductase / aromatase:	Equine reductase was obtained after chromatographic separation, by ω-aminohexyl-Sepharose 4B and adenosine 2´, 5´-diphosphate agarose, respectively, hydrophobic interaction and affinity columns. Equine cytochrom P450 aromatase was purified from equine microsomes, after its separation from reductase, by successive chromatographic steps.

5. Observations/analyses:

MTT assay	
Measurements:	The optical density was measured using a spectrophotometer at 560 nm for test and 640 nm for reference.
Radioimmuno assay (RIA)	
Measurements:	The conversion of androstenedione to E1 by the aromatase complex was measured in cell supernatants by radioimmunoassay (RIA). The aromatase activity was expressed in relation to the protein concentration that was evaluated in cell extracts using bovine serum albumin as standard
RT-PCR	
Measurements:	Quantitation of mRNA by RT-PCR using M-MLV-RT (Moloney murine leukemia viruse reverse transcriptase). The absence of DNA contamination in RNA samples was

checked in controls without M-MLV-RT.

All PCR reactions were performed using an ABI Prism 7000 Sequence Detection System.

Tritiated water release assay

Measurements: Microsomal aromatase activity was evaluated by tritiated water release from radiolabeled substrate [1β-3H]-androstenedione. This method based on the stereo specific release of 1β-hydrogen from the androstenedione substrate, which froms tritiated water during aromatisation.

Aromatase activity was determined by measuring the radioactivity of the 0.5 mL aqueous phase.

Spectral studies:

Measurements: Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of H+-NADPH at 550 nm for 2 min at 37 C using a Kontron-Uvikon 860 spectrophotometer.
The absorbance of purified equine aromatase in the presence or absence of glyphosate or Roundup was recorded from 375 to 475 nm with a spectrophotometer.

The spectra of aromatase with glyphosate or Roundup alone were subtracted from the incubation spectrum.

Statistics for all tests: All data are presented as the mean ± SE. The experiments were repeated three times in triplicate unless otherwise indicated. Statistically significant differences were determined by a Student *t*-test using significance levels of 0.01 and 0.05.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study design is insufficient for risk assessment of real exposure concentrations. Methodological deficiencies (no controls were included). Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- Glyphosate at non-cytotoxic concentrations in this test system was demonstrated to have no effects on aromatase activity.
- Likewise, did not affect mRNA levels after 18 hours treatment at ≤ 0.1% glyphosate.
- Roundup aromatase activity measurements are confounded by surfactant effects on microsomes.

- The *in vitro* test system is non-validated
- Physiologically irrelevant concentrations tested
- Testing surfactant-like substances in such systems is now recognized to be not valid.

Response 2 – summarized from the French Ministry of Agriculture and Fish, Committee for Study of Toxicity (2005)

- Major methodological gaps.
- JEG3 cells, a choriocarcinoma human cell line (average of 70 chromosomes vs 46 in normal human cells).
- Concentrations of Roundup used in the various experiments considered to be extremely high.
    - In consideration of limiting factors (oral absorption, 30%; skin absorption, 0.3%; rapid elimination kinetics), such levels would involve considerable human exposure, or several dozen liters of Roundup diluted at 2%.
    - concentrations of Roundup that trigger an effect on aromatase (0.5% - 2%) are at least 1000 times more effective than those of known aromatase inhibitors, such as azole derivatives
- Study design does not make it possible to show the influence of the adjuvants, nor synergism of adjuvants and glyphosate.
- Multiple non-specific effects of surfactant agents on a broad range of cellular targets not discussed.
- No comparison with comparable surfactant agents intended for household use.
- multiple instances of bias in its arguments and its interpretation of the data.
- The authors over-interpret their results in the area of potential health consequences for humans (unsuitable references, non-sustained in vitro-in vivo extrapolation, etc.).

Author(s)	Year	Study title
Benachour, N. Sipahutar, H. Moslerni, S. Gasnier, C. Travert, C. Seralini, G. E.	2007	Time- and dose-dependent effects of roundup on human embryonic and placental cells. Archives of Environmental Contamination and Toxicology Volume: 53 Pages: 126-133

Abstract*

Roundup® is the major herbicide used worldwide, in particular on genetically modified plants that have been designed to tolerate it. We have tested the toxicity and endocrine disruption potential of Roundup (Bioforce®) on human embryonic 293 and placental-derived JEG3 cells, but also on normal human placenta and equine testis. The cell lines have proven to be suitable to estimate hormonal activity and toxicity of pollutants. The median lethal dose (LD_{50}) Of Roundup with embryonic cells is 0.3% within 1 h in serum-free medium, and it decreases to reach 0.06% (containing among other compounds 1.27 mM glyphosate) after 72 h in the presence of serum. In these conditions, the embryonic cells appear to be 2-4 times more sensitive than the placental ones. In all instances, Roundup (generally used in agriculture at 1-2%, i.e., with 21-42 mM glyphosate) is more efficient than its active ingredient, glyphosate, suggesting a synergistic effect provoked by the adjuvants present in Roundup. We demonstrated that serum-free cultures, even on a short-term basis (1 h), reveal the xenobiotic impacts that are visible 1-2 days later in serum. We also document at lower non-overtly toxic doses, from 0.01% (with 210 µM glyphosate) in 24 h, that Roundup is an aromatase disruptor. The direct inhibition is temperature-dependent and is confirmed in different tissues and species (cell lines from placenta or embryonic kidney, equine testicular, or human fresh placental extracts). Furthermore, glyphosate acts directly as a partial inactivator on microsomal aromatase, independently of its acidity, and in a dose-dependent manner. The cytotoxic, and potentially endocrine-disrupting effects of Roundup are thus amplified with time. Taken together, these data suggest that Roundup exposure may affect human reproduction and fetal development in case of contamination. Chemical mixtures in formulations appear to be underestimated regarding their toxic or hormonal impact.

* Quoted from article

MATERIALS AND METHODS

Cytotoxicity assay

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: Monsanto,(Antwerp, Belgium)
Purity:	Glyphosate: not reported Roundup Bioforce® : 360 g/L acid glyphosate (equivalent to 480 g/L of isopropylamine salt of glyphosate
Lot/Batch #:	not reported
Homologation:	Roundup Bioforce® 9800036

2. Vehicle: Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France)

3. Test system / cells:

Cell cultures:	Human embryonic kidney (HEK) 293 cell line (ECACC 85120602) choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308)
Species:	Human
Source:	CERDIC (Sophia-Antipolis, France)
Cell line maintenance:	phenol red-free EMEM containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (mix of penicillin, streptomycin, and fungizone), and 10% fetal calf serum (Biowhittaker, Gagny, France). The JEG3 cell line was supplemented with 1 mM sodium pyruvate.
Culture conditions:	Temperature: 37°C Atmosphere: 5% CO_2, 95% air 48 h

4. Test method:

MTT assay	Assessment of cell viability
Guideline:	None guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	24-well plates, washed with serum-free EMEM
Test conditions:	A 2% solution of Roundup and an equivalent solution of glyphosate were prepared in EMEM and the pH was adjusted to about 5.8. From these stock solutions successive solutions were prepared in serum-free EMEM or serum-containing EMEM. The assays were conducted in 24-well plates. HEK 293 cells or JEG3 cells were grown to 80 % confluence, washed with serum-free EMEM and then exposed to various concentrations of Roundup Bioforce ® or the equivalent concentrations of glyphosate, in serum-free or serum-containing EMEM for 1, 24, 48 or 72 h. Afterwards cells were washed with serum-free EMEM and incubated with 250 µL MTT for 3 h at 37°C. per well. Then 250 µL of 0.04 N-hydrochloric acid containing isopropanol were added to each well, the plates were shaken. Measurements were done at 560 nm for test substance wells and at 720 nm for reference wells.
Dose levels:	0.01, 0.05, 0.1, 0.5, 0.8, 1, 2% of Roundup or equivalent concentrations of glyphosate in serum-free EMEM or serum-containing EMEM
Cells per well:	50000
Exposure duration:	1, 24, 48, and 72 h
Replicates per dose level:	9

5. Observations/analyses:

Measurements:	Cell viability
Statistics:	All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of $p < 0.01$ or $p < 0.05$.

Aromatase activity inhibition

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: Monsanto,(Anvers, Belgium)
Purity:	Glyphosate: not reported Roundup Bioforce® : 360 g/L acid glyphosate (equivalent to 480 g/L of isopropylamine salt of glyphosate
Lot/Batch #:	not reported
Homologation:	Roundup Bioforce® 9800036

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell culture:	HEK 293 cell line (ECACC 85120602)
Species:	Human
Source:	CERDIC (Sophia-Antipolis, France)
Tissue for microsome preparation #1:	full-term placentas of young healthy and non-smoking women
Species:	Human
Source:	Centre Hospitalier Régional de Caen (France)
Tissue for microsome preparation #2:	Equine testis
Species:	Horse
Source:	Not reported
Microsome preparation:	Human placental and equine testicular microsomes: Tissue preparation was done by differential centrifugations. All steps were conducted at 4°C. Tissues were washed with 0.5 M KC1, homogenized in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM Dithiothreiol DTT, and centrifuged at 20,000*g*. The supernatant was then ultracentrifuged at 100,000*g*, and the final pellet was washed twice, dissolved in the same buffer containing 20% glycerol, and stored at -70 C.

4. Test methods:

Study type:	Measurement of aromatase activity by tritiated water release assay Measurement of reductase a1ctivity in purified reductasee Moieties from equine testicular microsomes
Guideline:	Non-guideline assays
GLP:	No
Guideline deviations:	Not applicable
Test conditions:	Tritiated water release assay: 293 cells were transfected with human aromatase cDNA and exposed to nontoxic concentrations of glyphosate alone or Roundup. Human placental microsomes were incubated with various concentrations of glyphosate alone or Roundup. Reductase activity: Equine testis microsomes or the purified

reductase moieties were incubated with or without Roundup

Aromatase inhibition:

Equine testicular microsomes were pre-incubated with a saturating concentration (i.e. 11.6%) or without Roundup.

Dose levels: For aromatase activity:

Glyphosate: < 0.2%
Roundup Bioforce®: 1% of product

Test substance solutions were prepared in EMEM (for 293 cells) and in 50 mM Tris-maleate buffer, pH 7.4 or without pH adjustment (microsomes)

In addition for aromatase and reductase activity:

Roundup at IC_{50} (=)

Exposure duration: Tritiated water release assay:

293 cells: 24 h
human placental microsomes: 15 min

Reductase activity:

Equine testicular microsomes: 15 min

Aromatase inhibition (pre-incubation):

Equine testicular microsomes: 30 min

Replicates per dose level: 9

5. Observations/analyses:

Measurements: Aromatase and residual aromatase activity was determined with the tritiated water release assay. Radioactivity of released tritiated water was assessed by liquid scintillation counting.

Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of H^+-NADPH at 550 nm for 2 min at 20 C using a Kontron-Uvikon 860 spectrophotometer.

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study report has several reporting deficiencies in the methods section (e.g. test conditions for the pH- and temperature dependent assay not reported). There is no information on the suitability of the used HEK 293 cell line for assessment of hormonal activity. Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – GTF

- Glyphosate at and above relevant concentrations for this test system was demonstrated to have no effects on aromatase activity.
- Roundup aromatase activity measurements are confounded by surfactant effects on microsomes.
- Comparable research to Richard et al (2005), but with an additional cell line, HEK 293, derived from aborted human embryo kidneys, transformed by inserting adenovirus DNA.
- Excessively high doses tested, not environmentally relevant for human health or environmental risk assessment.
- Aromatase production within the steroidogenesis pathway. Therfore, aromatase inhibition would be detected in the steroidogenesis assay. The OECD multi-laboratory validation of the steroidogenesis assay evaluated glyphosate, demonstrating no impact on the steroidogenesis pathway (Hecker et al., 2010).

Response 2 – summarized from Williams et al. (2012)

- pH of test system not adjusted to physiologically appropriate levels
- Negative controls were not pH adjusted to appropriate levels
- Confounding surfactant effects due to cell membrane damage render data generated with formulated products in this test system null.

Author(s)	Year	Study title
Benachour, N. Seralini, G. E.	2009	Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chemical Research in toxicology Volume: 22 Pages: 97-105

Abstract*

We have evaluated the toxicity of four glyphosate (G)-based herbicides in Roundup formulations, from 10(5) times dilutions, on three different human cell types. This dilution level is far below agricultural recommendations and corresponds to low levels of residues in food or feed. The formulations have been compared to G alone and with its main metabolite AMPA or with one known adjuvant of R formulations, POEA. HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. All R formulations cause total cell death within 24 h, through an inhibition of the mitochondrial succinate dehydrogenase activity, and necrosis, by release of cytosolic adenylate kinase measuring membrane damage. They also induce apoptosis via activation of enzymatic caspases 3/7 activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis), and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. G provokes only apoptosis, and HUVEC are 100 times more sensitive overall at this level. The deleterious effects are not proportional to G concentrations but rather depend on the nature of the adjuvants. AMPA and POEA separately and synergistically damage cell membranes like R but at different concentrations. Their mixtures are generally even more harmful with G. In conclusion, the R adjuvants like POEA change human cell permeability and amplify toxicity induced already by G, through apoptosis and necrosis. The real threshold of G toxicity must take into account the presence of adjuvants but also G metabolism and time-amplified effects or bioaccumulation. This should be discussed when analyzing the in vivo toxic actions of R. This work clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®; AMPA
Active substance(s):	Glyphosate
Source of test items:	Glyphosate: Sigma-Aldrich, France Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (produced by Monsanto, all available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: not reported Roundup Express®: 7.2 g/L (R7.2) Bioforce® or Extra 360: 360 g/L (R360) Grands Travaux®: 400 g/L (R400) Grands Travaux plus®: 450 g/L (R450)

Homologation:	Roundup Express®: 2010321 Bioforce® or Extra 360: 9800036 Grands Travaux®: 8800425 Grands Travaux plus®: 2020448
Test item:	AMPA (aminomethylphosphonic acid)
Source:	Sigma-Aldrich (Saint Quentin Fallavier, France)
Lot / Batch #:	Not reported
Purity:	Not reported
Test item:	Polyethoxylated tallowamine (POEA)
Source:	Pr. R. Bellé (UMR 7150 CNRS/UPMC, Station Biologique de Roscoff, France)
Lot / Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Primary cell culture:	HUVEC (human primary cells of the umbilical vein cord endothelial cells)
Source:	Lonza
Culture conditions:	Specific endothelial growth medium EGM-2 SingleQuots (CC-4176) containing hEGF, hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), FBS (fetal bovine serum), VEGF, hFGF-B, R3-IGF-1, ascorbic acid, and heparin. Cells were grown in 48-well plates over a period of 24 h at 37 °C (5% CO_2, 95% air) to a confluence of 80%. Afterwards they were washed with serum-free EGM-2.
Cell lines:	Human embryonic kidney 293 cell line (ECACC 85120602) Human choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308)
Source:	CERDIC (Sophia-Antipolis, France)
Culture conditions:	Phenol red-free Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France) containing 2 mM glutamine, 1% nonessential amino acid, 100 U/mL antibiotics (a mix of penicillin, streptomycin, and fungizone; Lonza), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France), and 10% FBS (PAA, les Mureaux, France). The JEG3 cell line was supplemented with 1 mM sodium pyruvate. 50000 cells were grown at 37°C (5% CO_2, 95% air) over a 48 h period to 80% confluence and were washed with serum-free EMEM.

4. Test methods:

MTT assay:	Assessment of cell viability
ToxiLight® assay:	Bioluminescent assay for quantitative measurement of cell membrane damage
Caspase-Glo® 3/7 assay	Assessment of caspase activity or apoptosis induction
Microscopy:	Assessment of cell viability due to cell morphology
Guideline:	Non-guideline assays
GLP:	No

Guideline deviations:	Not applicable
Cell treatments for all tests:	Cells were exposed to various dilutions of the four Roundup formulations, glyphosate, AMPA and POEA in serum-free medium for 24 hours. In another case, cells were incubated with glyphosate, AMPA, and POEA mixtures by pairs at the final nontoxic dilution on SD (succinate dehydrogenase) of 0.5% on the human cell lines (293 or JEG3) and 0.05% on the human primary cells (HUVEC) in comparison to Roundup Bioforce or Extra 360.
Dose levels:	Roundup formulations, glyphosate, AMPA and POEA: 14 concentrations ranging from 10 ppm to 2 % Additional AMPA concentrations: 4, 6, 8 and 10% POEA concentrations. 1 and 5 ppm Combined exposures of G, AMPA and POEA mixtures: For the two cell lines, the first mixture was the combination of glyphosate (0.4999%) with POEA (0.0001%); the second was the combination of glyphosate (0.4%) with AMPA (0.1%), and the third was AMPA (0.4999%) plus POEA (0.0001%). Combined exposures of G, AMPA and POEA mixtures: For the primary HUVEC cells, the first mixture was glyphosate (0.04999%) with POEA (0.0001%); the second was glyphosate (0.04%) with AMPA (0.01%), and the third was AMPA (0.04999%) plus POEA (0.0001%).
Test conditions:	MTT assay: After treatment for 24 h the supernants were recovered for the ToxiLight bioassay, and adherent cells were washed with serum-free medium and incubated with 200 µL MTT per well. The plates were incubated for 3 h at 37°C. Afterwards 200 µL of 0.04 N-hydrochloric acid containing isopropanol were added, the plates were shaked. Optical density was measured at 570 nm. ToxiLight assay: After 24 h exposure the 50 µL of the above mentioned supernants were added to a 96-well plate and incubated under agitation with 50 µL AK detection reagent (AKDR) for 15 minutes protected from light. The luminescence was measured using a luminometer at 565 nm. Serum-free medium served as negative control. Serum-free medium served as negative control. The positive control was the active reagent AKDR mixed with cells treated in serum-free medium. Caspase-Glo® 3/7 assay: This assay was used for caspase activity or measurement of apoptosis induction. After treatment of 50 µL cell cultures to various dilutions of test items as described above, 50 µL/well of Caspase-Glo® 3/7 reagent was added and plates were incubated for 15 minutes at room temperature protected from light before luminescence was measured. Serum-free medium served as negative control. The positive control consisted of the active reagent mixed with cells treated in serum-free medium. The luminescence was measured using a luminometer at 565 nm. Cell Microscopy: At the end of the 24 h treatments, the serum-free medium was removed, and cells were fixed in absolute ethanol –chloroform – acetic acid (6:3:1, v/v/v) for 1 day at -

20°C. Each well was washed with PBS (pH 7.4) and incubated with 1 µg/mL DAPI solution. Staining of DNA with DAPI was examined using a fluorescence microscope.

