UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES,
AND TOXIC SUBSTANCES

MEMORANDUM

Date: 28-DEC-2009

Subject: **Glyphosate.** Public Comments Regarding the Health Effects Division's (HED's) Human-Health Assessment Scoping Document in Support of Registration Review of 3-JUN-2009. **HED's Response to Public Comments.**

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Decision No.: 421134
Registration No.: N/A
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Regulatory Action: Registration Review
Risk Assessment Type: N/A
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MRID No.: N/A
40 CFR: §180.364

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Executive Summary

HED previously completed a human-health risk assessment scoping document for glyphosate to support the Registration Review of glyphosate (Memo, J. Langsdale *et al.*, 3-JUN-2009; D362745). In that memo, HED evaluated the status of the human-health assessments for

glyphosate to determine if sufficient data are available and if any updates are required to support Registration Review. HED considered the most recent human-health risk assessment for glyphosate (Memo, J. Tomerlin, 29-Sep-06, D321992); the most recent human-health risk assessment for glyphosate applied to transgenic crops (Memo, T. Bloem, 18-Mar-08, D345923); updates to its toxicity, exposure, and usage databases; and the most updated Agency science policy and risk assessment methodologies to determine the scope of work necessary to support Registration Review. In addition, HED conducted an open search to look for new literature relevant to the human-health risk assessment.

"Beyond Pesticides," "The Center for Food Safety," "Monsanto," and "Gilles-Eric Séralini" have submitted responses to the Glyphosate Docket (Docket ID: EPA-HQ-OPP-2009-0361) regarding HED's human-health risk assessment scoping document for glyphosate.

CONCLUSIONS/RECOMMENDATIONS

The Agency thanks "Beyond Pesticides," "The Center for Food Safety," "Monsanto," and "Gilles-Eric Séralini" for their comments regarding HED's human-health risk assessment scoping document for glyphosate. The Agency has considered these comments and will utilize them, along with any newly available data, during the Registration Review of glyphosate.

DETAILED CONSIDERATIONS

Beyond Pesticides to the Office of Pesticide Programs (OPP) Regulatory Public Docket (7502P). "Registration Review; Glyphosate Docket Opened for Review and Comment. Docket Number: EPA–HQ–OPP–2009–0361." 21 September 2009.

"Beyond Pesticides" Comment: Human Exposures to Glyphosate Pose Unacceptable Risks

HED's Response: The letter from "Beyond Pesticides" cited additional studies in the open literature which associate glyphosate exposure with various adverse health outcomes including attention deficit syndrome and hyperactivity disorder (ADHD), non-Hodgkin's lymphoma (NHL), and hairy cell leukemia.

The Agency thanks "Beyond Pesticides" for its comments regarding human exposures to glyphosate. The Agency is aware of the studies referenced by "Beyond Pesticides" and will consider the importance of these results in addition to other studies identified in the open literature during the Registration Review of glyphosate. The Agency intends to evaluate this type of research and, when appropriate, consider the inferences drawn across studies in the risk assessment process, including identification of health effects relevant to the human population, and the magnitude and direction of exposure-response associations observed in observational epidemiologic research relating to glyphosate exposure.

While preparing the scoping document, the Agency did not synthesize results from across the open literature for use in risk assessment. The Agency did summarize results of the Agricultural Health Study (AHS), as it is considered to be among the best epidemiologic cohorts available to study glyphosate-health outcome associations. As additional studies evaluating glyphosate use and incident cancer and non-cancer health effects become available in the AHS, the Agency will

closely evaluate the results in the context of the risk assessment process. As the risk assessment matures and specific questions are identified, the Agency will perform a targeted examination of the open literature and provide conclusions in the final risk assessment.

"Beyond Pesticides" Comment: Roundup Formulations Are Toxic, Yet Go Unevaluated

HED's Response: The Agency thanks "Beyond Pesticides" for its comments regarding Roundup formulations. Between October 2009 and February 2010, EPA is issuing test orders/data call-ins for the first group of 67 chemicals, which contains 58 pesticide active ingredients and 9 inert ingredients, that they be screened under the Endocrine Disruptor Screening Program (EDSP). This list of chemicals was selected based on the potential for human exposure through pathways such as food and water, residential activity, and certain post-application agricultural scenarios. This list should not be construed as a list of known or likely endocrine disruptors. Glyphosate is among this group of 58 pesticide active ingredients on the initial list to be screened under EDSP and the Agency will be issuing the Test Order in January, 2010. The Agency will review the EDSP Tier 1 data and or "other scientifically relevant information" submitted in response to test orders. Based on this review the Agency will determine the need for additional testing. For further information on the status of the EDSP, the policies and procedures, the list of 67 chemicals, the test guidelines and the Tier 1 screening battery, please visit our website: http://www.epa.gov/endo/.

"Beyond Pesticides" Comment: FQPA 10x Factor Must be Reinstated

HED's Response: The Agency thanks "Beyond Pesticides" for its comments regarding the FQPA Safety Factor (FQPA SF). The Agency has considered the comments made by "Beyond Pesticides," however the Agency does not believe that the FQPA 10X SF should be reinstated for glyphosate. In its 2006 risk assessment for glyphosate, the FQPA SF was reduced by the Agency to 1X for several reasons. These include the absence of any increased susceptibility of the young to the effects of glyphosate in developmental and reproductive toxicity studies in rats or rabbits; the absence of any neurotoxic effects in submitted studies in mice, rats, rabbits, or dogs; the lack of any scientific support for a developmental neurotoxicity study; and the conservative dietary exposure assessment that did not underestimate the potential exposures of infants and children to glyphosate. The Agency's decision was consistent with previous decisions that the Agency has made for other pesticides, the results of non-GLP studies conducted with glyphosate and published in the biomedical literature, as well as an independent safety evaluation and risk assessment for both glyphosate and Roundup published by Williams *et al.* (2000) in which the authors concluded, "under present and expected conditions of new use, there is no potential for Roundup herbicide to pose a health risk to humans" (p. 160).