Replicates per dose level: 3

5. Observations/analyses:

Measurements: Cell viability, membrane damage, apoptosis induction, cell morphology

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems. EPA Test Guideline OCSPP 890.1200 specifically notes that microsomes are denatured by detergents (i.e. surfactants) and that all glassware should be thoroughly rinsed. No positive controls were included.

2. Relevance of study: **Not relevant** (Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants)

3. Klimisch code: **3**

Response – summarized from the French Agency for Food Safety (AFSSA, 2009)

- Cell lines used present characteristics which may be at the source of a significant bias in the interpretation of the results.
- Experiments were conducted with 24 hours exposure in a medium without serum, which could lead to disturbance of the physiological state of the cells.
- The glyphosate used in the study is glyphosate acid, whereas in the preparations tested it is in the form of an isopropylamine salt. No precise information is given about the pH of test concentrations except the highest dose.
- No mention of any positive evidence for the apoptosis test.
- Cytoxicity and induction of apoptosis may due to pH and/or variations in osmotic pressure on cell survival at the high doses tested.
- Surfactant (tensoactive) effects and increased osmolality are known to increase membrane permeability, causing cytotoxicity and induction of apoptosis.
- Conclusions are based on unvalidated, non-representative cell models (in particular tumour or transformed cell lines) directly exposed to extremely high product concentrations in culture conditions which do not observe normal cell physiological conditions.
- No new information is presented on mechanism of action of glyphosate and preparations containing glyphosate.
- The authors over-interpret their results with regard to potential health consequences for humans, based in particular on an unsupported *in vitro–in vivo* extrapolation
- The cytotoxic effects of glyphosate, its metabolite AMPA, the tensioactive POAE and other glyphosate-based preparations proposed by Benachour and Seralini do not add any pertinent

new facts which call into question the conclusions of the European assessment of glyphosate or those of the national assessment of the preparations.

Author(s)	Year	Study title
Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M. C., Seralini, G. E	2009	Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology Volume: 262 Number: 3 Pages: 184-191

Abstract*

Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic in vivo regulatory studies. We measured cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), plus genotoxicity (comet assay), anti-estrogenic (on ERα, ERβ) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in food, feed or in the environment has thus to be considered, and their classifications as carcinogens/mutagens/reprotoxics is discussed.

* Quoted from article

MATERIALS AND METHODS

Cytotoxicity assays

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®
Active substance(s):	Glyphosate
Source of test items:	Glyphosate: Sigma-Aldrich, France Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: not reported Roundup Express®: 7.2 g/L (R7.2) Bioforce® or Extra 360: 360 g/L (R360) Grands Travaux®: 400 g/L (R400) Grands Travaux plus®: 450 g/L (R450)

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell cultures:	Hepatoma cell line HepG2, breast cancer cell line MDA-

MB453-kb2

Species:	Human
Source:	HepG2: ECACC, Salisbury, UK MDA-MB453-kb2: ATCC, Molsheim, France
Culture conditions HepG2:	Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum
Culture conditions MDA-MB453-kb2:	Leibovitz-15 (L15) medium supplemented with 10% foetal calf serum. Cells were incubated at 37°C and the medium was removed every 48 h.

4. Test methods:

MTT assay:	Assessment of cell viability of HepG2 cells
ToxiLight® assay:	Bioluminescent assay for measurement of cell membrane damage of HepG2-cells
Alamar Blue® assay:	Assessment of cell viability of HepG2 cells
Caspase-Glo® 3/7 assay	Assessment of caspase activity or apoptose induction
Neutral red assay:	Assessment of cell viability of MDA-MB453-kb2 cells
Guideline:	Non-guideline assays
GLP:	No
Guideline deviations:	Not applicable
Test conditions:	MTT assay: 2% Roundup Bioforce® and an equivalent solution of glyphosate to Roundup Bioforce were prepared in serum-free medium and adjusted to pH 5.8. From these stock solutions consecutive dilutions up to 10^{-7} were used for measurement. Assays were conducted in 48-well plates. After treatment for 24 h the supernants were recovered for the ToxiLight bioassay, and adherent cells were washed with serum free medium and incubated with 120 µL MTT per well. The plates were incubated for 3 h at 37°C. Afterwards 120 µL of 0.04 N-hydrochloric acid containing isopropanol were added, the plates were shaked. Measurements were done at 570 nm. ToxiLight assay: After 24 h exposure the 50 µL of the above mentioned supernants were added to a 96-well plate and incubated with 50 µL AK detection reagent (AKDR) for 15 minutes protected from light. The luminescence was measured using a luminometer at 565 nm. Serum-free medium served as negative control. The positive control was the active reagent AKDR mixed with cells treated in serum-free medium. Alamar Blue assay: About 30000 HepG2 cells per well were grown for 24 h in 96-well plates and then exposed to 250 µL of test substance solutions for 24 h (at pH 7.4). Afterwards 100 µL of Alamar Blue solution was added to each well and incubated for 2 h at 37°C. The optical density was measured at 540 and 620 nM. The viability was expressed as percentage of the control results (medium only). Caspase-Glo® 3/7 assay: This assay was used for caspase activity or measurement of apoptose induction. Cells were exposed to R450 for 24 or 48 h in 96-well plates. Afterwards

	50 µL/well of Caspase-Glo® 3/7 reagent was added and plates were incubated for 45 minutes at room temperature protected from light before luminescence was measured. Serum-free medium served as negative control. The positive control consisted of the active reagent mixed with cells treated in serum-free medium. Neutral red assay: about 50000 MDA-MB453-kb2 cells were seeded in 24-weel plates and grown for 24 h at 37°C. Afterwards cells were exposed to test substance solutions for 24 h. Cells were washed and incubated with neutral red solution for 3 h at 37°C. After a further washing the viability was assessed by fluorescence measurement.
Dose levels:	Glyphosate: not reported Roundup Express®: 7.2 g/L Bioforce® or Extra 360: 360 g/L Grands Travaux®: 400 g/L Grands Travaux plus®: 450 g/L
Replicates per dose level:	4 x 3 replicates

5. Observations/analyses:

Measurements:	Cell viability, membrane damage, apoptose induction
Statistics:	All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

Genotoxicity test

1. Test material:

Test item:	Grands Travaux®
Active substance(s):	Glyphosate
Source of test items:	Grands Travaux® (available on the market)
Lot/Batch #:	Not specified
Purity:	400 g/L

2. Vehicle and/or positive control: medium / Benzo[a]pyrene 50 µM

3. Test system / cells:

Cell cultures:	Hepatoma cell line HepG2
Species:	Human
Source:	HepG2: ECACC, Salisbury, UK
Culture conditions HepG2:	Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

4. Test methods:

Study type:	Single-cell gel electophoresis assay (Comet assay)
Guideline:	Non-guideline assay The assay was conducted according to the method developed by Singh et al., 1988, with some modifications for cell preparation (Valentin-Severin et al., 2003).

	(Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 84–191. Valentin-Severin, I., Le Hegarat, L., Lebon, A.M., Lhuguenot, J.C., Chagnon, M.C., 2003. Use of hepG2 cell line for direct or indirect mutagens screening: comparative investigations between comet and micronucleus assay. Mut. Res. 536, 79-90)
GLP:	No
Guideline deviations:	Not applicable
Dose levels:	1, 2.5, 5, 7.5, 10 ppm
Exposure duration:	24 h
Replicates per dose level:	3 x 2 replicates
Analysed cells per replicate:	100

5. Observations/analyses:

Measurements:	Observed nuclei were classified into 4 classes: 0 (undamaged), 1 (minimum damage), 2 (medium damage) and 3 (maximum damage)
Statistics:	All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

<u>Aromatase disruption</u>

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®
Active substance(s):	Glyphosate
Source of test items:	Glyphosate: Sigma-Aldrich, France Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: not reported Roundup Express®: 7.2 g/L Bioforce® or Extra 360: 360 g/L Grands Travaux®: 400 g/L Grands Travaux plus®: 450 g/L

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells:

Cell cultures:	Hepatoma cell line HepG2
Species:	Human
Source:	HepG2: ECACC, Salisbury, UK
Culture conditions HepG2:	Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum

4. Test methods:

Study type:	Measurement of aromatase activity by tritiated water release assay, semi-quantitative RT-PCR
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Test conditions:	Tritiated water release assay: HepG2 cells were exposed to non-toxic concentrations of glyphosate alone or Roundup. RT-PCR: HepG2 cells were exposed to non-toxic concentrations of glyphosate alone or Roundup. RNA was extracted and reverse transcribed (using 200 U MMLV-RT at 42°C for 60 min). The resulting cDNA was subjected to RT-PCR.
Dose levels:	Glyphosate: 0.06, 0.2, 0.3% Roundup Express®: 0.3, 0.5, 0.8% of product Bioforce® or Extra 360: 0.08, 0.1, 0.3% of product Grands Travaux®: 0.001, 0.003, 0.005 % of product Grands Travaux plus®: 0.005, 0.007 % of product
Exposure duration:	24 h
Replicates per dose level:	4 x 3 replicates

5. Observations/analyses:

Measurements:	Tritiated water release assay: radioactivity of released tritiated water was assessed by liquid scintillation counting. RT-PCR: Aromatase mRNA levels were normalised with control gene GAPDH and analysed photographically.
Statistics:	All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01 or 0.05.

<u>Anti-estrogenic and anti-androgenic effects</u>

1. Test material:

Test item:	Glyphosate, Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus®
Active substance(s):	Glyphosate
Description:	
Source of test items:	Glyphosate: Sigma-Aldrich, France Roundup Express®, Bioforce® or Extra 360, Grands Travaux®, Grands Travaux plus® (available on the market)
Lot/Batch #:	Not specified
Purity:	Glyphosate: Roundup Express®: 7.2 g/L Bioforce® or Extra 360: 360 g/L Grands Travaux®: 400 g/L Grands Travaux plus®: 450 g/L

2. Vehicle and/or positive control: Medium / ICI 182 x 780 (10^{-8}M) and Nilutamide (10^{-6} M)

3. Test system / cells:

Cell cultures:	Hepatoma cell line HepG2, breast cancer cell line MDA-

MB453-kb2

Species: Human

Source: HepG2: ECACC, Salisbury, UK
MDA-MB453-kb2: ATCC, Molsheim, France

Culture conditions HepG2: Phenol red-free EMEM containing 2 mM L-glutamin, 1% non-essential amino acid, 100 U/mL antibiotics (mix of penicillin, streptomycin, fungizone), 10 mg/mL liquid kanamycin, 10% fetal bovine serum
For anti-estrogenic activity, HepG2 cells were grown in phenol red-free MEM

Culture conditions MDA-MB453-kb2: Leibovitz-15 (L15) medium supplemented with 10% foetal calf serum. Cells were incubated at 37°C and the medium was removed every 48 h.

4. Test methods:

Gene-receptor tests with luciferase activity measurement

Guideline: Non-guideline assays

GLP: No

Guideline deviations: Not applicable

Test conditions: Anti-estrogenic activity test: 120000 HepG2-cells per well were grown at 37°C (5% CO_2, 95% air) in MEM supplemented with 2 mM glutamine, 1% non-essential amino-acis and 10% of dextran-coated charcoal foetal calf serum in 24-well plates. After 24 h the cells were transfected with a mixture of 5 different plasmids (ERE-TK, hERα, hERβ, pCMVβGal and psG5) and incubated for 1 h at 37°C (5% CO_2, 95% air). Afterwards the medium was removed and replaced by1 mL of medium without foetal calf serum and incubated for further 24 h. Cells were co-treated with the test substance solutions and β-estradiol (10^{-8} M). ICI 182 x 780 (10^{-8} M) served as positive control. At the end of treatment cells were lysed with Reporter lysis buffer and frozen at -80°C for at least 30 min, and prepared for activity measurements.

Anti-androgenic activity test: 50000 MDA-MB-453-kb2 cells per well were grown in 24-well plates in L-15 medium without phenol-red supplemented with 5% dextran-charcoal fetal calf serum at 37°C without CO_2. After 24 h the medium was removed and cells were washed with PBS and exposed to Roundup solutions in co-treatment with DHT (4 x 10^{-10} M). Nilutamide (10^{-6} M) was used as positive control. After 24 h cells were lysed and luciferase activity was measured.

Dose levels: Anti-estrogenic activity test:
Glyphosate: 0.1, 0.2, 0.3%
Roundup Express®: 0.1, 0.2, 0.3% of product
Bioforce® or Extra 360: 0.05, 0.1, 0.15, 0.2% of product
Grands Travaux®: 0.00025, 0.0005, 0.00075, 0.001 % of product
Grands Travaux plus®: 0.001, 0.002, 0.003 % of product

Anti-androgenic activity test:
Glyphosate: 0.05, 0.1, 0.15%
Roundup Express®: 0.05, 0.1, 0.15, 0.2% of product
Bioforce® or Extra 360: 0.01, 0.02, 0.03, 0.04, 0.05% of

product
Grands Travaux®: 0.00005, 0.0001, 0.00015, 0.0002 % of product
Grands Travaux plus®: 0.001, 0.002, 0.003, 0.004 % of product

Replicates per dose level: 3 x 3 replicates

5. Observations/analyses:

Measurements: Anti-estrogenic activity test: Luciferase and β-galactosidase activities and protein level.

Luciferase activity for each treatment group was normalised to β-galactosidase activity and protein level (Luc x Prot/Gal) and compared to the control (17 β-estradiol) set at 100%.

Anti-androgenic activity test: Luciferase activity were measured and reported as a percentage of the data obtained with the androgen DHT

Statistics: All data were reported as mean ± standard error. Statistical differences were determined by Student t-test using significant levels of 0.01.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Due to reporting deficiencies (e.g. correlation between concentration used in toxicity tests and concentrations used in comet assay) assessment of results difficult. Exceedingly high doses above the limit dose for this study type. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems.

2. Relevance of study: **Not relevant**: Excessive doses exceed typical *in vitro* limit doses. *In vitro* test system is inappropriate with surfactants.

3. Klimisch code: **3**

Response 1 – summarized from Williams et al. (2012)

- Glyphosate demonstrated no significant anti-estrogenic potential
- Glyphosate demonstrated some anti-androgenic potential at lower concentrations, but not as doses increased and therefore results are considered unrelated to treatment
- Four glyphosate based formulations demonstrated both estrogenic and androgenic activity.
- Results are confounded due to surfactants within the formulated products tested, which affect cell membrane integrity and produces false findings.

Response 2 – summarized from BfR Review (2009)

- Numerous methodological flaws are noted.
  - Test substance(s) not characterized
  - Source of materials for cell culture not provided.
  - Dosing concentrations not well described

  - Serum free media only appropriate for short term (3-4 hour) *in vitro* exposures.
  - pH control of dilutions not clear.
  - Osmolality of test solutions not reported.
  - Electrophoresis parameters insufficiently or inaccurately reported.
- Numerous reporting deficiencies are noted.
  - Influence of serum-free cell culturing on endpoints can not be determined
  - Incomplete data reporting, in cluding β-galactosirase activity, cototoxicity for select assays.
  - Positive control data not reported.
  - Confusion between maximum residue levels verses systemic concentrations in humans.