The "Beyond Pesticides" comments referenced in vitro, epidemiological, and biomonitoring studies with glyphosate that were published in the biomedical literature. The Agency has reviewed these studies and believes that these studies do not indicate that infants and children are more susceptible than adults to glyphosate. The comments from "Beyond Pesticides" included the following statement: "Richard, *et al.* reports that glyphosate is toxic on human placental JEG3 cells within 18 hrs with concentrations lower than those used in agriculture." The Agency believes that results of in vitro studies like Richards *et al.* (2005) are not relevant to human health risk assessment because the concentrations tested are very high and are therefore not

representative of expected human exposures. The authors also acknowledged this when they stated, "The physiologic significance of these effects can be questioned, in regard to the concentration used" (p. 719). The Agency also believes that in vitro studies which are not performed in a whole animal do not mimic real-life physiological processes which limit absorption of compounds into the body, and include extensive metabolism and excretion of absorbed compounds. The results of the whole animal studies reviewed by the Agency indicate that there is no evidence of increased susceptibility of offspring to glyphosate.

"Beyond Pesticides" also referenced an epidemiological study that "found that preconception exposures to glyphosate moderately increased the risk for spontaneous abortions in mothers exposed to glyphosate products." The Agency disagrees with this statement. The study (Arbuckle *et al.* 2001) found a weak association between preconception exposure to glyphosate and increased spontaneous abortion [odds ratio (OR)=1.4]. It also found that exposure to glyphosate decreased (not increased) the risk of spontaneous abortion before 12 weeks in women exposed to glyphosate after conception (OR=0.8), thereby pointing to a beneficial role for glyphosate on the developing fetus. The Agency does not believe that this study is supportive of a convincing association between glyphosate exposure and spontaneous abortion. Many of the results reported in the study were not statistically significant, as acknowledged by the author, and the study had several other limitations which limit its application to human health risk assessment such as: "Because dose information was not available, misclassification of exposure is likely. Many factors including the pesticide formulation, application conditions, handling practices, and interindividual differences in absorption, distribution, metabolism, and excretion of the products or metabolites will lead to variability in the degree of exposure. Because the farmers used many different pesticides during the study and our sample size was limited, findings may be unreliable, particularly for multiple pesticide interactions... Because the analyses were designed to generate, not to test, hypotheses, and multiple comparisons were conducted, results should be interpreted with care and tested in other studies" (p. 855).

The "Beyond Pesticides" letter also stated that a "Farm Family exposure study found that all but one of the 79 children evaluated had detectable concentrations of glyphoste in their urine." Acquavella *et al.* (2004) was a biomonitoring study in which glyphosate levels were monitored in the urine of pesticide applying farmers and their families in two U.S. states. Urinary concentrations for farmers who applied glyphosate ranged from <1-233 ppb, and some farmers had no detectable levels. The highest levels in urine were in farmers who did not wear protective clothing (rubber gloves) during use or who improperly handled (i.e. spilled) glyphosate on the day of use. Urinary levels in children were much lower and ranged from <1-29 ppb. How this data might correlate with potential health effects associated with glyphosate exposure reported in epidemiology studies will be considered more fully in the anticipated risk assessment.

"Beyond Pesticides" Comment: Human Incidents Are Too High

HED's Response: The Agency thanks "Beyond Pesticides" for its comments regarding human incidents. The Agency will search the National Poison Data System (NPDS), the California Pesticide Illness Surveillance Program, and the National Institute of Occupational Safety and Health's Sentinel Event Notification System for Occupational Risks (NIOSH SENSOR) for additional glyphosate poisoning incident data in the course of registration review.

Freese, Bill. From The Center for Food Safety to Office of Pesticide Programs OPP Regulatory Public Docket (7502P). "Registration Review; Glyphosate Docket Opened for Review and Comment. Docket Number: EPA–HQ–OPP–2009–0361." 21 September 2009.

"Center for Food Safety" Comment: IV. Assessment of Human Health Impacts of Glyphosate and its Formulations

HED's Response: The Agency thanks the "Center for Food Safety" for its comments regarding human health impacts of glyphosate. Please refer to HED's response to the "Beyond Pesticides" comment entitled "Human Exposures to Glyphosate Pose Unacceptable Risks" for a discussion about additional studies in the open literature which associate glyphosate exposure with various adverse health outcomes such as ADHD, NHL, and hairy cell leukemia.

The Agency thanks the "Center for Food Safety" for its comments regarding human incidents. Please refer to HED's response to the "Beyond Pesticides" comment entitled "Human Incidents Are Too High" for a discussion about the databases the EPA will search for additional glyphosate poisoning incident data in the course of the risk assessment process.

The Agency thanks the "Center for Food Safety" for its comments regarding the FQPA SF. The Agency has considered the comments made by the "Center for Food Safety," however the Agency does not believe that the FQPA 10X SF should be reinstated for glyphosate. In its 2006 risk assessment for glyphosate, the Agency reduced the FQPA SF for glyphosate to 1X for several reasons which are outlined above in HED's response to the "Beyond Pesticides" comment entitled "FQPA 10x Factor Must be Reinstated."

The Agency thanks the "Center for Food Safety" for its comments on the tolerances presented in Table 6 in the Human-Health Scoping Document. The tolerances will be updated to reflect 40 CFR §180.364 in the final risk assessment document.

Adams, Stephen from Monsanto Company to OPP Regulatory Public Docket (7502P). "Docket ID: EPA-HQ-OPP-2009-0361; Comments on the Registration Review of Glyphosate." 21 September 2009.

"Monsanto" Comment: Glyphosate Incorrectly Linked to Organophosphate Insecticides and Cholinesterase Inhibitors

HED's Response: HED recognizes that glyphosate is best described as a phosphono amino acid herbicide.