Author(s)	Year	Study title
Clair, E., Mesnage, R., Travert, C., Seralini, G.E.	2012a	A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells *in vitro*, and testosterone decrease at lower levels Toxicology in Vitro Volume: 26 Number: 2 Pages: 269-279

Abstract*

The major herbicide used worldwide, Roundup, is a glyphosate-based pesticide with adjuvants. Glyphosate, its active ingredient in plants and its main metabolite (AMPA) are among the first contaminants of surface waters. Roundup is being used increasingly in particular on genetically modified plants grown for food and feed that contain its residues. Here we tested glyphosate and its formulation on mature rat fresh testicular cells from 1 to 10000 ppm, thus from the range in some human urine and in environment to agricultural levels. We show that from 1 to 48 h of Roundup exposure Leydig cells are damaged. Within 24–48 h this formulation is also toxic on the other cells, mainly by necrosis, by contrast to glyphosate alone which is essentially toxic on Sertoli cells. Later, it also induces apoptosis at higher doses in germ cells and in Sertoli/germ cells co-cultures. At lower non toxic concentrations of Roundup and glyphosate (1 ppm), the main endocrine disruption is a testosterone decrease by 35%. The pesticide has thus an endocrine impact at very low environmental doses, but only a high contamination appears to provoke an acute rat testicular toxicity. This does not anticipate the chronic toxicity which is insufficiently tested and only with glyphosate in regulatory tests.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Bioforce® and glyphosate
Active substance(s):	Glyphosate
Description:	Not reported
Source:	Glyphosate: Sigma-Aldrich (Saint Quentin Fallavier, France) Roundup Bioforce®: not reported
Lot/Batch #:	Not reported
Purity:	Glyphosate: not reported Roundup Bioforce®: 360 g/L acid glyphosate (corresponding to 100%)
Homologation:	Roundup Bioforce® 9800036

2. Vehicle and/or positive control:

Dulbecco Modified Eagle's Medium/Ham F12 Medium (DMEM; Biotech GmbH, Dutscher, Brumath, France)

3. Test system / cells / animals:

Species:	Rat
Strain:	Sprague-Dawley

Source:	Janvier, Le Genest-Saint-Isle, France or University Centre of Biological Resources, Caen, France
Age of test animals at study initiation:	70 days ± 5
Sex:	male
Body weight:	Not reported
Acclimation period::	Not reported
Diet/Food:	Standard food, *ad libitum*
Water:	Water, *ad libitum*
Housing::	Not reported
Environmental conditions:	Temperature: 20 ± 22°C Humidity: not reported Air changes: not reported 12-hour light/dark cycle
Cell Culture:	Leydig, Sertoli and germ cells
Species:	Rat
Source:	Sprague-Dawley rats
Cell line maintenance:	DMEM/Ham F12 nutrient medium (1:1, v/v) supplemented with or without hGC (human homolog of LH physiologically involved in endocrine regulation of Leydig cells) for Leydig cells culture and with serum replacement 3 for Sertoli and germ cells.
Culture conditions: :	Temperature: 32°C Atmosphere: 5% CO_2, 95% air

4. Test methods:

Bioluminescent ToxiLight ™ bioassay:	Cytotoxicity assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96 or 24-well plates
Test conditions:	Before the assay, cells were treated with different dilutions of Roundup Bioforce ® or glyphosate ± 1 UI/mL of hCG during different exposure time points. The adenylate kinase detection reagent (AKDR) was prepared in a buffer (5 g/10 mL). Subsequently 50 mL of supernatant were transferred to an opaque black 96-well plate. 50 µL of AKDR reagent were put into each well. The plates were then left under agitation for 15 min in the dark, and light was measured using a luminometer.
Dose levels:	Not exactly specified; several concentrations from 0 – 1.0% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium
Cells per well:	10^5 per well in 96-well plates and 3 x 10^5 per well in 24-well plates
Exposure duration:	3, 6, 9, 12, 18, 24 or 48 h
Replicates per dose level:	9

Caspase –Glo™ 3/7 assay:	Cytotoxicity assessment, apoptose assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96 -well plates
Test conditions:	Before the assay, cells were treated with different dilutions of Roundup Bioforce® or glyphosate ± 1 UI/mL of hCG during different exposure time points. The Caspase-Glo® 3/7 reagent was prepared in a buffer. After 30 min at room temperature, 50 µL of Caspase-Glo® 3/7 reagent was added to 50 µL of culture medium containing the cells previously treated. After shaking the plate 15 min, an incubation period of 45 min at ambient temperature in the dark was required to stabilize the signal before luminescence measurement with a luminometer was performed.
Dose levels:	Not exactly specified; several concentrations from 0 – 1.0% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium
Cells per well:	10^5 per well in 96-well plates
Exposure duration:	3, 6, 9, 12, 18, 24 or 48 h
Replicates per dose level:	9
DAPI-labelling:	Apoptose assessment
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	24 -well plates
Test conditions:	After 24 h incubation with various dilutions of the test substances, 24-well plates were centrifuged and the medium was removed slowly. Leydig cells were fixed for a day in ab absolute ethanol-chloroform–acetic acid (6:3:1, v/v/v) at -20 °C. The wells were rinsed with PBS (pH7.4) and incubated with 1µg/mL of a solution containing DAPI during 30 min. Each well was washed with water and then observed with a microscope using a fluorescent mode.
Dose levels:	0.05, and 1 % of Roundup Bioforce® and 1% of glyphosate in DMEM/Ham F12 medium
Cells per well:	30000 per well in 24-well plates
Exposure duration:	24 h
Replicates per dose level:	9
3β-hydroxysteroid dehydrogenase (3β-HSD) activity:	Assessment of testosterone production
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	96-well plates

Test conditions:	Leydig cells were exposed to different concentrations of the test substances. Afterwards the wells containing the pretreated cells and 3β-HSD reagent containing DHEA (substrate), NAD (cofactor), NBT and nicotinamide were incubated at 37 °C for 45-60 min**.** Subsequently, as soon as the cells were stained, a solution of 10% acetic acid was added to solubilise the previously formed formazan crystals. The 3β-HSD activity was then measured by reading the optical density of each well at 560 nm (formazan) through a plate reader.
Dose levels:	Not exactly specified; several concentrations from 0 – 0.1% dilutions of Roundup Bioforce® or equivalent concentrations of glyphosate in DMEM/Ham F12 medium
Cells per well:	Not reported
Exposure duration:	24 h
Replicates per dose level:	9
Radioimmunoassay (RIA) of testosterone:	Assessment of testosterone production
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	Not reported
Test conditions:	The RIA was carried out on Leydig cells by competition and stopped using the method of activated charcoal. 200 µL of unlabeled testosterone standard solution, phosphate buffer or culture supernatant were incubated with 100 µL of radioactive testosterone and 100 µL of rabbit anti-testosterone antibody. After 30 min at ambient temperature the mixture was placed at 4 °C until the next day. Afterwards 500 µL of charcoal/dextran (50%/5%) was added and the mix incubated at 4 °C. Finally, the tubes were centrifuged (10 min at 2400 rpm at 4 °C) and the radioactivity counted.
Dose levels:	0, 0.0001, 0.0005, 0.001, 0.0025, 0.005, 0.0075 and 0.01 % dilutions of Roundup Bioforce® or glyphosate in DMEM/Ham F12 medium
Cells per well:	Not reported
Exposure duration:	24 h
Replicates per dose level:	9
Real time PCR:	Measurement of mRNA expression of aromatase, androgen receptor and estrogen receptor α- and β.
Guideline:	Non-guideline assay
GLP:	No
Guideline deviations:	Not applicable
Plate culture:	6-well plates
Test conditions:	After exposure of Leydig cells with the test substances cell pellets were treated with Trizol for the cell degradation. The chloroform was added to recover the aqueous phase containing the RNA. RNA precipitation was done by adding isopropanol and washing by adding 70% ethanol.

	250 ng of RNA , 200 U of MMLV-RT (Moloney murine leukemia virus reverse transcriptase), 0.2 g of random primers, 500 mM of each dNTP and 20 U of recombinant RNasin® were incubate 90min at 37°C to obtain cDNA, The reaction was stopped by 5 min at 75 °C. The polymerase chain reaction was performed on cDNA using the method GoTaq® qPCR Master Mix (Promega). The PCR conditions were an initial step at 95 °C for 3 min, then 40 cycles of 30 s at 95°C abd 60°C for 60 s. mRNA levels of aromatase, estrogen receptor α and β and androgen receptor were normalized using the L19 control gene.
Dose levels:	0, 0.001, 0.005 and 0.01 % dilutions of Roundup Bioforce® or glyphosate in DMEM/Ham F12 medium
Cells per well:	Not reported
Exposure duration:	24 h
Replicates per dose level:	9

6. Observations/analyses:

Measurements:	Citotoxicity of Roundup Bioforce® or glyphosate measured through adenylate kinase activities; measurements of caspases 3 and 7 (key-caspases of apoptosis) in cell cultures by means of bioluminescence-based method; study of chromatin condensation by DAPI-labelling; measurement of 3β-HSD activity; changes in testosterone production secreted from Leydig cells in medium
Statistics:	All data are present as means ± SEM. Statistically significant differences from controls were determined by an ANOVA test followed by Bonferroni post-test with $p<0.001$ (****), $p<0.005$ (***), $p<0.01$ (**) and $p<0.05$ (*).

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline *in vitro* test with methodological (i.e. no positive controls included) and reporting deficiencies (e.g. dose levels not always specified).

2. Relevance of study: **Not relevant** (Due to reliability. In addition, *in vitro* data, do not reflect real *in vivo* exposure situations, and therefore not relevant for human risk assessment purposes.)

3. Klimisch code: **3**

Response - GTF

This publication presents no new findings relevant to the current discussions of glyphosate safety. It is clear from the previous work of Seralini and others that surfactants can injure or kill cells when applied to exposed cells living in a Petri-dish environment. It also is not surprising that injured cells demonstrate activation of injury-response systems or suffer from a general decline in a wide variety of cellular functions, including hormone production in cells which normally serve that function. The concentrations used in these experiments are not relevant to human exposures to glyphosate and the experimental system used is not relevant to whole animal outcomes. Importantly, the alleged impacts on endocrine function have not been observed in animal studies of glyphosate or other components of glyphosate formulations at

relevant concentrations. Authors state that the lowest concentration of glyphosate tested was 50 ppm, several orders of magnitude higher than an anticipated human intake (based on pharmacokinetics described in Anadon et al., 2009) following worst case dietary exposure at the ADI.

The experiments reported in this publication involve two additional cell types; Leydig and Sertoli cells from rat testes. However, Petri dish experiments in a laboratory are not representative of exposures to a living animal and are not informative about real-world risks to humans. Instead, these experiments demonstrate what we already know – substances, soaps, detergents of surfactants, can injure unprotected cells *in vitro*.

Author(s)	Year	Study title
Hokanson, R. Fudge, R. Chowdhary, R. Busbee, D.	2007	Alteration of estrogen-regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate. Human & Experimental Toxicology Volume: 26 Pages: 747-752

Abstract*

Gene expression is altered in mammalian cells (MCF-7 cells), by exposure to a variety of chemicals that mimic steroid hormones or interact with endocrine receptors or their co-factors. Among those populations chronically exposed to these endocrine disruptive chemicals are persons, and their families, who are employed in agriculture or horticulture, or who use agricultural/horticultural chemicals. Among the chemicals most commonly used, both commercially and in the home, is the herbicide glyphosate. Although glyphosate is commonly considered to be relatively non-toxic, we utilized *in vitro* DNA microarray analysis of this chemical to evaluate its capacity to alter the expression of a variety of genes in human cells. We selected a group of genes, determined by DNA microarray analysis to be dysregulated, and used quantitative real-time PCR to corroborate their altered states of expression. We discussed the reported function of those genes, with emphasis on altered physiological states that are capable of initiating adverse health effects that might be anticipated if gene expression were significantly altered in either adults or embryos exposed *in utero*.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate formulation
Source:	Unknown retail supplier
Purity:	Not reported
Concentration:	15% home use preparation

2. Vehicle and/or positive control:

SFBS medium / no positive control

3. Test system/cells:

Cell line:	MCF-7
Source:	American Type Culture Collection (Rockville, MD, USA)
Growing medium:	MEM (minimal essential medium), phenol red-free MEM
Source:	Gibco (Gaithersburg, MD, USA)
Culture conditions:	Not reported
Further materials:	17β-estradiol (E2) (Sigma, St. Louis, USA), fetal bovine serum (FBS) (Summit Biotechnology, USA) RZPD microarray chips (Deutsches Ressourcencentrum für Genomforschung GmbH, Berlin, Germany) Roche´s cDNA synthesis kit (Roche) Real time PCR kit (ABI, NJ, USA); ABI 7500 Real-Time PCR

system thermocycler (ABI, USA)

4. Test method:

Study type:	*In vitro* DNA microarray analysis, quantitative real-time PCR (qrtPCR)
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Dose levels:	0.1, 0.01, 0.001 or 0.0001% dilutions of the glyphosate stock solution containing 15% glyphosate.
Duration of exposure:	18 h
Exposure:	MCF-7 cells were grown in MEM in T-150 vented culture flasks. Upon reaching 60% confluency, the medium was removed and replaced with phenol red-free MEM containing 10% stripped fetal bovine serum (SFBS), to reduce the E2 availability to the cells. After a growing period of 24 hours the cells were treated with glyposate concentrations at 0.1, 0.01, 0.001 or 0.0001% dilutions of the stock solution (i.e. 15% glyphosate) with or without 3 x 10^{-10} M E2 for 18 hours.
DNA micro array:	Microarray analysis was performed in commercially available microarray slides. After 18 h exposure cells were harvested and RNA was purified. Closed DNA (cDNA) was generated from the isolated RNA using Roche´s cDNA synthesis kit. Cyanine-5 and cyanine-3-labeled anti-sense RNA was generated and hybridized using Wellmer´s protocol. The labelled RNA was loaded with a labelled control sample onto the array slides. Array slides were scanned in an Axon Genepix 4000B. Details of the hybridisation and scanning procedures were not reported.
qrtPCR:	Test was conducted in semi-skirted 96 well PCR plate using a commercially available PCR system
Replicates per gene of interest:	3

5. Observations/analyses:

Measurements:	Scan of microarray slides, quantitative rt-PCR
Statistics:	Statistical analysis utilized one-way ANOVA followed by Dunnett's test to analyse differences between control and chemically treated samples, with $P < 0.05$ considered to be statistically significant.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Not acceptable *in vitro* methods for test mixtures containing surfactant. Well documented study publication which meets basic scientific principles, but surfactants are inappropriate test substance in cell lines.

2. Relevance of study: **Not relevant** Temporal altered gene expression is not a biomarker for toxicity, but rather, may be within the range of normal biological responses of homeostasis. *In vitro* cytotoxicity of surfactants, however, is a significant confounder in data interpretation. Data do not reflect real *in vivo* exposure situations, and therefore not relevant for human risk assessment

purposes.)

3. Klimisch code: **3**

Response - GTF

- Relevance of altered gene expression in a cell line derived from a breast cancer should not be extrapolated to reflect human health endpoints.
- Altered gene expression should not be confused with adverse health outcomes. Rather altered gene expression may equally be considered a biological response within the range of normal homeostasis.
- The authors describe a “bewildering array” of possible human health endpoints, which are conspicuously absent in the vast glyphosate toxicology data base.
- The concluding sentence, with implications of both adult and foetal cell damage, lack biological plausibility when considering glyphosate *in vivo* ADME, kinetics and toxicology data.

IN VIVO DART/ED PUBLICATIONS

Author(s)	Year	Study title
Yousef, M.I., Salem, M.H., Ibrahim, H.Z., Helmi, S., Seehy, M.A., Bertheussen, K.	1995	Toxic Effects of Carbofuran and Glyphosate on Semen Characteristics in Rabbits. Journal of Environmental Science and Health. Part B. Volume: 30 Number: 4 Pages: 513-534

Abstract*

The present study was undertaken to investigate the effect of chronic treatment with two sublethal doses of Carbofuran (carbamate insecticide) and Glyphosate (organophosphorus herbicide) on body weight and semen characteristics in mature male New Zealand white rabbits. Pesticide treatment resulted in a decline in body weight, libido, ejaculate volume, sperm concentration, semen initial fructose and semen osmolality. This was accompanied with increases in the abnormal and dead sperm and semen methylene blue reduction time. The hazardous effect of these pesticides on semen quality continued during the recovery period, and was dose-dependent. These effects on sperm quality may be due to the direct cytotoxic effects of these pesticides on spermatogenesis and/or indirectly via hypothalami-pituitary-testis axis which control the reproductive efficiency.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate (N-(phosphonomethyl) glycine)-containing pesticide Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate)-containing pesticide
Active substance(s):	Glyphosate Carbofuran
Source:	Glyphosate: Monsanto Company, USA Carbofuran: Brichima S.P.A., Italy, Brifur
Purity:	Not reported
Lot/Batch #:	Not reported

2. Vehicle: Gelatine capsule

3. Test animals:

Species:	Rabbit
Strain:	New Zealand white
Source:	Not reported
Age of test animals at study initiation:	8 months
Sex:	Male
No. of rats:	20
Body weight:	2863 ± 59.8 g

Acclimation period:	Not reported
Diet/Food:	Ration pellets consisting of 48% berseem hay (Trifolium alexandrinum), 18% wheat bran, 16% ground corn, 14% soybean meal, 3% molasses, 0.5% salt, and 0.5% vitamins. Feed provided *ad libitum*
Water:	Water provided *ad libitum*
Housing:	Individually in cages
Environmental conditions:	Temperature: Not reported Humidity: Not reported Air changes: Not reported Light/dark cycle: Not reported

4. Test system:

Study type:	Toxic Effects of Carbofuran and Glyphosate on Semen Characteristics in Rabbits.
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	18 weeks
Pre-exposure period:	6 weeks
Duration of exposure:	6 weeks
Recovery period:	6 weeks
Dose levels:	1st group – control group; 2nd group – 1/100 LD_{50} carbofuran; 3rd group – 1/10 LD_{50} carboruran; 4th group – 1/100 LD_{50} glyphosate; 5th group – 1/10 LD_{50} glyphosate The doses of the pesticides were calculated according to the animals' body weight on the day before dosing. (The LD_{50} values of both pesticides were not reported. Dose levels were not reported as mg/kg bw/day.
Animals per dose group:	4 animals per group
Administration:	Given orally into a gelatine capsule

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	Measured weekly in the morning before access to feed and water
Collection of test material:	Semen was collected once a week from all animals and continued throughout the 18-week experimental period
Measurement:	Volume of each ejaculate; Determination of seminal initial fructose was carried out directly after collection; Methylene blue reduction time (MBRT) was measured using

	methylene blue semen mixture in a capillary tube; Assessment of live, dead and abnormal spermatozoa were performed using an eosin-nigrosine blue staining mixture; Evaluation of sperm concentration by the improved Neubauer hemocytometer slide using weak eosin solution; Semen osmolality was determined by measuring the freezing point depression by using Osmete A (Precision Systems Inc., Sudbury, Mass., USA).
Food- and water consumptions:	Not reported
Haematology:	Not done
Clinical chemistry:	Not done
Urine analysis:	Not done
Sacrifice/pathology:	Not reported
Organ weights:	Not reported
Histology and morphometry:	Not reported
Statistics:	Data were analysed by generalized linear model procedure, Statistical Analysis System (SAS, 1984). The level of significance was reported as $P < 0.05$.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comment:	Non-GLP, non-guideline study with major reporting deficiencies. Dose-levels poorly defined as 1/10 and 1/100 LD_{50}. Purity of the test substances, source of animals, environmental conditions, mortality, and clinical signs not reported. No testis and epididymis weights were determined or reported and no histopathological examination conducted. In addition, stability and homogeneity assessment of test substance preparations were not done or not reported. Rabbits have low body weights at study start, suggesting impaired health status.
2. Relevance of study:	**Not relevant** (Due to very low confidence in study conduct and the inadequacy of reporting)
3. Klimisch code:	**3**

Response – summarized from Williams et al. (2000)

- Numerous serious deficiencies in the design, conduct, and reporting of this study which make the results uninterpretable.
- Only four rabbits per treatment group were used, and therefore statistics are questionable.
- Rabbits appeared to be small for their age; at study start (32 weeks) tested animals had 16-25% lower body weight than historical weights for commercially bred animals of the same age and strain.
- Low body weights as study start suggest compromised health status of the animals at initiation.
- Dose levels were not quantified.
- Purity of glyphosate and composition of the glyphosate formulation were not reported.
- Inadequate description of test material administration.
- Improper semen collection technique reported.