"Monsanto" Comment: General Comments Regarding Public Health and Pesticide Epidemiology Data and Evaluation of the OPP Incident Data System (IDS) Data

HED's Response: The Agency thanks "Monsanto" for its comments regarding human incidents. The Hawkins memorandum (12-MAR-2009; Attachment 7 in Memo, J. Langsdale *et al.*, 3-JUN-2009, D362745) reviewed the incident information that was retrieved from the Agency's IDS to determine if there is a pattern or trend that merits further consideration during the preliminary risk assessment phase of the registration review process for glyphosate. A large majority of the incident reports were classified as moderate cases that involved dermal effects such as blisters,

rash, pruritus, skin irritation, hives, welts, sores, burning skin, and peeling skin and neurological effects such as shaking, loss of coordination, tingling, neuropathy, ataxia, and numbness. A pattern exists in that many of the dermal cases were due to accidental splashing of the product and leakage onto the hands. The Agency will search the NPDS, the California Pesticide Illness Surveillance Program, and NIOSH SENSOR databases for additional glyphosate poisoning incident data in the course of the risk assessment process.

The "Monsanto" letter commented on the use of case reports to establish causal relationships between a chemical and exposure symptoms. In terms of the focus on case reports in IDS that may be attributable to products containing only one active ingredient, the Agency uses this search criterion when performing the IDS data query to avoid potential synergistic effects. If an unusual or "sentinel" event is observed during the data query, the Agency will attempt to verify the presence of only one active ingredient in the product formulation, if the information allows.

"Monsanto" Comment: Comments Related to the Agricultural Health Study

HED's Response: "Monsanto" articulates two main points concerning recent publications from the AHS in relation to potential glyphosate health effects in the human population: 1) persons who reported ever using glyphosate were not observed to have increased risk of cancer overall or an increased risk of most common cancer sub-types; however in comparison with those who have never used the chemical, ever users of glyphosate were observed to have a non-significant 2-fold increased risk of multiple myeloma [odds ratio (OR) 2.6, 95% confidence interval (95% CI) 0.7-9.4]; and, 2) a recent AHS study reported a non-significant decreased risk of developing monoclonal gammopathy of undetermined significance (MGUS), a condition which precedes multiple myeloma, in association with ever-use of glyphosate.

The Agency thanks "Monsanto" for its comments regarding the AHS. The Agency is aware of the AHS publications referenced by "Monsanto" and notes that both publications report preliminary findings based upon a small number of glyphosate exposed multiple myeloma cases and MGUS cases, respectively. Study authors acknowledged the work must be replicated before conclusions regarding any causal association, or lack thereof, can be determined. As research progresses and additional study results are made available, the Agency will consider this information in the glyphosate regulatory review and risk assessment process.

Monsanto Comment: Comments on the U.S. Tolerances Reported in the Human-Health Assessment Scoping Document

HED's Response: The Agency thanks "Monsanto" for its comments on the tolerances presented in Table 6 in the Human-Health Scoping Document. The tolerances will be updated to reflect 40 CFR §180.364 in the final risk assessment document. HED notes the increase in poultry, meat from 0.10 ppm to 4 ppm in 40 CFR §180.364. The Registration Division (RD) will be notified of this discrepancy and 40 CFR §180.364 will be corrected to reflect the appropriate tolerance of 0.10 ppm for poultry, meat.

Monsanto Comment: Clarification of the Need for a New Residential Exposure Risk Assessment

HED's Response: The Agency thanks "Monsanto" for the clarification regarding Roundup®

Weed & Grass Killer Super Concentrate. The Agency recognizes that Roundup® Weed & Grass Killer Super Concentrate, EPA Reg. No. 71995-25, was not a new product registered in October 2008, but rather was a new alternative formulation under this registration. As stated in the human-health scoping document, a new residential exposure risk assessment is required which reflects the use rate of 10.5 lb acid equivalents per acre (ae/A) for Roundup® Weed & Grass Killer Super Concentrate (EPA Reg. No. 71995-25).

Monsanto Comment: Regulation of Aminomethylphosphonic Acid (AMPA)

HED's Response: HED recognizes "Monsanto's" comment that it does not believe it is necessary to revisit the regulation of AMPA residues. However, the decision that AMPA need not be regulated, regardless of levels observed in foods or feeds, may be revisited during the registration review process.

Séralini, Gilles-Eric. Email to Carol Stangel. "Re: EPA's Response re: Glyphosate." 02-OCT-09.

Séralini Comment: "I was aware of the letter of August 12th 2009 your received from EPA (from Dr. Debra Edwards, Director Pesticide Programs) in Washington, about my recent paper on glyphosate based herbicides, which have been proved to be human cellular endocrine disruptors."

HED's Response: The Agency thanks "Gilles-Eric Séralini" for the comments regarding glyphosate. Please refer to HED's response to the "Beyond Pesticides" comment entitled "Roundup Formulations Are Toxic, Yet Go Unevaluated" for a discussion about the test orders/data call-ins EPA is issuing for pesticide active ingredients and inert ingredients that they be screened under the EDSP.

HED General Comments about Inert Ingredients and Surfactants

Several of the responses the Agency received regarding HED's human-health risk assessment scoping document for glyphosate specifically addressed inert ingredients and surfactants. Pesticide products contain both "active" and "inert" ingredients. The terms "active ingredient" and "inert ingredient" are defined by the federal law that governs pesticides (Federal Insecticide, Fungicide, and Rodenticide Act [FIFRA]). An active ingredient is one that prevents, destroys, repels, or mitigates a pest, or is a plant regulator, defoliant, desiccant, or nitrogen stabilizer.

All other ingredients in a pesticide product are called "inert ingredients." An inert ingredient means any substance (or group of similar substances) other than an active ingredient that is intentionally included in a pesticide product. Called "inerts" by the law, the name does not mean non-toxic.

Pesticide products often contain more than one inert ingredient. Inert ingredients play key roles in the effectiveness of pesticides. Examples include inerts that prevent caking or foaming, extend product shelf-life, or solvents that allow herbicides to penetrate plants.

Like pesticides, inert ingredients are also subjected to complete evaluation of health, environment, and ecological effects. EPA evaluates the inerts to ensure that it will not have unreasonable adverse effects on humans, the environment, and non-target species. Inert ingredients are permitted in pesticide products once the safety of the inert ingredients established by the EPA. Like pesticides, inert ingredients are also subjected to tolerance reassessment. EPA conducted tolerance reassessments for inert ingredients in 2006. Several groups of surfactants were reassessed in 2009. For further information about EPA's review of inert ingredients, please visit our website: http://www.epa.gov/opprd001/inerts/.