- Report is unclear whether control animal sham handling was undertaken, a critical factor in stress related outcomes in this species.
- Food consumption of test and control groups not adequately reported.
- Variability not adequately reported for endpoint measurements in test and control groups, preventing statistical analysis to support the author's conclusions.
- Dose-responses not observed, despite the wide dose spread.
- Sperm concentrations of all groups within normal ranges for this strain of rabbit.
- No meaningful conclusions can be drawn from this publication.

Author(s)	Year	Study title
Daruich, J. Zirulnik, F. Gimenez, M. S.	2001	Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses Environmental Research Volume: 85 Pages: 226-231

Abstract*

To prevent health risk from environmental chemicals, particularly for progeny, we have studied the effects of the herbicide glyphosate on several enzymes of pregnant rats. Glyphosate is an organo-phosphorated nonselective agrochemical widely used in many countries including Argentina and acts after the sprout in a systemic way. We have studied three cytosolic enzymes: isocitrate dehydrogenase-NADP dependent, glucose-6-phosphate dehydrogenase, and malic dehydrogenase in liver, heart, and brain of pregnant Wistar rats. The treatment was administered during the 21 days of pregnancy, with 1 week as an acclimation period. The results suggest that maternal exposure to agrochemicals during pregnancy induces a variety of functional abnormalities in the specific activity of the enzymes in the studied organs of the pregnant rats and their fetuses.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Herbycigon
Active substance(s):	Glyphosate
Source:	Herbycigon: M.F. L. S.R.L., Argentinia
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle: Tap water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	National University of San Luis, Argentina
Age of test animals at study initiation:	Not reported
Sex:	Females
Body weight:	210-230 g
Acclimation period:	1 week
Diet/Food:	20 g of stock laboratory diet (elaborated at Cargill) per day; ingredients: meat flour, bone and meat flour, fish meal, blood flour, soybean meal, toasted soybean, soy expeller, sunflower flour, cotton flour, peanut meal, animal fat, corn, wheat, sorghum, oat, barley, wheat bran, rice bran, gluten meal, vitamins A, E, B, D3, K3, and B12, niacin, pantothenic acid, choline, ascorbic acid, bone ash, salt, calcium carbonate, oyster, manganese oxide, zinc oxide, ferrous sulfate, copper oxide, sodium selenite, iodine, and cobalt.

Water:	35 ml of potable water per day
Food and water for control group:	Low water and food (10 ml and 10 g, respectively)
Housing::	After mating, individually in cages
Environmental conditions:	Temperature: 22-25°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Enzymatic activity of cytosolic enzymes in pregnant rats and fetuses
Guideline:	No
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	21 days during pregnancy
Dose levels:	0 (tap water), glyphosate solution 0.5% w/v in tap water (dose: 0.2 ml glyphosate/ml water), glyphosate solution 1% w/v in tap water (dose: 0.4 ml glyphosate/ml water)
Animals per test substance group:	8
Animals per control group:	Tap water control group: 8 Low water and low food control group: 6 The latter group received only 10 g food and 10 mL tap water per day. This treatment began in the second week after the high-dose group exhibited a decreased water- and foodintake.
Administration:	The test substance was prepared as solution in tap water. 35 mL of the test substance preparations were provided in water bottles per day and animal
Mating:	Female rats at the proeatrus stage were housed for one night with fertile males. Fertilisation was assumed by the presence of spermatozoa in the vaginal smear. That day was designated as gestation day 1.

5. Observations/analyses:

Analyses of test material preparations:	Not reported
Measurements:	Enzymatic activity of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, malic dehydrogenase
Mortality:	Not reported
Clinical signs:	Not reported
Maternal body weight:	Measured daily
Food- and water consumptions:	Measured daily
Test substance intake:	Not reported
Haematology:	Not reported
Clinical chemistry:	Not reported
Urine analysis:	Not reported
Sacrifice/pathology:	On day 21 of gestation, rats were anesthetisied with

	diethylether. Each foetus was delivered by rapid hysterectomy, identified, weighed and then killed by decapitation. Maternal and foetal livers, hearts, and brains were immediately removed, washed in a cold saline solution, and stored at -20°C until analysis. Foetal organs were pooled.
Tissue sample processing:	Livers, hearts, and brains (0.5g/1 ml buffer) were homogenised in an Ultra Turrax with 0.5 M Tris-HClbuffer, pH 7.4 containing 1 mM dithiothreitol. Cytosolic fractions were obtained by ultra centrifugation.
Measurements (enzymatic assays):	Enzymatic activities of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and maic dehydrogenase were measured in the supernant by the determination of the rate of NADPH formation at 340 nm in a spectrometer. The results were expressed as µmol NADP/min/mg protein. Protein concentration was measured by Biuret reaction.
Organ weights:	Liver, hearts and brains of maternal females
Histopathology:	Not done.
Statistics:	Signifficant differences among means were considered at a level of $P < 0.05$ and identified by one-way ANOVA, Kolmogorov-Smirnov, and Newman-Keul procedures. In all the cases the variances were homogeneous.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comment:	Basic data given, however, the study is performed with methodological and reporting deficiencies (unknown exposure levels, only cytosolic enzymes measured, inappropriate controls, lack of consistent dose-response data).
2. Relevance of study:	**Not relevant** (Due to reliability. In addition, study was performed with a glyphosate formulation (commercialised in Argentina) and not with glyphosate)
3. Klimisch code:	**3**

Response 1 – GTF

- Test substance administration is poorly described, but rough calculations on approximate surfactant intake show excessively high and unrealistic exposures when compared to DART systemic parental and reproductive/developmental NOAEL values for POEA formulation surfactants.
    - For the low dose group, based on 360 g/L glyphosate solution containing 18% surfactant, 0.1 mL glyphosate (conservatively assumed to be the formulation)/mL water = 0.018 mL surfactant/mL water. Assuming water consumption of 10 mL/day surfactant intake = 0.18 mL per rat per day. Assuming surfactant density of 1 g/mL and 250 gram rat, surfactant low dose = 720 mg/kg/day.
    - Conservative high surfactant dose estimate = 1440 mg/kg/day
    - Conservative estimate of surfactant intake is at least one order of magnitude greater than parental and DART NOAEL values reported in Williams et al. (2012).

Response 2 – summarized from Williams et al. (2012)

- Test substance and doses not adequately described.

- Inappropriate control groups.
- Results suggest that the effect of treatment on body and organ weights may be due to reduced food and water intakes.
- A consistent effect of treatment was not observed and dose-response relationships were generally lacking
- The information gathered may be misleading because the enzymes monitored are found in both the cytosol and mitochondria.
- Food restriction affects the activity of many enzymes, including those examined in this study.
- Same comments apply to Bueret et al. (2005; on-line version 2004), in which test group dams showed a 23% reduction in food consumption, 21% reduction in water consumption and 42% reduction in body weight gain versus controls.

Author(s)	Year	Study title
Dallegrave, E. Mantese, F. D. Coelho, R. S. Pereira, J. D. Dalsenter, P. R. Langeloh, A.	2003	The teratogenic potential of the herbicide glyphosate-Roundup® in Wistar rats Toxicology letters Volume: 142 Pages: 45-52

Abstract*

The aim of this study was to assess the teratogenicity of the herbicide glyphosate-Roundup(R) (as commercialized in Brazil) to Wistar rats. Dams were treated orally with water or 500, 750 or 1000 mg/kg glyphosate from day 6 to 15 of pregnancy. Cesarean sections were performed on day 21 of pregnancy, and number of corpora lutea, implantation sites, living and dead fetuses, and resorptions were recorded. Weight and gender of the fetuses were determined, and fetuses were examined for external malformations and skeletal alterations. The organs of the dams were removed and weighed. Results showed a 50% mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in 15.4, 33.1, 42.0 and 57.3% of fetuses from the control, 500, 750 and 1000 mg/kg glyphosate groups, respectively. We may conclude that glyphosate-Roundup(R) is toxic to the dams and induces developmental retardation of the fetal skeleton.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup ®
Active substance:	Glyphosate
Source:	Monsanto of Brasil
Lot/Batch #:	BS 1096/98
Concentration:	360 g/L
Surfactant Class:	Polyoxyethyleneamine (POEA)
Concentration:	18% (w/v) (POEA)

2. Vehicle: Distilled water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Department of Pharmacology, Instituto de Ciencias Basicas da Saude, Brazil
Age of test animals at study initiation:	90 days
Sex:	Male and virgin female
Body weight:	200-280 g
Acclimation period:	Not reported
Diet/Food:	Laboratory rat chow, *ad libitum*
Water:	Water , *ad libitum*

Housing:	Polyethylene (65 x 25 x 15 cm) home cages, with sawdust-covered floors
Environmental conditions:	Temperature: 22 ± 2°C Humidity: not reported Air changes: not reported 12-hour light/dark cycle

4. Test system:

Study type:	Developmental toxicity study
Guideline:	Refers to the EPA (Environmental Protection Agency), 1996. Guidelines for Reproductive Toxicity Risk Assessment-EPA/630/R-96/009, Washington, USA, pp. 1-163. (reproductive toxicity protocols; segment II).
GLP:	no
Guideline deviations:	Reduced allowed mating time
Duration of study:	From day 6 up to 15 of gestation
Dose levels:	0 (water), 500, 750 or 1000 mg/kg glyphosate-Roundup® diluted in water
Animals per dose group	Sixty pregnant rats were divided into four groups (n=15±1 per group).
Administration:	Test substance preparations were prepared by diluting the Roundup-formulation with appropriate volumes of distilled water. Applications were done once daily by oral gavage Dosing volume: 10 mL/kg bw
Mating:	3 females were placed in a cage with one male during the dark period. Females showing sperm in the vaginal sperm on the following morning were housed individually. The other females were returned to the cage of the same male, each dark period for 15 consecutive days.

5. Observations/analyses:

Test substance preparations:	Not reported
Mortality:	Assessed, but details (e.g. time points, etc) not specified.
Clinical signs:	Not reported
Body weight:	Maternal body weights were determined daily during pregnancy and lactation periods. Offspring body weights were determined in weekly intervals from lactation to puberty
Body weight gain:	The body weight noted at day 0 (sperm positive smear) in parent females was considered as 100 %. The differences observed during the study with regard to this parameter were expressed as relative weight gain.
Food- and water consumptions:	In three day intervals during pregnancy. Data presented as relative intakes without reference to how data were normalized.
Test substance intake:	Not applicable
Sacrifice/pathology:	On day 21 of gestation dams were anesthetized with a combination of 5 mg/kg bw xylazine and 90 ,g/kg bw ketamine injected intramusculary and subjected to caesarean section. The uterus was removed and weighed with its contents.

Organ weights:	The weights of the following organs were determined and relative organ-to-body weights were calculated. Maternal: heart, lungs, liver, spleen and kidney
Developmental parameters:	Number of living and death foetuses, number of implantation sites, corpora lutea, resorptionssex of pups, sex-ratio, external malformations and skeletal alterations. Reported errors include more foetuses than implantation sites in one dose group. Note artifacts from atypical fixing and staining of foetal skeletons may have caused skeletal damage.
Statistics:	Parametric data, expressed as mean ± S.E.M., were analyzed by repeated measure ANOVA or one-way ANOVA, followed by the Duncan test when appropriate. The non-parametric data, expressed as proportion or percentage, were analyzed by the x^2-test. Differences were considered to be statistically significant when $P<0.05$.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comment:	Study design similar to US-EPA and OECD 414, with deviations (e.g. group size, inadequate dosing period) and reporting deficiencies. In addition, some methodological deficiencies (e.g. histopathological methods)
2. Relevance of study:	Relevant study type for investigating developmental endpoints, but questionable relevance of this specific study based on low reliability of data and interpretation. Test material was a formulated product, not glyphosate.
3. Klimisch code:	**3**

Response 1 – GTF

- This non-guideline prenatal developmental toxicity study with a POEA containing formulation may be compared directly with the test guideline and GLP compliant POEA rat prenatal developmental toxicity study, in which the same POEA surfactant maternal NOAEL was 15 mg/kg/day, and developmental NOAEL was considered the highest dose tested, 300 mg/kg/day.
- Approximate calculated exposures to the either glyphosate or POEA surfactant in the formulation can not be verified because the publication is unclear whether doses are based on the glyphosate content or actual formulation.
    - If based on dose levels of 500, 750 or 1000 mg/kg formulation, surfactant doses are 90, 135 and 180 mg/kg/day, well in excess of systemic maternal NOAEL value of 15 mg/kg/day reported by Williams et al. (2012).
    - If based on dose levels of 500, 750 or 1000 mg/kg glyphosate technical acid (versus the salt form in the formulated product), surfactant doses are even more extreme, approximately 250, 375 and 5000 mg/kg/day, well in excess of systemic maternal NOAEL value of 15 mg/kg/day reported by Williams et al. (2012).
- This publication reports excessively high and unrealistic exposures to the POEA surfactant in the tested formulation.
- While reporting weight gain in an atypical manner as relative %, actual reported mean body weight gains for mid and high dose groups align with the control group, while the low dose group body weight gain is approximately 20% less than the control group, indicating significant maternal toxicity in the low dose group. This significant non-dose related toxicity brings the quality and accuracy of this study into question.

Response 2 – summarized from Williams et al. (2012)

- Non-guideline prenatal developmental toxicity study design.
- Test material an unspecified commercial formulation "Roundup," which was reported to consist of 360 g/L glyphosate and 18% (w/v) POEA.
- Treatment doses unclear as to whether glyphosate or formulation concentrations.
- 15 rats per group, significantly lower than the recommended minimum of 20 litters per group in OECD 414.
- High dose group was further reduced to 7 pregnant dams due to maternal deaths.
- Few data presented in the publication.
- Unusual data presentation for body weight, food intake and water consumption, all a relative numbers without any reference to normal values.
- Fetal findings are presented as percentages or unsubstantiated mean values throughout the article, which complicates interpretation.
- Further investigation data presented notes a number of reporting errors (see Williams et al., 2012, Table 3). For example, in the 750-mg/kg/d treatment group, more fetuses than implantation sites were reported.
- Reports a dose-related increased incidence of skeletal alterations.
- Unusual methods described to fix and stain the fetal skeletons for evaluation, which may have led to artifacts that were falsely categorized as alterations (use of a a proteolytic enzyme which may have digested peptide bonds in the bone matrix). The reported skeletal alterations showed an extremely high prevalence of incomplete ossification of various bone structures, which are signs of a developmental delay that correct themselves within a brief period.
- treatment during gestation days 6-15 rather that to full term as per current test OECD 414 guidelines
- "Based on the use of these questionable methods, and the obviously flawed reporting of data, it is not possible to draw any conclusions regarding the developmental effects of "Roundup" treatment from this article. Furthermore, because a commercial formulation was used, it is not possible to attribute any observed effects to glyphosate specifically."

Author(s)	Year	Study title
Dallegrave, E. Mantese, F. D. Oliveira, R. T. Andrade, A. J. M. Dalsenter, P. R. Langeloh, A.	2007	Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats Archives of Toxicology Volume: 81 Pages: 665-673

Abstract*

Glyphosate is the active ingredient and polyoxyethyleneamine is the surfactant present in the herbicide Roundup (R) formulation commercialized in Brazil. The aim of this study was to assess the reproductive effects of glyphosate-Roundup (R) on male and female offspring of Wistar rats exposed during pregnancy and lactation. Dams were treated orally with water or 50, 150 or 450 mg/kg glyphosate during pregnancy (21-23 days) and lactation (21 days). These doses do not correspond to human exposure levels. The results showed that glyphosate-Roundup (R) did not induce maternal toxicity but induced adverse reproductive effects on male offspring rats: a decrease in sperm number per epididymis tail and in daily sperm production during adulthood, an increase in the percentage of abnormal sperms and a dose-related decrease in the serum testosterone level at puberty, and signs of individual spermatid degeneration during both periods. There was only a vaginal canal-opening delay in the exposed female offspring. These findings suggest that in utero and lactational exposure to glyphosate-Roundup (R) may induce significant adverse effects on the reproductive system of male Wistar rats at puberty and during adulthood.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup ®
Active substance(s):	Glyphosate
Source:	Monsanto of Brazil
Lot/Batch #:	Not reported
Concentration:	360 g/L
Surfactant:	Polyoxyethyleneamine (POEA)
Concentration:	18% (w/v) POEA

2. Vehicle: Distilled water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Department of Pharmacology, Federal University of Rio Grande do Sul, Brazil
Age of test animals at study initiation:	90 days
Sex:	Male and female
Body weight:	250-350 g
Acclimation period:	Not reported
Diet/Food:	standard lab rat chow (Nuvital®, Curitiba/PR, Brazil), *ad*

	libitum
Water:	Water, *ad libitum*
Housing::	Polyethylene (65 x 25 x 15 cm) home cages with sawdust-covered floors
Environmental conditions:	Temperature: 22 ± 2°C
	Humidity: not reported
	Air changes: not reported
	12-hour light/dark cycle

4. Test system:

Study type:	Reproductive toxicity
Guideline:	None
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	21-23 days during pregnancy;
	21 days during lactation
Dose levels:	0 (water), 50, 150, 450 mg/kg glyphosate-Roundup®
Mating:	3 females were placed in a cage with one male during the dark period. Females showing sperm in the vaginal sperm on the following morning were housed individually. The other females were returned to the cage of the same male, each dark period for 15 consecutive days.
Animals per dose group:	Sixty primigravid female rats were randomly divided into 4 groups of 15 animals each
Administration:	Test substance preparations were prepared by diluting the Roundup-formulation with appropriate volumes of distilled water.
	Applications were done once daily by oral gavage
	Dosing volume: 10 mL/kg bw

5. Observations/analyses:

Test substance preparations:	Not reported
Mortality:	Assessed, but details (e.g. timepoints, etc) not specified.
Clinical signs:	Assessed, but details (e.g. timepoints, etc) not specified.
Body weight:	Maternal body weights were determined daily during pregnancy and lactation periods.
	Offspring body weights were determined in weekly intervals from lactation to puberty.
Body weight gain:	The body weight noted at day 0 (first period day) in parent females was considered as 100 %, for each period. The differences observed during the study with regard to this parameter were expressed as relative weight gain.
Food- and water consumptions:	Not done
Test substance intake:	Not applicable
Haematology:	Not done
Clinical chemistry:	Not done
Hormone levels:	For determination of testosterone levels, blood was collected at termination, and the serum was removed. The samples were

	analysed in duplicate using a double-antibody according to the standard protocol for the radioimmunoassay (RIA) with Diagnostic Products Corporation testosterone kits.
Urine analysis:	Not done
Litter data:	Litter size, number of living and dead pups, viable pups, sex ratio (male/female)
Offspring development:	The development of offspring was assessed daily from lactation until puberty. The following characteristics were assessed: ears unstuck, fur emergence, incisor eruption, eye opening, testis descent (by scrotum palpation starting after the 15th postnatal day), preputial separation (by manually retracting the prepuce with gentle pressure after the 30th postnatal day) and opening of the vaginal canal (after the 30th postnatal day)
Sacrifice/pathology:	Males: One male from each litter (n = 15/group) was randomly selected for assessment of treatment-related systemic and reproductive effects at puberty (age: 65 days) and adulthood (age: 140 days). Selected males were sacrificed by thiopental anaesthesia followed by diaphragm incision. Females: One female from each litter (n = 15/group) was randomly selected for assessment of treatment-related systemic and reproductive effects at puberty (age: 65-70 days) and adulthood (age: 140 days).
Organ weights:	The weights of the following organs were determined and relative organ-to-body weights were calculated. Males: heart, lungs, liver, spleen, kidneys, adrenal glands and brain; testis, epididymis, seminal vesicle with coagulating glands (without fluid) and prostate Females: heart, lungs, liver, spleen, kidneys, adrenal glands and brain; uterus, oviducts and ovaries
Histopathology:	Five testes per dose group were fixed in Bouin´s solution immediately after removal, embedded in paraffin, sectioned at 3 µm and stained with hematoxylin/eosin. 20 essentially round seminiferus tubules per testis were analysed microscopically. The following parameters were assessed: tubule diameter, percentage of seminiferus tubules with complete spermatogenesis, presence of degenerating, sloughed and/or infiltrating cells, and absence of tubular lumen and of elongated spermatids.
Reproductive toxicity assessment:	Relative weight of the reproductive organs expressed as percentage of body weight and of reproductive indices, including sperm number per epididymis tail, daily sperm production, sperm transit, sperm morphology, testis morphology and serum testosterone level. Spermatid and sperm counts were determined.
Statistics:	Parametric data, expressed as mean ± standard error (SEM), were analyzed by repeated measure ANOVA or one-way ANOVA, followed by the Bonferroni test when appropriate. The nonparametric data, expressed as proportion or percentage, were analyzed by the chi-square test. Differences were

considered statistically significant when $P < 0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment: Study that does not comply with any test guideline. Reporting deficiencies. Conflicting results include decreased testes weights but increased testosterone levels in high dose. Questionable micrograph quality and interpretation may be artifacts of processing techniques. Conclusions not consistent with findings when viewed in light of dose-response or historical data for this strain of rat.

2. Relevance of study: **Not relevant** based on lack of dose-response, contradicting findings and unreliable data quality)

3. Klimisch code: **3**

Response 1 – GTF

- This non-guideline reproductive toxicity study with a POEA containing formulation may be compared to the DART NOAEL values for POEA surfactants reported in Williams et al. (2012).
- Approximate calculated exposures to the either glyphosate or POEA surfactant in the formulation can not be verified because the publication is unclear whether doses are based on the glyphosate content or actual formulation.
- If based on dose levels of 50, 150 or 450 mg/kg formulation (18% POEA), surfactant doses are 9, 27 and 81 mg/kg/day. In this case, doses are in the range of NOAEL values reported by Williams et al. (2012).
- Based on dose levels of 50, 150 or 450 mg/kg glyphosate technical acid (versus the salt form in the formulated product), POEA surfactant doses would be approximately 25, 75 and 225 mg/kg/day. In this case the low/mid doses are in the range of NOAEL values and the high dose exceeds NOAEL values reported by Williams et al. (2012).
- The findings reported by Dallegrave et al. (2007) are contrary to the GLP and guideline compliant studies reviewed by Williams et al. (2012), in which no effects on testis morphology, sperm parameters or testosterone levels were evident.

Response 2 - summarized from Williams et al. (2012)

- Non-guideline prenatal developmental-reproductive toxicity study design.
- Test material an unspecified commercial formulation "Roundup," which was reported to consist of 360 g/L glyphosate and 18% (w/v) POEA.
- Treatment doses unclear as to whether glyphosate or formulation concentrations.
- Maternal toxicity was not observed.
- Reproductive outcomes (number of pups, sex ratio, etc.) and pup weights unaffected.
- Statistical increased percentage of abnormal sperm in male offspring at the low but not medium or high dose offspring, suggesting a random finding
- Non-dose-related delay in vaginal opening in females within the normal physiological range for the species and in line with historical control data.
- Non-dose-related early preputial separation in the high dose males within the normal physiological range for the species and in line with historical control data.
- Contrary to expected outcome of early preputial separation, a statistical decrease in blood testosterone levels was also observed at puberty for high dose males.
- Decreased testosterone level was no longer evident at adulthood
- No dose-related findings in adult sperm production parameters

- Investigators fail to mention enlarged interstitial cells in the micrographs, suggesting limited experience conducting such histological examinations.
- The other reported histological interpretations, reduction in elongated spermatids and the presence of vacuolization at puberty and degeneration of the tubular lumen at adulthood, may be attributable to an artifact of tissue processing rather than exposure related effects.
- Multiple guideline study types and a subchrionic National Toxicology Program study do not report the testicular anomalies described by Dallegrave et al. (2007).

Author(s)	Year	Study title
Romano, R.M. Romano, M.A. Bernardi, M.M. Furtado, P.V. Oliveira, C.A.	2010	Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Archives of Toxicology Volume: 84 Pages: 309-317

Abstract*

Glyphosate is a herbicide widely used to kill weeds both in agricultural and non-agricultural landscapes. Its reproductive toxicity is related to the inhibition of a StAR protein and an aromatase enzyme, which causes an in vitro reduction in testosterone and estradiol synthesis. Studies in vivo about this herbicide effects in prepubertal Wistar rats reproductive development were not performed at this moment. Evaluations included the progression of puberty, body development, the hormonal production of testosterone, estradiol and corticosterone, and the morphology of the testis. Results showed that the herbicide (1) significantly changed the progression of puberty in a dose-dependent manner; (2) reduced the testosterone production, in semineferous tubules' morphology, decreased significantly the epithelium height ($P < 0.001$; control = 85.8 ± 2.8 µm; 5 mg/kg = 71.9 ± 5.3 µm; 50 mg/kg = 69.1 ± 1.7 µm; 250 mg/kg = 65.2 ± 1.3 µm) and increased the luminal diameter ($P < 0.01$; control = 94.0 ± 5.7 µm; 5 mg/kg = 116.6 ± 6.6 µm; 50 mg/kg = 114.3 ± 3.1 µm; 250 mg kg = 130.3 ± 4.8 µm); (4) no difference in tubular diameter was observed; and (5) relative to the controls, no differences in serum corticosterone or estradiol levels were detected, but the concentrations of testosterone serum were lower in all treated groups ($P < 0.001$; control = 154.5 ± 12.9 ng/dL; 5 mg/kg = 108.6 ± 19.6 ng/dL; 50 mg/dL = 84.5 ± 12.2 ng/dL; 250 mg/kg = 76.9 ± 14.2 ng/dL). These results suggest that commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Transorb
Active substance(s):	Glyphosate
Source:	Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil
Purity:	480 g/L of glyphosate (648 g/L as isopropylamine salt)
Lot/Batch #:	Not reported

2. Vehicle: Water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Not reported
Age of test animals at study initiation:	21 days
Sex:	Male
No. of rats:	68

Body weight:	Not reported
Acclimation period:	Not reported
Diet/Food:	Commercial balanced mixture for rats
Water:	Mineral water available *ad libitum*
Housing:	Not reported
Environmental conditions:	Temperature: 23 ± 1°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Evaluation of endocrine disruption potential of glyphosate formulation by assessment of rats prepubertal reproductive development.
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	From postnatal day (PND) 23 until PND53
Dose levels:	Control group – deionized water; 5, 50 or 250 mg/kg of body weight of glyphosate-Roundup Transorb
Animals per dose group:	4 treatment groups, 17 animals per group
Animal selection	No mention of avoiding selection of siblings within the same group to control for possible litter effects
Administration:	The glyphosate-Roundup Transorb was diluted in a watery suspension and administered once a day, by gavage; Dosing volume: 0.25 mL/100 g of body weight, Application time: between 7 and 8 a.m. each day

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	The experimental design was composed of random blocks, with the formation factor of these blocks as the body weight at the PND23. All the animals were weighed, and the average and standard deviation were calculated. The animals having body weights lower or higher than two standard deviations from the average were removed from the experiment.
Determination of puberty age:	Evaluation of the balanopreputial separation was made, which consists of the separation of the preputial membrane and the externalization from the glands of the penis. The assessment, which included gentle tissue manipulation, was performed once per day from the PND33 and was completed at the time of the balanopreputial separation. No discussion on whether this was a blinded procedure to avoid bias.
Hormone measurements:	Hormone concentrations of testosterone, estradiol and

	corticosterone in the serum were measured by radioimmuno-assay (RIA) from commercial kits (Testosterone Total Coat-A-Count, Estradiol Coat-A-Count and Coat-A-Count Corticosterone in rats, DPC, Los Angeles, CA, USA).
Food- and water consumptions:	Not reported
Haematology:	Not done
Clinical chemistry:	Not done
Urine analysis:	Not done
Sacrifice/pathology:	On PND 53. No details reported.
Organ weights:	The testes (right and left) and the adrenal glands (right and left) were weighed in absolute values and then transformed to relative weights as mg/100 g of body weight at PND53.
Histology and morphometry:	The testes and adrenal glands of all 68 animals werefixed in Bouin´s solution for 8 h, treated with alcohol, embedded in paraffin and prepared as stained laminas with hematoxylin and eosin. Laminas were analysed by light microscopy (40x and 100 x magnification). The linear morphometry from the seminiferous tubules were analysed by determining the tubular diameter (measured from the basal lamina to the basal lamina in the opposite direction), seminiferous epithelium (from the basal lamina to the neck of the elongated spermatids) and luminal diameter. Micrographs presented are of poor quality with artifacts such as shrinkage. Considered together with the natural variably in spermatogenesis of pubescent rats, the accuracy of morphometric data comes into question. For each tubule, the averages were calculated for the measurements indicated and, then, the average of each Weld was also calculated. The measurement for each animal was obtained through measure of all the analyzed Welds.
Statistics:	The variables under study were first submitted to tests of normality from Kolmogorov–Smirnov and homocedasticity by the test of Bartlet. When some of the premises of parametric testing were not obtained, non-parametric tests were chosen for subsequent averages and tests. Statistical differences were considered significant when the value of P was lower than 0.05. The values were expressed in mean (x) and standard error of the mean (±SEM). Data analysis of daily weights was performed through the two-way analysis of variance for repeated measures (MANOVA) by a general linear model (GLM). The weights were compared between diVerent groups and diVerent ages, considering the evolution expected by the body growth. The day and the weight of the complete balanoprepucial separation were compared among the groups using non-parametric analyses by the Kruskal–Wallis method followed by the post hoc Dun test. The testis and the adrenal weights were analyzed by the Kruskal–Wallis followed by the post hoc Dunn test, or by using a one-way analysis of variance (ANOVA) followed by the post hoc Tukey test. The testis measures of tubular diameter and

epithelium depth, as well as the serum concentrations of testosterone, estradiol and corticosterone, were analyzed by the ANOVA followed by the Tukey test.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Study with methodological and reporting deficiencies or conflicting findings. Eg, increased relative testicular weights, but decreased testosterone measurements.

2. Relevance of study: Relevant study type for investigating male reproductive endpoints, but questionable relevance of this specific study based on low reliability of data and interpretation. Test material was a formulated product, not glyphosate.

3. Klimisch code: **3**

A comprehensive review, pointing out a significant number of issues with this publication, was undertaken by experts in reproductive and developmental toxicology and endocrinology; William R. Kelce, M.S., Ph.D, Fellow ATS; James C. Lamb, IV, Ph.D, DABT and Fellow ATS; John M. DeSesso, Ph.D, Fellow ATS. Their critique is referenced in Doc L and included in Appendix K and their summary is quoted below.

> "To the uninformed reader, this manuscript by Romano et al. appears to demonstrate that exposure to Roundup Transorb alters testosterone levels and testis morphology. In this respect, the importance of these data to the scientific literature can be grossly over-interpreted by the uninformed reader. Upon closer examination, the authors have failed to provide robust data to support their conclusion that the "commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats". The authors failed to measure many of the key parameters in the validated pubertal male assay protocol by Stoker et al., (2000a) and hence generated data that were internally inconsistent, incomplete or in error. The results lack the scientific rigor necessary to support a definitive scientific conclusion and certainly do not equal or offset previous large, definitive and GLP-compliant studies concluding that Roundup and glyphosate do not affect reproductive development."

Author(s)	Year	Study title
Romano, M.A. Romano, R.M. Santos, L.D. Wisniewski, P. Campos, D.A. de Souza, P.B. Viau, P. Bernardi, M.M. Nunes, M.T. de Oliviera, C.A.	2012	Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression Archives of Toxicology Volume: 86 Number: 4 Pages: 663-673

Abstract*

Sexual differentiation in the brain takes place from late gestation to the early postnatal days. This is dependent on the conversion of circulating testosterone into estradiol by the enzyme aromatase. The glyphosate was shown to alter aromatase activity and decrease serum testosterone concentrations. Thus, the aim of this study was to investigate the effect of gestational maternal glyphosate exposure (50 mg/kg, NOAEL for reproductive toxicity) on the reproductive development of male offspring. Sixty-day-old male rat offspring were evaluated for sexual behavior and partner preference; serum testosterone concentrations, estradiol, FSH and LH; the mRNA and protein content of LH and FSH; sperm production and the morphology of the seminiferous epithelium; and the weight of the testes, epididymis and seminal vesicles. The growth, the weight and age at puberty of the animals were also recorded to evaluate the effect of the treatment. The most important findings were increases in sexual partner preference scores and the latency time to the first mount; testosterone and estradiol serum concentrations; the mRNA expression and protein content in the pituitary gland and the serum concentration of LH; sperm production and reserves; and the height of the germinal epithelium of seminiferous tubules. We also observed an early onset of puberty but no effect on the body growth in these animals. These results suggest that maternal exposure to glyphosate disturbed the masculinization process and promoted behavioral changes and histological and endocrine problems in reproductive parameters. These changes associated with the hypersecretion of androgens increased gonadal activity and sperm production.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Transorb
Active substance(s):	Glyphosate (isopropylamine salt)
Source:	Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil
Purity:	480 g/L of glyphosate (648 g/L isopropylamine salt)
Lot/Batch #:	Not reported

2. Vehicle: Water

3. Test animals:

Species:	Rat
Strain:	Wistar
Source:	Not reported

Age of test animals at study initiation:	90 days
Sex:	Female
No. of rats:	12
Body weight:	Not reported
Acclimation period:	Not reported
Diet/Food:	Commercial balanced mixture for rats, *ad libitum*
Water:	Water available *ad libitum*
Housing:	Not reported
Environmental conditions:	Temperature: 23 ± 1°C Humidity: Not reported Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Glyphosate effects on the reproductive development of male offspring
Guideline:	Non-guideline study
GLP:	No
Guideline deviations:	Not applicable
Duration of exposure:	From gestational day 18 to postnatal day (PND) 5
Dose levels:	Control group – deionised water; 50 mg/kg bw of glyphosate
Animals per dose group:	2 treatment groups, animals per group – not reported
Administration:	Roundup Transorb was diluted in a watery suspension and administered once a day by gavage from Gestation Day 18 to Post Natal day 5; Dosing volume: 0.25 mL/100 g bw, Application time: between 7 and 8 a.m. each day