References

Williams GM, Kroes R, Munro IC. 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul Toxicol Pharmacol 31(2 Pt 1):117-65.

Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE. 2005. Differential effects of glyphosate and Roundup on human placental cells and aromatase. Environ Health Perspect 113(6):716-20.

Arbuckle TE, Lin Z, Mery LS. 2001. An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. Environ Health Perspect 109(8):851-7.

Acquavella, J. F., *et al.* 2004. Glyphosate Biomonitoring for Farmers and Their Families: Results from the Farm Family Exposure Study. Environmental Health Perspectives 112(3):321-326.

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Objet transmission de documents

Paris, le 29 janvier 2007

Monsieur,

Pour répondre à votre demande, je vous adresse, ci-joint, l'extrait du procès-verbal de la commission d'étude de la toxicité du 14 décembre 2005, relatif à l'avis rendu par cette commission sur la publication scientifique intitulée :

« *Differential effects of glyphosate and Roundup on human placental cells and aromatase. Richard S., Moslemi S., Sipahutar H., Benachour N. Seralani G.E., Environ. Health Perspect., 2005*

Vous souhaitant bonne réception de ces documents, je vous prie d'agréer, Monsieur, l'expression de mes salutations distinguées.

L'Ingénieur en chef du Génie Rural,
des Eaux et des Forêts,
Sous-Directeur de la Qualité et de la Protection des Végétaux

José MATHURIN

P.J. : 9 feuillets

Instruction de la saisine
de la Commission d'étude de la toxicité par la DGAL sur l'article

« Differential effects of glyphosate and Roundup on human placental cells and aromatase.
Richard S., Moslemi S., Sipahutar H., Benachour N. Seralani G.E.,
Environ. Health Perspect., 2005 (sous presse ; online 24 February 2005)

1. Documents reçus :

1.1. Courrier de la SSM du 21 Nov. 2005 : nomination du rapporteur de la saisine

1.2. Courrier de la DGAL du 14 avril 2005 (référence illisible : 050 ??51): demande à Monsanto Agriculture France SAS d'observations sur l'article en référence pour communication à la Commission Européenne et au pays rapporteur (Allemagne)

1.3. Courrier de Monsanto du 26 avril 2005 (référence ILG/YF/206) : copie à la DGAL du dossier envoyé à la DG Sanco et à l'Allemagne (courrier du 22 avril 2005 et pièces jointes :

- 1.3.1. Publication de Richard S et al, 2005
- 1.3.2. Réponse de Monsanto du 2 mars 2005 sur la publication de Richard S. et al (rédacteur : Donna R. Farmer, Daniel A. Goldstein, Monsanto, St Louis, Missouri)
- 1.3.3 Publication de Marc J. et al, « *Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation* », Chem. Res. Toxicol., 2002, 15, 326-331
- 1.3.4. Réponse de Monsanto du 2 mars 2005 sur la publication de Marc J. et al (document confidentiel pour usage strictement interne à la firme)
- 1.3.5. Abstract PubMed de la publication d'Amouroux I. et al, *« Mechanisms of cytotoxicity by cosmetic ingredients in sea urchin eggs »*, Arch. Environ. Contam. Toxicol., 1999, 36 (1), 28-37.
- 1.3.6 Commentaires de Fellous et al. du 21 mars 2005 sur la publication de Richard S. et al.

1.4. Courrier de la DGAL du 20 mai 2005 (référence 0500204) : copie du dossier de Monsanto à la SSM faisant état de la saisine du président de la Com Tox en vue de l'examen de la publication de Richard S. et al. et de la réponse de Monsanto pour Juin 2005

1.5. Courrier de la DGAL du 27 Mai 2005 (référence 0500254) : report de l'examen de la saisine (à inscrire à l'ordre du jour de décembre 2005)

1.6. Courrier de la DGAL du 12 Octobre 2005 (référence 0500561) : transmission à la SSM de la copie électronique de deux documents et des commentaires de l'Allemagne sur la publication de Richard S.

1.7. CD ROM contenant la version électronique des documents 1.3., 1.3.1., 1.3.2., 1.3.3., 1.3.4., 1.3.6., et de la publication complète d'Amouroux I. et al.

2. Analyse de la publication « *Differential effects of glyphosate and Roundup on human placental cells and aromatase"* (Richard et al, Environ. Health Perspect., 2005)

2.1. Résumé des données :

2.1.1. La toxicité du Glyphosate (origine Sigma-Aldrich) et du Roundup (origine « commerciale » ; 360 g/L de glyphosate), seuls ou en association, est testée sur une lignée de cellules placentaires humaines JEG3 en mesurant la viabilité cellulaire (test MTT), et l'activité aromatase *in vitro* (dosage radio-immunologique) avec recherche du mécanisme la modulant (quantification des ARNm), après exposition pendant 1, 18, 24 ou 48h à des concentrations dites « représentatives » des usages recommandés (2% de Roundup ou concentration équivalente de Glyphosate avec ajustement du pH) ou inférieures à celles-ci. En complément, l'activité aromatase de microsomes issus de placentas de femmes non fumeuses et de testicules de cheval a été mesurée dans les mêmes protocoles d'exposition, et l'activité des enzymes purifiés issus de testicule de cheval a été déterminée (études spectrales, mesure de l'activité NADPH réductase).

2.1.2. Les résultats indiquent :

2.1.2.1. une **diminution significative de la viabilité** des cellules JEG3 (peu de données chiffrées, multiples courbes !),

- 2 fois plus importante pour le Roundup que pour le glyphosate,
- dépendante de la durée d'exposition : objectivable pour 1h et triplant pour 18h d'exposition ...
- dépendante de la concentration : premier point de chute de survie (environ 20%) pour 18h d'exposition à 0.2% de Roundup et 0.8% de glyphosate, et viabilité nulle pour 18h d'exposition à 0.4% de Roundup et 1.9% de glyphosate),
- non explicable par l'acidité des solutions (pH =5.8)
- augmentée pour le glyphosate en présence de 0.1% de Roundup (pour toutes les concentrations de glyphosate).

2.1.2.2. une **inhibition significative de l'activité aromatase** des cellules JEG3 (peu de données chiffrées, multiples courbes)

- uniquement pour le Roundup pour 18 heures d'exposition à partir de concentration de 0.01% (IC50 ≈ 0.04%) alors qu'une exposition d'une heure augmente l'activité d'environ 40% quelle que soit la concentration (gamme testée : 0.01 à 0.2%)
- présente pour le glyphosate qu'en cas d'addition de Roundup à 0.02% (2 concentrations testées : 0.18 et 0.38% de glyphosate
- attribuée à un effet sur l'expression du gène *CYP19* (chute des ARNm)

2.1.2.3. une **inhibition significative de l'activité aromatase microsomale** des cellules humaines (placenta) et équines (testicule)

- 3 fois plus importante pour le Roundup (IC50 = 0.6%) que pour le glyphosate
- identique dans les 2 modèles cellulaires testés
- attribuée à une interaction directe du glyphosate sur le site actif (études spectrales)
- associée à une moindre diminution de l'activité NADPH réductase (IC50 = 5%)

2.1.3. Dans leurs commentaires de ces résultats, les auteurs de la publication soulignent les points suivants :

2.1.3.1. L'effet sur la viabilité des cellules JEG3 (10 fois plus important pour le Roundup que pour le glyphosate) a pu être mis en évidence du fait :

- de l'utilisation d'un milieu de culture sans sérum, assurant une meilleure biodisponibilité et optimisant de ce fait la détection de l'effet
- de longues durées d'exposition, autorisant une « *action génomique* » et une bio-accumulation (avec plusieurs références à l'appui d'effets toxiques ou génotoxiques et d'une accumulation du Roundup : Peluso M *et al.* 1998, Lioi MB *et al*, 1998, Mitchell DG et al,1987, Vigfusson NV *et al*,1980 et Yousef MI *et al*, 1995)

2.1.3.2. L'inhibition de l'aromatase des cellules JEG3 est induite par des expositions de durée suffisante à des concentrations non toxiques de Roundup uniquement (la stimulation observée pour une courte durée d'exposition étant supposée due à une augmentation de la perméabilité membranaire et une meilleure biodisponibilité en substrat, induites par la présence des adjuvants du Roundup).

2.1.3.3. Cette inhibition de l'aromatase, qui relèverait d'une action directe sur l'enzyme, est confirmée par les mesures sur microsomes humains et équins qui révèlent en outre un potentiel inhibiteur du glyphosate (4 fois inférieur à celui du Roundup).

2.1.3.4.L'addition de faibles concentrations de Roundup au glyphosate conférerait à ce dernier un potentiel cytotoxique et inhibiteur de l'aromatase, la biodisponibilité étant facilitée par les adjuvants.

2.1.4. Les auteurs concluent donc :

- qu'il existe un potentiel de perturbation endocrinienne inductible chez les mammifères par des concentrations de glyphosate 100 fois inférieures à celles des usages agricoles.

- que l'action directe du glyphosate sur l'aromatase pourrait expliquer certains effets reprotoxiques observés *in vivo* en s'appuyant notamment sur plusieurs références bibliographiques rapportant des problèmes de grossesse chez des utilisateurs d'herbicides à base de glyphosate (Savitz DA *et al*, 2000), une perturbation du cycle cellulaire dans l'oeuf d'oursin (Marc J. 2002) ou encore de l'expression post-transcriptionnelle d'une protéine régulatrice de la stéroïdogénèse dans les cellules de Leydig tumorales de souris (Walsh LP. *et al*, 2000)

2.2. Commentaires :

2.2.1. Sur le fond, cette publication, qui tente de mettre en avant une suspicion d'effets reprotoxiques au travers d'un mécanisme potentiel de perturbation endocrinienne, présente plusieurs **lacunes méthodologiques majeures** :

2.2.1.1. Une grande partie des expérimentations *in vitro* ont été menées sur cellule JEG3, lignée cellulaire humaine tumorale (potentiel démontré par greffe sur souris nude) dérivée d'un choriocarcinome présentant un caryotype hypertriploïde (70 chromosomes en moyenne) avec 5 chromosomes très remaniés et inclassables et un seul chromosome X. Ces cellules ont la capacité de transformer les précurseurs des stéroïdes en oestrone et en oestradiol. Un tel profil caryotypique peut conférer une spécificité de réponse au modèle cellulaire de sorte qu'aucune conclusion définitive ne peut être avancée sans recourir à une

validation de la reproductibilité des observations dans d'autres lignées cellulaires, notamment non placentaires.

2.2.1.2. Bien que prétendues représentatives voire 10 fois inférieures à celles des usages agricoles, les concentrations de Roundup utilisées dans les diverses expérimentations de cette publication doivent en fait être considérées comme extrêmement élevées, s'agissant de l'exposition directe de cellules ou de fractions sub-cellulaires.

- D'après les diagrammes, une l'altération de la viabilité cellulaire apparaît à partir de concentrations de l'ordre de 0.2 % et l'inhibition de l'aromatase pour les concentrations de l'ordre de 0.02% de Roundup, ce qui correspond respectivement à 700 et 70 mg/L de glyphosate. Compte des facteurs limitants que représentent l'absorption orale (30%), l'absorption cutanée (0.3%), la cinétique d'élimination (moins de 1% de résidus tissulaires à 7 jours), de telles teneurs impliqueraient des expositions humaines considérables, soit plusieurs dizaines de litres de Roundup dilué à 2%.

- dans son commentaire de la publication, le pays rapporteur (Allemagne) souligne que les concentrations de Roundup déclenchant un effet sur l'aromatase (0.5 - 2%) sont au moins 1000 fois plus efficaces que celles des inhibiteurs connus de l'aromatase, tels que les dérivés azolés.