5. Observations/analyses:

Test substance preparations:	Stability, achieved concentrations, homogeneity not reported
Mortality:	Not reported
Clinical signs:	Not reported
Body weight:	The pups were weighted at PND21 (weaning), PND30, PND40 and PND60 to compare the body growth between the groups.
Sexual partner preference:	The sexual partner preference was assessed at PND 60 by exposing male offspring from treated and non-treated mothers to female stimulus rats (i.e. ovariectomised female rats that were treated with estradiol (50 µg/kg bw s.a. 54 h before the test) and progesterone (2 mg/kg bw s.c., 6 h before test)).
Sexual behaviour:	Sexual behaviour was assessed at PND 60 by exposing the male rats to an oestrus-induced female for 40 min. Several parameters were assessed incl. Number of mounts, intromission, ejaculatory intervals, number of attempted mounts).
Determination of puberty age:	Evaluation of the balanopreputial separation (separation of the preputial membrane and externalization from the glands of the

	penis). The assessment (including gentle tissue manipulation) was performed once per day from the PND33 and was completed at the time of the balanopreputial separation.
Hormone measurements:	Hormone serum concentrations of testosterone, estradiol in the serum were measured by radioimmunoassay-assay (RIA) from commercial kits (Coat-A-Count, DPC, Los Angeles, CA, USA). The serum FSH and LH measurements were determined by chemiluminescence immunoassay using Luminex xMAP technology (Milliplex MAP rat pituitary panel, Billerica, MA, USA).
Pituitary hormone levels:	mRNA-levels of LH, FSH and GH were assessed by real-time PCR in homogenised pituitary tissues. Protein expression of LH, FSH and GH was assessed in homogenised pituitary tissues using Western-blot analysis.
Food- and water consumptions:	Not reported
Haematology:	Not reported
Clinical chemistry:	Not reported
Urine analysis:	Not reported
Sacrifice/pathology:	Not reported
Organ weights:	The testes, epididymides and seminal vesicle were weighed, and the values were converted to relative weights of mg/100 g bw at PND60. The epididymis was previously divided into three segments: caput, corpus and cauda. The seminal vesicle was weighted with fluid (undrained) and after fluid removal (drained).
Sperm evaluation:	At PND 60, the sperm counts were determined. Testes and epididymes (capus, corpus, cauda) were weighed. The tunica albuginea was removed from the testes, and the parenchyma was homogenized. The samples were then diluted 10 times in saline, and the mature spermatids resistant to homogenization were counted using a hemocytometer. Daily sperm production was calculated. The segments of the epididymis were cut with a scissor, homogenized, diluted and counted. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for the parts of the epididymis were calculated. The mean time for sperm transit through the epididymis was calculated.
Histology and morphometry:	The testes were fixed in Bouin's solution for 8 h, treated with alcohol and embedded in paraffin, and were prepared as stained laminas with hematoxylin and eosin. Laminas were analysed by light microscopy (40x and 100 x magnification). Linear morphometry of the seminiferous tubules were analyzed by determining the tubular diameter, seminiferous epithelium length and luminal diameter. For each tubule, the averages were calculated for the measurements indicated, and the average of each field was also calculated. The measurement for each animal was obtained by measuring all the analyzed fields.
Statistics:	First the Kolmogorov–Smirnov tests for normality and the

Bartlett test for homoscedasticity. For analysis of body growth the multi-way analysis of variance for repeated measures (MANOVA) by a general linear model (GLM) was used. Weights were compared between different groups and ages, considering the expected changes with age. The sexual behavior and day of PPS were compared among the groups using the Mann–Whitney *U* test. Weights of seminal vesicle (drained and undrained) were compared by paired Student's *t-test.* All other parameters were analyzed by Student's *t-test*. Statistical differences were considered significant when the value of P was < 0.05. Values were expressed as means and the standard error of the mean (±SEM) for parametric and interquartile ranges of nonparametric analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Non-guideline, non-GLP study meeting scientific principles. Unusual and short dosing regiment commencing towards the end of pregnancy (GD18, rather than GD6 as per OECD Test Guidelines 414) through post natal day 5. *In vivo* study with reporting deficiencies (detailed strain description, source of animals, housing conditions, no information if clinical signs were assessed, stability and homogeneity assessment of test substance preparations, no of male offspring evaluated in individual tests evaluated). A number of atypical endpoints evaluated.

2. Relevance of study: Not relevant (due to questionable dosing regimen and atypical array of endpoints measured

3. Klimisch code: **3**

This quality and value of this follow up study to Romano et al. (2010) is consistent with their previous publication. Selective literature citations in the introduction frame the basis for this research as endocrine disruption potential, referring mostly to the publications from Seralini laboratory, previously discussed. The concluding sentences inaccurately cite published *in vitro* research (Richard et al., 2005) as evidence that "women occupationally exposed to this herbicide have reproductive disorders".

From the outset, the study design and endpoint selection are not consistent with other research in the field of developmental and reproductive toxicology, suggesting a lack of experience in this well published and studied discipline. Dosing was very limited to dams, starting on gestation day 18, well after organogenisis, through post natal day 5. No controls for litter effects appear to be reported, confounding interpretation of results.

Any glyphosate exposure to offspring either before or after parturition is questionable. ADME studies with glyphosate clearly demonstrate poor absorbtion via the gastrointestinal tract, rapid excretion of systemic doses via urine and a lack of bioaccumulation. Restricted placental transfer for glyphosate is documented in an *ex vivo* human perfusion system, in which the three other compounds tested (caffeine, benzoic acid and antipyrine) demonstrated much greater transfer kinetics across the placenta (Mose et al., 2008). Given the physico-chemical properties and *in vivo* kinetics of glyphosate, exposure to offspring during lactation should be considered negligible, if any.

With the very short window of maternal exposure, biological plausibility of any test substance related effects in the mature offspring is questionable. However, the normal variability of some unusual or atypical endpoint measurements, such as "sexual partner preference" along with mRNA and protein expression, is not known. Of particular concern, however, are differences in critical endpoints for control animals reported in Romano et al. (2010) compared to Romano et al. (2012); these include increased day of preputional sepratarion (PPS) of control male rate (37 days in 2010; 47 days in 2012), body weight at day of PPS (146 grams in 2010; 245 grams in 2012), serum testosterone concentrations (155 ng/dL in 2010; 63 ng/dL in 2012), estradiol concentrations (32 pg/mL in 2010; 1.4 pg/mL in 2012), subular diameter (266 μm in 2010; 479 μm in 2012), epithelial height (86 μm in 2010; 92 μmin 2012) and luminal height (94 μm in 2010; 257μm in 2012). Therefore, results are difficult to interpret, particularly for relevance to human health risk assessment. The merits of this publication should be placed in context with the quality of the authors' previous published research (Romano et al., 2010), as critiqued by experts in DART and ED above.

EPIDEMIOLOGY DART/ED PUBLICATIONS

Author(s)	Year	Study title
Arbuckle, T. E. Lin, Z. Mery, L. S.	2001	An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population Environmental Health Perspectives Volume: 109 Pages: 851-857

Abstract*

The toxicity of pesticides on human reproduction is largely unknown—particularly how mixtures of pesticide products might affect fetal toxicity. The Ontario Farm Family Health Study collected data by questionnaire on the identity and timing of pesticide use on the farm, lifestyle factors, and a complete reproductive history from the farm operator and eligible couples living on the farm. A total of 2,110 women provided information on 3,936 pregnancies, including 395 spontaneous abortions. To explore critical windows of exposure and target sites for toxicity, we examined exposures separately for preconception (3 months before and up to month of conception) and postconception (first trimester) windows and for early (< 12 weeks) and late (12–19 weeks) spontaneous abortions. We observed moderate increases in risk of early abortions for preconception exposures to phenoxy acetic acid herbicides [odds ratio (OR) = 1.5; 95% confidence interval (CI), 1.1–2.1], triazines (OR = 1.4; 95% CI, 1.0–2.0), and any herbicide (OR = 1.4; 95% CI, 1.1–1.9). For late abortions, preconception exposure to glyphosate (OR = 1.7; 95% CI, 1.0–2.9), thiocarbamates (OR = 1.8; 95% CI, 1.1–3.0), and the miscellaneous class of pesticides (OR = 1.5; 95% CI, 1.0–2.4) was associated with elevated risks. Postconception exposures were generally associated with late spontaneous abortions. Older maternal age (> 34 years of age) was the strongest risk factor for spontaneous abortions, and we observed several interactions between pesticides in the older age group using Classification and Regression Tree analysis. This study shows that timing of exposure and restricting analyses to more homogeneous endpoints are important in characterizing the reproductive toxicity of pesticides.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various pesticides (herbicides, insecticides, fungicides, miscellaneous)
Active substance(s):	Dicamba, glyphosate, 2,4-DB, 2,4-D, MCPA, atrazine, cyanazine, carbaryl, captan
Chemical families:	Phenoxy acetic acid (phenoxy herbicides), triazine, organophosphates, thiocarbamate

2. Vehicle and/or positive control: Not applicable

3. Test group:

Number of test persons:	2110
Age:	≤ 44 years
Sex:	Females
Inclusion criteria:	The couple had to be living year round on the study farm; The wife had to be 44 years of age or younger; At least one member of the couple had to be working on the farm

4. Test system:

Type:	Retrospective epidemiological study
Collection of data:	Questionnaire
Guideline:	Non-guideline study
GLP / GCP:	no

5. Observations / analyses:

Information in questionnaires:	Demographic and lifestyle information; Pesticides currently and historically used on the farm and around the home; Medical history; Complete reproductive history farm activities
Outcome of interest in analysis:	Self reported spontaneous abortion of less than 20 weeks' gestation
Subgroups created:	Spontaneous abortions of less than 12 weeks' and 12–19 weeks' gestation
Pesticide use:	Information from the farm operator husband, and wife to construct a history of monthly agricultural and residential pesticide use
Identification of pesticides:	Using a database of registered pesticide products in Canada
Dose levels:	Not reported
Grouping of pesticides:	Major classes of use: herbicides, insecticides, fungicides, and miscellaneous others (including those that could not be classified)
Number of reported pregnancies:	3936
Number of reported spontaneous abortions:	395
Number of reported early abortions:	226
Number of reported late abortions:	169
Analysed exposure to pesticides:	For two windows: - pre-conception, the 4-month period from 3 months before conception to the calendar month of conception (consistent with potential sperm-mediated effects); - post-conception, the 3-month period from the first calendar month after conception to the end of the first trimester (consistent with a fetotoxic effect)
Number of pesticide variables:	17
Number of possible risk factors for spontaneous abortions:	21
Statistics:	Crude odds ratios (ORs) using logistic regression for each combination of pesticide unit, exposure window, and gestational age at abortion category. To explore statistical interactions between the various pesticide units and other risk factors for spontaneous abortion, we used the Classification and Regression Tree (CART) method.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: No information about exposure duration, used glyphosate products and application rates. No information, if the subjects used more than one pesticide. Due to study design and evaluation methods, study results are not reliable.

2. Relevance of study: **Not relevant** (Study design is not suitable for assessment of glyphosate exposure).

3. Klimisch code: **3**

Response 1 – GTF

- This publication reports an "exploratory analysis" of pesticide exposure timing as a possible risk factor for spontaneous abortion.
- Pre-conception glyphosate exposure odds ratio for spontaneous abortion is considered of borderline significance (OR = 1.4).
- Post-conception glyphosate exposure was not associated with spontantous abortin (OR = 1.1).
- Authors note multiple limitations of the study relating to exposure
    - likely misclassification of pesticides
    - correct assignment of exposure window to pre- or/and post-conception
- This is one of several publications arising from the Ontario Farm Family Health Study (OFFHS), in which farm couples were asked to recall on-farm activities and pesticide usage over the last 5 years. Participants were also asked to recall all pregnancy outcomes (38% of which occurred more than 10 years earlier). This information was gathered via mail questionnaires with telephone follow-up for non-respondents.
- OFFHS information gathering methodology has high potential recall bias. Blair and Zahm (1993; referenced in Doc L, available in Doc K) report 60% accuracy when comparing self reported pesticide usage with purchasing records.
- OFFHS relied exclusively on maternal self-reports of adverse pregnancy outcomes, not all of which were confirmed via medical or other records.
- Three highly relevant confounding factors were not considered in the OFFHS questionnaire
    - history of previous spontaneous abortion(s);
    - maternal age; and
    - smoking.
- Lack of control for putative pesticide effect(s) and consideration use of multiple pesticides further compromise the utility of the data set.
- Arbuckle et al. (2001) Reported findings linked preconception use of phenosyacetic acids, triazines, glyphosate and thiocarbamates with weak but stastically significant spontaneous abortions.
- Authors considered the findings reported "hypothesis generating", and cautioned that "results should be interpreted with care and tested in other studies".
-

Response 2 – Summarized from Williams et al. (2012)

- 395 spontaneous abortions were reported out of 3936 pregnancies; rate of spontaneous aborting in Arbuckle et al. (2001) was 10%.
- The baseline rate of spontaneous abortions in the general populations is much higher, ranging from 12% to 25%.
- Recall bias is reflected in the recall of spontaneous abortion over the previous 5 years (64% of all spontaneous abortions reported) being much higher than the recall of those greater than 10 years prior to the survey (34% of all spontaneous abortions reported).
- Substantial exposure misclassification may have occurred (pre- versus post-conception) due to likely author extrapolation of exposure data.
- Strong confounding variables are not apparent in previous data analyses published by the authors of the OFFHS, and therefore odds ratios are crude.

- Published results fail to demonstrate a significant association of glyphosate exposure spontaneous abortion risk and therefore must be considered cautiously.

Author(s)	Year	Study title
Savitz, D.A. Arbuckle, T. Kaczor, D. Curtis, K.M.	1997	Male pesticide exposure and pregnancy outcome. American Journal of Epidemiology Volume: 146 Number: 12 Pages: 1025-1036

Abstract*

Potential health effects of agricultural pesticide use include reproductive outcomes. For the Ontario Farm Family Health Study, the authors sampled Ontario farms from the 1986 Canadian Census of Agriculture, identified farm couples, and obtained questionnaire data concerning farm activities, reproductive health experience, and chemical applications. Male farm activities in the period from 3 months before conception through the month of conception were evaluated in relation to miscarriage, preterm delivery, and small-for-gestational-age births. Among the 1,898 couples with complete data (64% response), 3,984 eligible pregnancies were identified. Miscarriage was not associated with chemical activities overall but was increased in combination with reported use of thiocarbamates, carbaryl, and unclassified pesticides on the farm. Preterm delivery was also not strongly associated with farm chemical activities overall, except for mixing or applying yard herbicides (odds ratio = 2.1, 95% confidence interval 1.0-4.4), Combinations of activities with a variety of chemicals (atrazine, glyphosate, organophosphates, 4-[2,4-dichlorophenoxy] butyric acid, and insecticides) generated odds ratios of two or greater. No associations were found between farm chemicals and small-for-gestational-age births or altered sex ratio. Based on these data, despite limitations in exposure assessment, the authors encourage continued evaluation of male exposures, particularly in relation to miscarriage and preterm delivery.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various pesticides (herbicides, insecticides, fungicides, livestock chemicals)
Active substance(s):	Glyphosate, atrazine, 2,4-DB, 2,4-D, MCPA, dicamba, carbaryl, and other pesticides
Chemical families:	Phenoxy herbicides, thiocarbamates, organophosphates, triazines

2. Vehicle and/or positive control: Not applicable

3. Test group:

Number of test persons:	2964 couples (initial inclusion) 1898 couples (complete response)
Age:	Females: ≤ 44 years
Sex:	Males/females
Inclusion criteria:	The couple had to be living year round on the study farm; The wife had to be 44 years of age or younger; At least one member of the couple had to be working on the farm
No. of pregnancies analyzed:	3984
Miscarriage cases due to glyphosate:	17

4. Test system:

Type:	Retrospective epidemiological study
Collection of data:	Questionnaire, telephone interview, interview
Guideline:	Non-guideline study
GLP / GCP:	no

5. Observations/analyses:

Information in questionnaires:	Mother's and father's age, education, jobs outside the farm (classified as potentially hazardous or nonhazardous), tobacco use, alcohol use, caffeine use, mother's language, ethnicity, religion, parity, per capita income, child's sex, interval between conception and the survey, and the month of conception.
Classification of pregnancies:	Singleton live births, multiple gestations (twins, triplets), miscarriage (recognized pregnancy loss before 20 weeks of completed gestation), stillbirth (pregnancy loss at 20 or more weeks of completed gestation), medically induced abortion, currently pregnant, or other (ectopic pregnancy, hydatidiform mole, unknown).
Criteria for classification of pregnancies:	Singleton live births were classified as preterm if they occurred before the completion of 37 weeks of gestation and small for gestational age (SGA) if they fell below the 10^{th} percentile of birth weight for gestational age based on Canadian percentiles. Sex ratio was defined as the proportion of males among singleton live births.
Analyzed outcomes of pregnancies:	Risk of miscarriage (pregnancies ending in miscarriages, singleton live births, induced abortions, and stillbirths, as well as current pregnancies of 20 or more weeks of gestation), preterm delivery (all live births and current pregnancies of 37 or more weeks of gestational age), and SGA births (all live births of known weight and gestational age), as well as sex ratio (all live births of known sex), not addressing stillbirths and other more rare outcomes due to insufficient numbers for analysis.
Farm activities:	Over the past 5 years; for each reported activity, months of the year were asked
Activities that involve direct pesticide exposure:	Mixing or applying crop herbicides, crop insecticides and fungicides, livestock chemicals, yard herbicides, and building pesticides.
Man's exposure classification:	Based on man's experiences in the time window of 3 months before conception to the time of conception. During that time window, specific to each pregnancy, we first determined whether he had engaged in any activities associated with direct pesticide exposure for 1 or more months. Defined 2 groups of activities: - chemical activity; - nonchemical activity + no activity.
Use of protective equipment:	Information gathered on date of use, but not specified to each of the chemical activities.
Pesticide use:	Information from farm operator (who may or may not have been the male partner) regarding the application of specific pesticides on the farm in the time period of interest.