2.2.1.3. Fait remarquable, si les effets du Roundup sur la viabilité cellulaire et l'inhibition de l'aromatase (mesurée sur cellules et microsomes) sont supérieurs à ceux du glyphosate (dépourvu notamment d'effet inhibiteur sur cellules), l'addition de très faibles teneurs de Roundup conférerait au glyphosate un effet cytotoxique significatif (en présence de 1% de Roundup) et inhibiteur de l'aromatase (en présence de 0.2% de Roundup), le phénomène étant attribué par les auteurs à une facilitation des effets du glyphosate par les adjuvants du Roundup. Une telle interprétation apparaît contestable, d'autant que les auteurs évoquent dans la conclusion un « *effet multiplicateur du Roundup sur l'effet endocrinien du glyphosate* », sachant que :

- Le protocole expérimental ne permet pas de démontrer l'influence des adjuvants et à fortiori un effet synergique avec le glyphosate, puisque le Roundup contient lui-même du glyphosate (il aurait été nécessaire de disposer de données indépendantes sur les adjuvants, le glyphosate et l'association).

- La toxicité des adjuvants n'est pas discutée, et aucune référence n'est faite aux résultats publiés dans ce domaine, notamment

    - Les multiples effets non spécifiques de surfactants sur un large spectre de cibles cellulaires, comme l'atteinte de l'intégrité ou de la perméabilité membranaire consécutive à l'induction d'un déséquilibre ionique (travaux d'Amouroux I et al[i] sur l'œuf d'oursin)
    - L'atteinte de la membrane mitochondriale induite par divers surfactants destinés aux usages domestiques (Farmer D.R. et al[ii])

- Aucune analyse de la cohérence des différents résultats ne semble menée, en mettant par exemple en perspective les effets du pH sur le modèle cellulaire utilisé (soulignant qu'ils ont ajusté le pH des solutions de glyphosate à celui des solutions de Roundup, soit un pH de 5.8, les auteurs indiquent que cette acidification ne peut expliquer qu'une diminution de 23 % de la survie cellulaire après 18 heures d'exposition, sans proportion avec celle induite dans les expérimentations ; il n'en reste pas moins, qu'1/4 de la toxicité des cellules relève de l'acidité !!!)

2.2.2. Sur la forme, cette publication comporte de **multiples biais d'argumentation et l'interprétation** des données :

2.2.2.1. Pour étayer le risque d'exposition de l'homme au glyphosate, les auteurs se réfèrent aux travaux d'Acquavella J.F. et al[iii], rapportant des niveaux d'exposition maximums au glyphosate de 0.004 mg/kg chez des agriculteurs et leur famille, soit des valeurs sans commune mesure avec de l'expérimentation.

2.2.2.2. Pour étayer les effets du glyphosate sur la reproduction et le développement, les auteurs se réfèrent aux travaux de Savitz DA et al[iv], rapportant une augmentation modérée du risque d'accouchements prématurés chez les épouses d'agriculteurs exposés, 3 mois avant le début de la grossesse, à une grande variété de pesticides dont l'atrazine, le glyphosate, des organo-phosphorés, le 2-4D…Dans une publication ultérieure (Arbuckle TE et al[v]) sur la même cohorte (« *Ontario Farm Family Health Studies* »), une augmentation, à la limite de la signification statistique, du risque de fausses couches tardives après exposition pré-conceptionnelle au glyphosate, est mise en évidence en l'absence d'ajustement pour d'éventuels facteurs de confusion, de sorte que ce résultat est considéré par les enquêteurs eux-mêmes, comme exploratoire compte tenu notamment des limitations relatives à la mesure des expositions qui ont été évaluées par questionnaire…des compléments d'investigation étant envisagés pour préciser les molécules incriminées en utilisant des marqueurs biologiques validés d'exposition.

2.2.2.3. Pour étayer le manque de connaissance sur le mécanisme d'action du glyphosate et la possibilité de multiples effets enzymatiques, les auteurs se réfèrent notamment à la publication de Williams GM et al[vi], qui consiste en une revue des études réglementaires et publiées sur le glyphosate et son métabolite principal (acide aminoethylphosphonique ou AMPA), ainsi que sur les formulations de Roundup et le principal agent surfactant utilisé dans celles-ci (« polyethoxylated tallow amine ou POEA). Cette synthèse qui examine pas à pas les différents volets toxicologiques, indique que « *l'évidence expérimentale montre que ni le glyphosate, ni l'AMPA ne s'accumulent dans aucun des tissus, qu'aucune toxicité significative ne se manifeste dans les études aiguës, subaiguës ou chroniques, quil n'existe pas de preuve convaincante de lésions directes de l'ADN in vitro ou in vivo et donc que le Roundup et ses formulants ne posent pas de risque en terme de production de mutations héréditaires/somatiques, ….que le glyphosate n'est pas cancérogène, que le glyphosate, l'AMPA et le POEA ne sont pas toxiques pour la reproduction ou le développement… que les études standardisées sur ces molécules ne montrent pas d'effet sur la modulation endocrinienne…* » et conclut donc que « *dans les conditions actuelles et envisageables d'usage, le Roundup ne pose pas de risque pour l'homme* » …La référence à cette publication apparaît donc aberrante !

2.2.2.4. Pour étayer leur préoccupations, les auteurs se réfèrent à la publication de Marc J et al[vii], rapportant une perturbation du cycle cellulaire induite par le Roundup dans l'oeuf d'oursin.

- Cette étude sur le Roundup (170 g/l de glyphosate isopropylamine) et le glyphosate (Cluzeau Info Labo, France) rapporte :

  - l'induction dose dépendante d'un retard de la première division de l'embryon d'oursin, significative après exposition pendant 6 heures à une concentration de 0.8% de Roundup, avec arrêt de la division pour une concentration de 1% (effet objectivable pour des expositions d'au moins 1 heure dans une fenêtre n'excédant pas 1 heure après la fertilisation).