Data analysis: Unadjusted risk ratios between the potential confounders and each of the four outcomes (miscarriage, preterm delivery, SGA, and sex ratio) were calculated, starting with finely stratified exposure variables. Based on the pattern of crude results, variables were eliminated and categories of variables were collapsed to retain only those variables and strata that yielded risk ratios of less than 0.8 or greater than 1.2. For each of the pregnancy outcomes, a logistic regression model was constructed that used the reduced set of variables and category levels. Additional variables were eliminated from the logistic regression models, and categories were collapsed or converted to continuous variables as appropriate.
For each of the four outcomes, risks and relative risks were generated, contrasting exposed to unexposed groups. Men with no activity or no chemical activity served as the referent, with various subsets of men defined by activity, use of protective equipment, and farm chemical use constituting the exposed groups. Adjusted odds ratios were calculated using logistic regression models with all the predictors of each outcome described above along with the exposure of interest.
Because multiple pregnancies per woman were included, the variance estimates from the logistic regression are expected to be slightly underestimated on average. Several logistic regression analyses were conducted based on generalized estimating equations, which account for the within-woman correlation across pregnancies.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: No information about exposure duration, used glyphosate products and application rates. No information, if the subjects used more than one pesticide. Due to study design and evaluation methods, study results are not reliable.

2. Relevance of study: **Not Relevant** (Study design is not suitable for assessment of glyphosate exposure).

3. Klimisch code: **3**

Response to Savitz, Arbuckle and the Onratio Farm Family Health Study (OFFHS) taken from monsanto.com
http://www.monsanto.com/products/Documents/glyphosate-background-materials/gly_reprooutcomes_bkg.pdf

Glyphosate is one of many pesticides mentioned in three epidemiological reports that examine possible links between on-farm pesticide use and reproductive outcomes. All three reports - Savitz et *al.* (1997) [category 'E' in this literature review], Curtis et *al.* (1999) [outside the scope of this literature review as per the introduction describing literature review categories], and Arbuckle et *al.* (2001) [previously reviewed publication] - use data from the Ontario Farm Family Health Study (OFFHS) (Arbuckle 1994). Savitz *et al.* (1997) investigated associations between reported pesticide use by males and pregnancy outcomes, specifically: miscarriage, pre-term delivery and small-for-gestational-age birth. Curtis *et al.* (1999) studied whether reported pesticide use by males or females was associated with delayed pregnancy,

while Arbuckle *et al.* (2001) looked for associations between reported pesticide use and spontaneous abortion.

The OFFHS was a questionnaire-type study in which farm couples were asked to recall on-farm activities and pesticide usage on the farm during the previous 5 years. They were also asked to recall all pregnancy outcomes, 38% of which occurred more than 10 years before the survey. The farm couples lived year-round on a farm and the OFFHS investigators employed mail questionnaires to collect information about pregnancy outcomes from the mothers. Telephone follow-up was employed for non-respondents.

In the study by Savitz et *al.,* a number of specific pesticides had weak statistical associations with miscarriages and pre-term deliveries, but pesticides tended not to be associated with small for gestational age births. There were no statistically significant findings for glyphosate. In the study by Curtis *et al.,* for farms on which glyphosate was used, there was no significant association for women being engaged in pesticide activities. For men, glyphosate use was associated with a slight, but statistically significant, decrease in time to pregnancy. The authors dismissed this finding, which was contrary to their hypothesis that pesticide exposure delayed pregnancy, as probably due to uncontrolled factors or chance. Arbuckle *et al.* (2001) found that reported preconception use of phenoxyacetic acids, triazines, glyphosate, and thiocarbamates were weakly, but statistically significantly, associated with spontaneous abortions. Post conception reported use was not associated with increased risk. The authors characterized the associations between pesticides and spontaneous abortions as "hypothesis generating" pending confirmation from other epidemiologic studies.

These studies are not convincing evidence of a relationship between glyphosate exposure and adverse pregnancy outcomes for a number of reasons:

1. Uncertainty about exposure
There was no actual exposure data per se in these three epidemiologic studies. Exposures were assumed based on questionnaire responses by study subjects about farm activities and pesticide use. This type of information can be inaccurate. For example, according to a study by the National Cancer Institute, self-reports of pesticide usage were found to be only 60 percent accurate when compared with purchasing records (Blair & Zahm 1993). Further increasing the potential for inaccuracy is the fact that study subjects were only asked about pesticide use for the 5 years before the OFFS survey. These responses were assumed to be applicable to the entire farming careers of study subjects, an assumption inconsistent with changes in agricultural practice. Lastly, basing exposure estimation on questionnaire responses has the potential to be influenced by what epidemiologists call "recall bias." This refers to the likelihood that families that experienced an adverse reproductive outcome are more likely to remember use of certain pesticides than families that had only normal births.
The most widely used pesticides, like atrazine, glyphosate, and 2,4-D, are most easily recalled and most likely to be over-reported.

2. Low biological plausibility
Biologic plausibility is an important criterion for deciding whether a reported statistical association between a pesticide and a disease is likely to be valid. Glyphosate, even at very high doses in chronic feeding studies, does not cause adverse reproductive outcomes in laboratory animals (USEPA 1993, WHO 1994). This makes statistical associations from epidemiologic studies less plausible.

3. Inaccuracy of reported pregnancy outcomes
The OFFHS study relied exclusively on maternal self-reports of adverse pregnancy outcomes with no medical or other validation. Generally, scientists place less confidence in reports of health outcomes that are not validated with medical records.

4. Confounding
A confounding factor is a cause of a disease that is correlated with another exposure being studied. Failure to control confounding factors, especially those that are strong causes of a disease, can create spurious associations between benign exposures and diseases. In the Arbuckle study, there were at least three

important potential confounding factors that were not controlled: history of previous spontaneous abortion, maternal age, and smoking. Even a weak correlation between these factors and use (or recall of use) of pesticides would produce spurious associations. In addition, in all three studies, the authors did not control the putative effect of one pesticide for the putative effects of other pesticides. So, for example, since farmers tend to use 4 or more pesticides each year, a disease that is associated with one pesticide will likely be associated with all, since their use patterns are correlated. In the absence of an analysis that controls for multiple pesticides, the best that can be said is that the findings for any individual pesticide might be due to its correlation with another pesticide.

In summary, three publications based on data collected in the OFFHS found associations between several pesticides and various adverse reproductive outcomes. There was no actual exposure data per se in these three epidemiologic studies. Exposures were assumed based on questionnaire responses by study subjects about farm activities and pesticide use. This type of information can be inaccurate. Glyphosate was not significantly associated with adverse reproductive outcomes in two of these studies (Savitz *et al.* 1997, Curtis *et al.* 1999). Glyphosate and other pesticides were weakly associated with spontaneous abortion in the study by Arbuckle (2001). However, the author did not control for important personal confounding factors or for multiple exposures and no actual exposure data was used, casting doubt on the validity of the findings in this study.

Biomonitoring data for glyphosate, collected as part of the Farm Family Exposure Study (FFES), provide assurance that human health effects related to glyphosate exposure are very unlikely. In the FFES, researchers from the University of Minnesota collected 5 days of urine samples from 48 farm families before, during, and after a glyphosate application (Mandel *et al.,* accepted for publication). Only 60% of farmers showed detectable exposure to glyphosate, with a 1 part per billion limit of detection, and the maximum estimated absorbed dose was 0.004 *mg/kg* (Acquavella *et al.,* 2004). For farmers who apply glyphosate 10 times per year for 40 years, this maximum dose is more than 30,000-fold less than the EPA reference dose1 of 2 mg/kg/day. For spouses, only 4% showed detectable exposures and the maximum systemic dose was 0.00004 mg/kg/day. Since glyphosate is not a reproductive toxic in high dose animal studies (USEPA 1993, WHO 1994) and since actual exposures on farms are so low, it is very unlikely that glyphosate would cause adverse reproductive outcomes for farmers or their spouses.

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Author(s)	Year	Study title
Garry, V. F. Harkins, M. E. Erickson, L. L. Long-Simpson, L. K. Holland, S. E. Burroughs, B. L.	2002b	Birth defects, season of conception, and sex of children born to pesticide applicators living in the Red River Valley of Minnesota, USA. Environmental Health Perspectives Volume: 110 Pages: 441-449

Abstract*

We previously demonstrated that the frequency of birth defects among children of residents of the Red River Valley (RRV), Minnesota, USA, was significantly higher than in other major agricultural regions of the state during the years 1989-1991, with children born to male pesticide applicators having the highest risk. The present, smaller cross-sectional study of 695 families and 1,532 children, conducted during 1997-1998, provides a more detailed examination of reproductive health outcomes in farm families ascertained from parent-reported birth defects. In the present study, in the first year of life, the birth defect rate was 31.3 births per 1,000, with 83% of the total reported birth defects confirmed by medical records. Inclusion of children identified with birth or developmental disorders within the first 3 years of life and later led to a rate of 47.0 per 1,000 (72 children from 1,532 live births). Conceptions in spring resulted in significantly more children with birth defects than found in any other season (7.6 vs. 3.7%). Twelve families had more than one child with a birth defect (n = 28 children). Forty-two percent of the children from families with recurrent birth defects were conceived in spring, a significantly higher rate than that for any other season. Three families in the kinships defined contributed a first-degree relative other than a sibling with the same or similar birth defect, consistent with a Mendelian inheritance pattern. The remaining nine families did not follow a Mendelian inheritance pattern. The sex ratio of children with birth defects born to applicator families shows a male predominance (1.75 to 1) across specific pesticide class use and exposure categories exclusive of fungicides. In the fungicide exposure category, normal female births significantly exceed male births (1.25 to 1). Similarly, the proportion of male to female children with birth defects is significantly lower (0.57 to 1; p = 0.02). Adverse neurologic and neurobehavioral developmental effects clustered among the children born to applicators of the fumigant phosphine (odds ratio [OR] = 2.48; confidence interval [CI], 1.2-5. 1). Use of the herbicide glyphosate yielded an OR of 3.6 (CI, 1.3-9.6) in the neurobehavioral category. Finally, these studies point out that a) herbicides applied in the spring may be a factor in the birth defects observed and b) fungicides can be a significant factor in the determination of sex of the children of the families of the RRV. Thus, two distinct classes of pesticides seem to have adverse effects on different reproductive outcomes. Biologically based confirmatory studies are needed.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Herbicides, insecticides, fumigants, fungicides
Active substance(s):	At least 15 different substances that were not further specified. (Only pesticide classes were assessed)
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Age of test persons:	Not reported
Sex:	Males and females

4. Test system:

Study type:	Epidemological study for the assessment of birth defects, season of conception, and sex of children born to pesticide applicators living in the Red River Valley of Minnesota, USA.
Collection of data:	Interview and questionnaire
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Inclusion criteria:	Farm families with live births fathered by a pesticide applicator
No. of live births with birth defects:	1532
No. of family participants:	695
No. of family with children:	536
No. of control persons:	None

5. Observations/analyses:

Working history:	All subjects
Detailed assessment of exposure:	Confounding variables such as maternal smoking, drinking, age, and chronic diseases such as diabetes and hypertension were examined. In this retrospective study, where possible, familial genetic history (pedigree), pregnancy medication use, and nonmedicinal drug use (including vitamins) were assessed in families with birth defects. Each certified pesticide applicator was initially interviewed by phone regarding current and past pesticide use in agriculture with specific attention to product name, years used, and the number of days per year applied. Approximately 6 months later, where possible, the subject was re-interviewed by written questionnaire to document common pesticide use by pesticide class, acreage treated, type of crop, and use of personal protective gear. Overlap between the two questionnaires was intentional to validate use of pesticides by class (herbicides, insecticides, fumigants, fungicides).
Statistics:	Regression analysis, two-sided *t*-tests, and analysis of variance methods were employed. Variables considered for regression analysis included mother's age, smoking status, alcohol use, and season of conception. Chronic diseases such as diabetes, pharmacologically treated hypertension, and arthritis and occupations other than agriculture were considered separately. Specific medication use during pregnancy and dietary information were not considered in our survey. Residence at a rural site (towns with populations <3,000) or on a farm during childhood (<18 years of age) was considered a factor in some

of these statistical analyses.

Conditional logistic regression analysis for matched studies was performed with SAS statistical program. Odds ratios and 95% confidence intervals were obtained. Both univariate and multivariate analyses were done. In the pooled analysis an adjustment was made for study, study area and vital status. When risk estimates for different pesticide exposures were analysed only subjects with no pesticide exposure were taken as unexposed, whereas subjects exposed to other pesticides were disregarded.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Epidemiological study with some methodological / reporting deficiencies (selection of study subjects, no information about exposure duration, exposure concentration, pesticide use frequency).

2. Relevance of study: **Not relevant** (Glyphosate not mentioned.)

3. Klimisch code: **3**

Response 1 – summary from Mink et al. (2011)

- Publication reports on different classes of pesticides and several birth defects and developmental outcomes.
- Paternal use of glyphosate was associated with parent-reported ADD/ADHD in children (OR = 3.6). Six out of 14 children with parent reported ADD/ADHD also reported exposure to glyphosate.
- Diagnoses of ADD/AHDH were not all confirmed. However, overall rate for the sample population (14/1532) was well below ADD/ADHD rates for the general population (7%).
- Variables in statistical model analyses were not reported.

Response 2 – summary from Williams et al. (2012)

- Health data obtained via parent reporting for 695 families via written questionnaire and confirmed where possible.
- Pesticide use information obtained initially via telephone then followed up by written questionnaire.
- Reproductive health outcomes for births occurring between 1968 and 1998 were obtained for 1532 live births. Over half the births occurred prior to 1978, approximately 20 years after study initiation.
- All pesticide use classes (herbicide only; herbicide and insecticide; herbicide, insecticide and fungicide; herbicide, insecticide and fumigant) were associated with birth defects.
- Authors state neurobehavioral disorder would not be considered based lack consistent diagnoses. However, a detailed analysis was conducted for ADD/ADHD.
- 43% (6/14) parent reported children with ADD/ADHD were associated with glyphosate formulation use.
- 14 cases of ADD/ADHD reported out of 1532 live births, which is substantially lower that the diagnosed incidence of 7% for the general population.
- No conclusions regarding glyphosate exposure and ADD/ADHD outcome can be drawn.
- No other glyphosate specific data were reported.

Author(s)	Year	Study title
Garry, V.F., Holland, S.E., Erickson, L.L., Burroughs, B.L.	2003	Male Reproductive Hormones and Thyroid Function in Pesticide Applicators in the Red River Valley of Minnesota Journal of Toxicology and Environmental Health, Part A Volume: 66 Number: 11 Pages: 965-986

Abstract*

In the present effort, 144 pesticide applicators and 49 urban control subjects who reported no chronic disease were studied. Applicators provided records of the season's pesticides used by product, volumes, dates, and methods of application. Blood specimens for examination of hormone levels were obtained in summer and fall. In the herbicide-only applicator group, significant increases in testosterone levels in fall compared to summer and also elevated levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the fall were noted. With respect to fungicide use, in an earlier cross-sectional epidemiologic study, data demonstrated that historic fungicide use was associated with a significant alteration of the sex ratio of children borne to applicators. As before, among current study subjects it was noted that historic fungicide use was associated with increased numbers of girls being born. Lower mean total testosterone concentrations by quartile were also correlated with increased numbers of live-born female infants. A downward summer to fall seasonal shift in thyroid-stimulating hormone (TSH) concentrations occurred among applicators but not among controls. Farmers who had aerial application of fungicides to their land in the current season showed a significant shift in TSH values (from 1.75 to 1.11 mU/L). Subclinical hypothyroidism was noted in 5/144 applicators (TSH values >4.5 mU/L), but not in urban control subjects. Based on current and past studies, it was concluded that, in addition to pesticide exposure, individual susceptibility and perhaps economic factors may play a supporting role in the reported results.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various herbicides, fungicides, and insecticides
Active substance(s):	Various active substances that were not specified in detail
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: None

3. Test group:

Species:	Human
Age of test persons:	Exposed group to herbicides: 43.5 y; non-exposed (no pesticide use during the relevant application season): 43.0 y; non-exposed (control): 41.8 y
Sex:	Exposed group: 144 males non-exposed group: 49 males

4. Test system:

Study type:	Epidemiological study to determine male reproductive hormones and thyroid function in pesticide applicators
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Data collection:	Interview and questionnaire
Duration of study:	Not specified
Application rate:	Not reported
Persons per group:	144 exposed; 49 non-exposed (control)
Application technique:	Ground, aerial, manual, and custom ground spraying and seed treatment
Test conditions:	The test group consisted of 144 randomly selected applicators residing in the Red River Valley (RRV). Exposure occurred during the applications. The non-exposed group consisted of 49 individuals selected as the volunteers from the community blood bank. Non-exposed controls were matched by age, health, and smoking status with the pesticide applicators. Control samples taken in summer and in fall were from different subjects!
Inclusion criteria:	No chronic disease, no chronic medication, herbicide use frequency >10 day/year, no use of fungicides, or fungicide use of < 5 days/year, no or < 5 agricultural pesticide application during the last year
Blood sampling:	Exposed group: blood samples were collected in summer and fall. Non-exposed group: Blood samples were collected and processed as for the exposed group.

5. Observations/analyses:

Clinical history:	All subjects
Exposure assessment:	Past pesticide use (incl. use frequency, application technique); During and at the end of the application season , detailed assessment of used pesticides, application rates, use frequency, application techniques etc., as well as a reproductive health history of the family.
Clinical signs:	Not performed
Body weight:	Not performed
Haematology:	Not performed.
Clinical chemistry:	LH and FSH levels; Total and free testosterone levels; TSH, total and free T4 levels
Urine analysis:	Not performed.
Other:	Offspring gender ratio
Statistics:	Urban control and pesticide applicator subject comparison groups were matched by age (within 5 yr) and smoking status. Within-group hormonal measurements from summer and fall were compared using paired t-tests. Between-group

comparisons were conducted using two-tailed t-tests for significance. The criterion for significance was set at $p<0.05$. Applicators and control subjects whose values exceeded the established normal clinical range (outliers) for these hormones were treated separately in our analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Epidemiological study with some methodological / reporting deficiencies (e.g. selection of control subjects/samples, no details of exposure). Documentation is insufficient for assessment.