- aucun effet du glyphosate pur sur la cinétique de la première mitose dans une gamme de concentration de 1 à 20 mM (correspondant respectivement à la quantité de glyphosate présente dans 0.1 – 2% de Roundup)

- l'induction d'un retard mitotique par le glyphosate (1 -10 mM) en présence de 0.2% de Roundup (sachant que le seul Roundup n'entraîne pas d'effet sur le cycle à cette concentration), ce qui conduit les auteurs à évoquer un effet synergique

- l'absence d'effet du Roundup sur la létalité ou de dommages sur le développement ultérieur des œufs (études cytologiques)

- l'absence d'effet *in vitro* du Roundup et du glyphosate sur d'activité du complexe CDK1/cycline B (contrôlant l'entrée en phase M du cycle) mais l'induction *in vivo* d'un retard d'activation de ce complexe comme le démontre la suppression complète de l'activité kinase H1 et la forte inhibition de la synthèse protéique (sans réduction de la synthèse de cycline B) en présence de 0.8% de Roundup. Cette observation conduit les auteurs à faire l'hypothèse d'une action du Roundup sur une protéine, encore méconnue, nécessaire à l'activation du complexe CDK1/cycline B !

- Ces résultats conduisent les auteurs à conclure qu'une altération du cycle cellulaire peut être induite par des concentrations de Roundup excédant largement celles des usages herbicides (les concentrations en résidus dans l'eau et les sols étant de l'ordre de la nanomole alors que celles induisant une perturbation du cycle sont de l'ordre de la millimole) ***mais...***:

- que cet écart de concentrations peut être partiellement compensé par les durées d'exposition très différentes (?)

- que la cible initiale chez l'embryon n'est pas encore identifiée et pourrait être affectée par des concentrations beaucoup plus basses de Roundup

- que 100% des cellules sont atteintes dans leur expérimentation alors que cancérogenèse procède à partir de quelques cellules d'où la possibilité d'un effet de concentrations beaucoup plus basses

- que les surfactants contenus dans le Roundup agissent sur le cycle cellulaire de façon synergique avec le glyphosate, ce qui indique l'existence d'un effet du seul glyphosate (?)

- Cette publication appelle différents types de critiques :

- seul de Roundup peut être considéré comme perturbant la cinétique du cycle cellulaire et les conséquences de cet effet restent obscures puisque les œufs ne présentent aucune anomalie décelable à l'examen microscopique et se développent normalement par la suite

- en l'absence d'effet du glyphosate sur le cycle, aucune conclusion valide ne peut être proposée sur l'existence d'un éventuel effet synergique avec les formulants du Roundup (seule une expérimentation comportant une étude des formulants, du glyphosate et du mélange formulants / glyphosate serait à même de le démontrer)

- la sensibilité du modèle d'embryon d'oursin à différents stress n'est pas discutée, notamment aux agents surfactants (Amouroux I et al, 1999)

- les conclusions tirées par les auteurs relèvent davantage de conjecture que de faits scientifiques avérés ; aucune place n'est réservée à la mise en perspective des

données toxicologiques publiées sur le glyphosate en particulier la mutagenèse et la cancérogenèse

- En conclusion, cette étude devrait être uniquement considérée comme susceptible d'apporter au plus, un éclairage sur un mécanisme d'action potentiel, mais dépourvu de pouvoir prédictif pour la cancérogenèse (un retard de cycle pouvant de ce point de vue être plutôt considéré comme bénéfique dans la mesure où il permet généralement à la cellule de mieux réparer des lésions !). De tels résultats ne peuvent remettre en cause l'ensemble des expérimentations attestant du caractère non mutagène et non cancérogène du glyphosate.

2.2.2.5. Pour étayer leur expérimentation sur l'aromatase, les auteurs se réfèrent à la publication de Walsch L.P. et al[xiii], montrant une inhibition de la stéroïdogenèse induite par le Roundup (180 g/l ; origine non spécifiée) dans une lignée cellulaire tumorale de cellules de Leydig de souris.

- Cette étude rapporte :

  - Une diminution dose dépendante de la synthèse de progestérone induite uniquement par le Roundup (CI_{50} = 24.4 ± 0.67 µg/mL) sans diminution concomitante de la synthèse des protéines ; par contre, le glyphosate (origine ?) ne perturbe ni la synthèse des stéroïdes, ni celle des protéines, dans une gamme de concentrations de 0 à 100 µg/mL (résultats non rapportés dans la publication), d'où l'hypothèse de cible (s) spécifique (s) au Roundup.

  - Une inhibition d'activités enzymatiques de la stéroïdogenèse (clivage - P450 de la chaîne latérale du cholestérol, 3 béta-hydroxystéroïde déshydrogénase) après 2 heures d'exposition à 25 µg/mL de Roundup, totalement réversible après cessation de l'exposition, mais insuffisante pour expliquer la chute de la synthèse des stéroïdes (résultat tiré d'expérimentations combinant la stimulation par une dibutyryl cAMP, un précurseur d'hydroxycholestérol ...). De plus, ni la teneur mitochondriale de ces enzymes, ni celle des ARNm correspondants ne sont diminuées en proportion suffisante pour expliquer l'inhibition de la stéroïdogenèse.

  - Par contre, le Roundup réduit significativement la teneur en protéine StaR[2], (impliquée dans le passage du cholestérol au travers de la membrane mitochondriale), sans réduire le taux d'ARNm correspondant, indiquant une perturbation de la régulation post-transcriptionnelle, en aval de l'activité kinasique, le Roundup ne diminuant pas l'activité de la phosphokinase A (PKA).

- Bien que le mécanisme par lequel le Roundup perturbe la régulation post-transcriptionnelle de la protéine StaR reste à élucider, les auteurs soulignent que ce phénomène résulte d'un ou plusieurs formulants, puisque le glyphosate n'altère pas la stéroïdogenèse.
- Ces résultats ont été remis en cause par la firme (document 1.3.2.) qui fait état de travaux[x] effectués en liaison avec les laboratoires de recherche académique (documents non fournis), montrant que la diminution de la synthèse de progestérone dans cette lignée MA-10 de cellules de Leydig relèverait d'une atteinte de la membrane mitochondriale ; ces résultats ont été récemment rappelés dans un abstract disponible sur internet (Farmer D.R. et al, 2005).