2. Relevance of study: Not relevant (Due to reliability. In addition, no direct assessment of glyphosate exposure was made).

3. Klimisch code: **3**

Response – GTF

- The publication brings little if any information on endpoints attributable to glyphosate.
- Given the subjects were pesticide applicators, little can be drawn from the findings other than perhaps certain endpoints which may be associated with this specific occupation exposed to multiple chemical substances.
- Of the 136 participants volunteering blood samples, only one individual (subject D) was noted with one abnormally high thyroid hormone levels associated with glyphosate use; thyroid stimulating hormone (FSH) was about double the normal range in the fall and thyroid stimulating hormone (TSH) higher than normal in the summer.
- Another individual (subject E) had abnormally high TSH levels associated with multiple pesticide usage of 12 different active ingredients.

Author(s)	Year	Study title
Bell, E.M. Hertz-Picciotto, I. Beaumont, J.J.	2001	A Case-Control Study of Pesticides and Fetal Death Due to Congenital Anomalies Epidemiology Volume: 12 Number: 2 Pages: 148-156

Abstract*

We examined the association between late fetal death due to congenital anomalies (73 cases, 611 controls) and maternal residential proximity to pesticide applications in ten California counties. A statewide database of all applications of restricted pesticides was linked to maternal address to determine daily exposure status. We examined five pesticide chemical classes. The odds ratios from logistic regression models, adjusted for maternal age and county, showed a consistent pattern with respect to timing of exposure; the largest risks for fetal death due to congenital anomalies were from pesticide exposure during the 3rd– 8th weeks of pregnancy. For exposure either in the square mile of the maternal residence or in one of the adjacent 8 square miles, odds ratios ranged from 1.4 (95% confidence interval = 0.8 – 2.4) for phosphates, carbamates, and endocrine disruptors to 2.2 (95% confidence interval = 1.3 – 3.9) for halogenated hydrocarbons. Similar odds ratios were observed when a more restrictive definition of nonexposure (not exposed to any of the five pesticide classes during the 3rd– 8th weeks of pregnancy) was used. The odds ratios for all pesticide classes increased when exposure occurred within the same square mile of maternal residence.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Various chemical groups – carbamates, halogenated hydrocarbons, phosphates, pyrethroids, and endocrine disruptors (total of 327 pesticides)
Active substance(s):	Various active substances incl. glyphosate
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: None

3. Test group:

Species:	Human
Age of test persons:	18 - >35y
Sex:	Exposed group: 73 females non-exposed group: 611 females

4. Test system:

Study type:	A Case-Control Study of Pesticides and Fetal Death Due to Congenital Anomalies
Guideline:	None

GLP / GCP:	No
Guideline deviations:	Not applicable
Duration of study:	Not applicable
Application rate:	Not reported
Persons per group:	73 exposed; 611 non-exposed (control)
Application technique:	Ground and aerial spraying
Test conditions:	The exposed group consisted of 73 selected cases which were located in the same square mile or surrounding square miles from an area where the pesticides were applied. Exposure occurred during 1-20, 1-13, and 3-8 weeks of pregnancy by ground or aerial spraying. The non-exposed group consisted of 611 healthy females not exposed to the specific pesticide during the relevant time period. None of the persons (exposed, non-exposed) were involved in application of pesticides.
Case identification:	Exposed group: identified congenital anomalies in foetuses from the death certificates. Late foetal deaths after week 20 were considered. Non-exposed group: normal births defined as livebirths with no congenital malformations.

5. Observations/analyses:

Clinical history:	Exposed and non-exposed persons
Clinical signs:	Exposed persons only
Body weight:	Not performed.
Haematology:	Not performed.
Clinical chemistry:	Not performed.
Urine analysis:	Not performed.
Statistics:	Stratified analyses were used to determine which covariates had potential to be confounders. The exposure prevalence among controls and the distribution of covariates by case-control status were assessed. Stratified odds ratios (ORs) were examined to screen for potential effect modifiers. Inclusion criteria for potential effect modifiers required that stratum-specific ORs differ by 100% or more. On the basis of the results of these stratified analyses, we included no interaction term in the model. Adjusted ORs and 95% confidence intervals (CIs) were calculated using logistic regression for those exposed according to the nine-TRS definition, and again for those exposed in the one-TRS definition, separately for each of the five pesticide classes. Separate analyses for ground and aerial modes of application were also completed for those exposed in the nine TRSs. These analyses were limited to those exposed to the specific pesticide class and mode of interest. For those who returned questionnaires, an analysis that adjusted for variables not available from the birth and death certificates was conducted.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Epidemological study with methodological deficiencies (e.g. glyphosate was included in the pesticide class of phosphates, thiophosphates, phosphonates, no differentiation between single and multiple exposures, correlation, if any, only to pesticide classes and not to specific active substances)

2. Relevance of study: **Not relevant** (No glyphosate-specific results.)

3. Klimisch code: **3**

Response – summary from Williams et al. (2012)

- Classes of pesticides were evaluated in this study, with glyphosate included as one of 47 active ingredients in the broad category of "phosphates/triphosphotates/phosphonates".
- Of the 47 active ingredients, many were organophosphate insecticide with known mammalian modes of action. The glyphosate mode of action is on the EPSPS enzyme in plants, which is not present in the animal kingdom.
- Given the very low volatility of glyphosate and the low potential for inhalation exposures to aerosol sprays up to two miles away from the subjects, systemic doses to glyphosate would be considered negligible.
- Mose et al., (2008) demonstrated a low perfusion rate of glyphosate across the placenta. Coupled with the known low dermal and gastrointestinal absorption of glyphosate and the rapid elimination of systemic doses of glyphosate in the urine, human *in utero* exposures would be extremely limited.
- The reported congenital anomalies associated with fetal death in Bell et al. (2001) can in no way be linked to glyphosate exposure.

Author(s)	Year	Study title
Aris, A. Leblanc, S.	2011	Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada. Reproductive toxicology Volume: 31 Pages: 528-533

Abstract*

Pesticides associated to genetically modified foods (PAGMF), are engineered to tolerate herbicides such as glyphosate (GLYP) and gluphosinate (GLUF) or insecticides such as the bacterial toxin bacillus thuringiensis (Bt). The aim of this study was to evaluate the correlation between maternal and fetal exposure, and to determine exposure levels of GLYP and its metabolite aminomethyl phosphoric acid (AMPA), GLUF and its metabolite 3-methylphosphinicopropionic acid (3-MPPA) and Cry1Ab protein (a Bt toxin) in Eastern Townships of Quebec, Canada. Blood of thirty pregnant women (PW) and thirty-nine nonpregnant women (NPW) were studied. Serum GLYP and GLUF were detected in NPW and not detected in PW. Serum 3-MPPA and CryAb1 toxin were detected in PW, their fetuses and NPW. This is the first study to reveal the presence of circulating PAGMF in women with and without pregnancy, paving the way for a new field in reproductive toxicology including nutrition and utero-placental toxicities.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test items / active substances:	Glyphosate;	
	Gluphosinate;	
	Bacillus thuringiensis;	
	AMPA (aminomethyl phosphoric acid)	
	3-MPPA (3-methylphosphinicopropionic acid)	
	Cry1Ab protein (Bt toxin)	
Purity:	Not reported	

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human	
Sex:	Female	
Age:	Pregnant woman:	32.4 ± 4.2 yr (mean)
	Non-pregnant women:	33.9 ± 4.0 yr (mean)
Number of test persons (pregnant):	30	
Number of control persons (non-pregnant):	39	

4. Test system:

Study type:	Maternal and foetal exposure to pesticides associated to genetically modified foods
Guideline:	Non-guideline study
GLP:	No

Guideline deviations:	Not applicable
Duration of study:	Not reported
Collection of data:	
Inclusion criteria:	Subjects were pregnant and non-pregnant women living in Sherbrooke, an urban area of Eastern Townships of Quebec, Canada. No subject had worked or lived with a spouse working in contact with pesticides. Eligible groups were matched for age and biody mass index (BMI)
BMI:	Pregnant woman: 24.9 ± 3.1 kg/m^2 (mean) Non-pregnant women: 24.8 ± 3.4 kg/m^2 (mean)
Exposure conditions:	It was assumed that the subjects were exposed due to the diet of herbicide-tolerant genetically modified crops.
Diet:	The diet taken is typical of a middle class population of Western industrialized countries. A food market-basket, representative for the general Sherbrooke population.
Additional factors:	Participants were not known for cigarette or illicit drug use or for medical condition (i.e. diabetes, hypertension or metabolic disease).

5. Observations/analyses:

Sampling:	Blood sampling was done before delivery for pregnant women or at tubal ligation for nonpregnant women and was most commonly obtained from the median cubital vein, on the anterior forearm.
Measurements:	Levels of GLYP, AMPA, GLUF and 3-MPPA were measured using gas chromatography–mass spectrometry (GC–MS). Cry1Ab protein levels were determined in blood using a commercially available double antibody sandwich (DAS) enzyme-linked immunosorbent assay.
Statistics:	PAGMP (pesticides associated to genetically modified plants) exposure was expressed as number, range and mean ± SD for each group. Characteristics of cases and controls and PAGMP exposure were compared using the Mann–Whitney U-test for continuous data and by Fisher's exact test for categorical data. Wilcoxon matched pairs test compared two dependent groups. Other statistical analyses were performed using Spearman correlations. Analyses were realized with the software SPSS version 17.0. A value of $P < 0.05$ was considered as significant for every statistical analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Exact levels of PAGMF, glyphosate or AMPA in the diets were not determined. It is not clear if the measured concentrations could have been resulted from other exposure routes.

2. Relevance of study: **Relevant with restrictions** (Provides real life actual exposure concentrations in humans. Data are limited due to the absence of any information on applied pesticides, application rates, etc.)

3. Klimisch code: 3

Response – Monsanto Letter to the Editor

Comment: Aris and Leblanc "Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada"

To the Editor,

We have reviewed the publication of Aris and Leblanc entitled "Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada", and wish to provide comment. The study has also been the subject of a regulatory review (FSANZ) which reached conclusions similar to our own. Findings for glyphosate and AMPA are consistent with previous publications (cquavella et al., 2003; Curwin et al., 2007), and levels detected are consistent with intakes far below any level of concern (Curwin et al., 2007). Glyphosate has not demonstrated reproductive or developmental toxicity in repeated mammalian studies. The recent inclusion of glyphosate in Tier-1 endocrine disrupter screening is the result of exposure potential, not evidence of endocrine disruption as implied by Aris and Leblanc.

Attempts to detect Cry proteins in the blood of GM-fed animals have been limited by methodological challenges and commercial immunoassay kits (as used in this study) did not produce valid results in porcine blood. An assay system validated for use in bovine blood failed to detect Cry1Ab (LOD 1 ng/mL) despite very much higher intake (as % diet or per kg body weight) than humans, making assay validation essential. The authors did not provide validation information for the Cry1Ab assay in human blood. A standard curve was said to span a range of 0.1–10 ng/mL, but no statistical limit of detection is reported. It appears that the authors have reported all signals above baseline as confirmed "detects", despite the fact that many samples have concentrations below the likely detection limit of this assay system based on our own experience. Thus, the number of Cry1Ab detects is likely overstated, probably significantly.

The antibody in the Agdia immunoassay kit is known to bind to other cry proteins, and can also bind to fragments derived from the intact protein. While protein digestion and absorption primarily takes place as mono to tri-peptides, small quantities of proteins or larger protein fragments are absorbed as a part of normal human physiology.

Cry1Ab and related proteins (which may interact in this assay system) are widely used in organic agriculture on foods intended for direct human consumption. Cry1Ab is present in GM maize intended primarily for animal feed and processing to food ingredients (corn syrup, starch, etc.), and human consumption is expected to be quite low. Further, very little corn is consumed by humans in a raw state, and cooking denatures Cry1Ab protein eliminating its biological (insecticidal) activity.

Although we believe that the reported rate of detection is elevated, it is possible that Cry1Ab (or fragments) can be found in some individuals with a sufficiently high intake and sensitive assay system. This must be put in proper perspective. Cry proteins as a class are exempt from tolerance (i.e. no maximal intake levels were set), indicating that any potentially achievable exposure raises no safety concern. The no-effect level for purified Cry1Ab in acute animal testing is 4000 mg/kg (highest level tested). For a theoretical 50 kg female, this is the equivalent of 200,000,000 _g of Cry1Ab protein. Detection of 1 ng/mL of Cry1Ab in the blood of a 50 kg female (assuming 20% extracellular fluid volume, as proteins generally do not distribute intracellularly) is crudely equivalent to 10 _g of total Cry1Ab – 20-million times less than a dose which has no discernable effect.

In short, results for glyphosate are unsurprising and raise no health concerns. Detections of Cry1Ab appear to be over-reported. Based upon the limited intake of Cry1Ab and the fact that little protein is absorbed intact, reported detections may be technical artifacts and at best represent protein fragments in addition to intact protein – the vast majority of which are expected to be biologically inactive after processing.

Cry1Ab has been subjected to extensive safety assessment accounting for human exposure with a large margin of safety. Contrary to Aris and Leblanc, available traits are approved for human consumption, even if not the primary intent of cultivation. Mammalian toxicity has not been demonstrated with Cry1Ab or related Cry proteins, and all of the women and infants were normal. The reported findings, even if they should prove to be correct, raise no safety concerns.
The authors are full-time employees of Monsanto company, a manufacturer of products incorporating glyphosate and Cry1Ab.

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Author(s)	Year	Study title
Benítez-Leite, S. Macchi, ML and Acosta, M.	2009	Malformaciones congénitas asociadas a agrotóxicos. Arch Pediatr Urug Volume: 80 Number: 3 Pages: 237-247

Abstract*

Introduction: exposure to pesticides is a known risk for human health. This paper describes the relationship between parental exposure and congenital malformations in the newborn. Objective: to study the association between exposure to pesticides and congenital malformations in neonates born in the Regional Hospital of Encarnación, in the Department of Itapúa, Paraguay. Materials and methods: a prospective case-controlled study carried out from March 2006 to February 2007. Cases included all newborns with congenital malformations, and controls were all healthy children of the same sex born immediately thereafter. Births outside the hospital were not counted. Exposure was considered to be any contact with agricultural chemicals, in addition to other known risk factors for congenital defects. Results: a total of 52 cases and 87 controls were analyzed. The average number of births each month was 216. The significantly associated risk factors were: living near treated fields (OR 2,46, CI95% 1,09-5,57, p<0,02), dwelling located less than 1 km (OR 2,66, CI95% 1,19-5,97, p<0,008), storage of pesticides in the home (OR 15,35, CI95% 1,96-701,63), p<0,003), direct or accidental contact with pesticides (OR 3,19, CI95% 0,97-11,4, p<0,04), and family history of malformation (OR 6,81, CI95% 1,94-30,56, p<0,001). Other known risk factors for malformations did not show statistical significance. Conclusion: the results show an association between exposure to pesticides and congenital malformations. Further studies are required to confirm these findings.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Several pesticides were assessed but not specified.
Active substance(s):	Several active substances were assessed but not specified.
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: None

3. Test group:

Species:	Human
Age of test persons:	Newborn babies (The exposed mothers had an average age of 25 years (range: 12-45 years)) (Age of mothers from the control group not specified)
Sex:	Males and females

4. Test system:

Study type:	Epidemiological study for developmental toxicity
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Duration of study:	11 months (between March 28, 2006 and February 28, 2007)
Collection of data:	Questionnaire
Test group:	2414 newborn babies
Control group:	Controls were all healthy children of the same sex born immediately after the study period (February 28, 2007): up to 87 newborns
Application rate:	The concentration of the pesticides to which the mothers had been exposed was not specified.
Exposure frequency:	Not assessed.
Application technique:	Mainly fumigation

5. Observations/analyses:

Clinical history:	Not performed.
Clinical signs:	All persons during pregnancy.
Body weight:	Not performed.
Haematology:	Not performed.
Clinical chemistry:	Not performed.
Urine analysis:	Not performed.
Evaluation:	The test group consisted of all newborns recorded in the Regional Hospital of Encarnación during the observation period. A total of 2414 cases were recorded (mean value: 216/month). The mothers of the newborn were asked several questions such as where they live, if they store pesticides at home, if they work with pesticides, etc…. The region of Itapúa has mainly soya cultivation. Paraguay was declared by the FAO as a place of concern, since big amounts of pesticides are yearly used (approx. 24 million L of pesticides per year). Population living in the area are exposed to these agrochemicals via many pathways (mother's home proximity to treated fields, workplace, or private use of pesticides). According to the statistic conducted with the mothers 55% of them lived in urban areas, 82% worked as housewife.
Exposure situation:	19.9% of the mothers had had direct contact or accidentally with pesticides, 28.8% of the fathers had been exposed. 42.3% of the asked mothers lived near treated fields. 15.3% had pesticides at home for private use.
Record of malformations:	22 different types of congenital malformations were recorded and statistically assessed, such as ear, hand or arm malformation.
Clinical history:	Not performed.
Clinical signs:	All mothers during pregnancy.
Body weight:	Not performed.

Haematology:	Not performed.
Clinical chemistry:	Not performed.
Urine analysis:	Not performed.
Malformation assessment:	All newborns were examined for malformations 22 different types of congenital malformations were recorded and statistically assessed, such as ear, hand or arm malformation.
Statistics.	Yes, odds ratio statistic.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comment:	Study design of epidemiological study for developmental toxicity insufficient for assessment, as well as methological and reporting deficiencies (no assessment to which pesticides / active substances the mothers were exposed, use frequency not specified, selection of control group after study period is questionable, no information on exposure situation of mother for this control group assessed, etc.)
2. Relevance of study:	**Not relevant** (The exposure to several pesticides was assessed in general, but no pesticide or active substance, including glyphosate, was specified or assessed)
3. Klimisch code:	**3**