3. Conclusion :

Les effets de perturbation endocrinienne du Roundup, voire du glyphosate avancés par Richard et al, de même que le potentiel de perturbation du cycle cellulaire et ses

[2] Steroidogenic Acute Regulatory Protein

conséquences mutagène et cancérogènes avancées par Marc J. et al n'apportent pas d'éléments pertinents pour démontrer une toxicité pour l'homme, sachant que :

3.1. Les conclusions ne reposent que sur des expérimentations *in vitro* portant sur des modèles cellulaires non validés, non représentatifs (lignées tumorales, œuf d'oursin) exposés directement à des concentrations supra-physiologiques des substances.

3.2. Un large spectre de d'études réglementaires de mutagenèse, de cancérogenèse, et de toxicité pour la reproduction ne permettent pas de mettre en évidence d'effet du glyphosate aux plus fortes concentrations testées. L'U.E. a d'ailleurs utilisé un facteur de sécurité de 100 sur la base d'autres effets observés dans l'étude à long terme sur le rat, pour fixer la DJA du glyphosate (0.3 mg/kg).

3.3. Aucune étude épidémiologique ne permet d'incriminer directement le glyphosate ou les formulations de Roundup en matière d'effets sur la reproduction.

3.4. Fait remarquable, le Roundup apparaît plus «actif » que le glyphosate sur les divers paramètres biologiques mesurés. Ce phénomène est également observé dans d'autres modèles cellulaires utilisés pour examiner le cycle cellulaire (œuf d'oursin) ou la synthèse des hormones stéroïdiennes (cellules tumorales de Leydig). De telles observations conduisent, à l'évidence, à mettre en cause l'effet des surfactants sur les membranes cellulaires et/ou mitochondriales, d'autant que plusieurs publications en démontrent la nocivité sur un grand nombre de paramètres biologiques. Il est donc hautement probable, comme le suggèrent certains auteurs eux –mêmes, que l'exposition directe de cellules à ces formulants puisse expliquer l'ensemble des effets constatés dans toutes ces expérimentations *in vitro*.

3.5. Les auteurs sur-interprètent leurs résultats en matière de conséquences sanitaires potentiels pour l'homme (références inadéquates, extrapolation in vitro-in vivo non étayée…)

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[i] Amouroux I., Pesando D., Noël H., Girard J-P. Mechanisms of cytotoxicity by cosmetic ingredients in sea urchin eggs, Arch. Environ. Contam. Toxicol., 36, 28-37, 1999

[ii] Farmer D.R., Levine S.L., Heydens W.F., Garnett R., Han Z., Papadopoulos V., Mitochondrial mediated effects of surfactant on MA-10 cells steroidogenesis, Abstracts/Toxicological Letters, 158S (2005) – S258

[iii] Acquavella J.F., Bruce H., Alexander B.H., Mandel J.S., Gustin C., Baker B., Champan P., Bleeke M., Glyphosate biomonitoring for farmers and their families: results from the farm family exposure study. Environ. Health Perspect., 112: 321-326, 2004

[iv] Savitz D.A., Arbuckle T., Kaczor D., Curtis K.M., Male pesticides and pregnancy outcome, Am. J. Epidemiol., 146, 1025-1036, 2000

[v] Arbuckle T., Linz Z., Mery L., An explanatory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. Environ. Health Perspect., 109: 851-857, 2001

[vi] Williams G.M., Kroes R., Munro I.C., Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans, Regul. Toxicol. Pharmacol., 31: 117-165, 2000

[vii] Marc J., Mulner-Lorillon O., Boulben S., Hureau D., Durand G., Bellé R., Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. Chem. Res. Toxicol., 15: 326-331, 2002

[viii] Walsch L.P., McCormick C., Martin C., Stocco D.M., Roundup inhibits steroidogenesis by disruptiong steroidogenic acute regulatory (StAR) protein expression, Envrion. Health Perspect., 108: 769-776, 2000

[ix] Levine S.L., Farmer D.R., Heydens W.F., Han Z., Wall C., Papadopoulos V., Non-specific alteration of steroidogenesis in vitro by supra-physiological levels of surfactant, Society of Environmental Toxicology and chemistry, 22[nd] annual meeting abstracts, 2003

[8] Heydens W.F., Levine S.L., Farmer D.R., Ha, Z., Wall C., Papadopoulos V., Non-specific alteration of steroidogenesis in Ma-10 Leydig cells by supra-physiological concentrations of the surfactant in Roundup herbicide, Toxicologist, 131, 2003

Enquiry into the referral
of the Committee for the Study of Toxicity by the DGAL regarding the article

"Differential effects of glyphosate and Roundup on human placental cells and aromatase."
Richard S., Moslemi S., Sipahutar H., Benachour N., Seralani G.E.,
Environ. Health Perspect., 2005 (in the press; online 24 February 2005)

1. Documents received:

1.1. Letter from the SSM dated 21 Nov. 2005; appointment of the referral reporter

1.2. Letter from the DGAL (Directorate General for Food) dated 14 April 2005 (illegible reference: 050 ??51): asking Monsanto Agriculture France SAS for comments on the referenced article for sending to the European Commission and to the reporting country (Germany)

1.3. Letter from Monsanto dated 26 April 2005 (reference ILG/YF/206): copy for the DGAL of the dossier sent to DG Sanco and to Germany (letter dated 22 April 2005) and enclosed documents:

1.3.1. Publication by Richard S. et al. 2005

1.3.2. Reply from Monsanto dated 2 March 2005 regarding the publication by Richard S. et al. (written by: Donna R. Farmer, Daniel A. Goldstein, Monsanto, St. Louis, Missouri)

1.3.3. Publication by Marc J. et al., *"Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation"*, Chem. Res. Toxicol., 2002, 15, 326-331

1.3.4. Reply from Monsanto dated 2 March 2005 regarding the publication by Marc J. et al. (confidential document strictly for internal use in the company only)

1.3.5. PubMed abstract of the publication by Amouroux I. et al., "*Mechanisms of cytotoxicity by cosmetic ingredients in sea urchin eggs*", Arch. Environ. Contam. Toxicol., 1999, 36 (1), 28-37.

1.3.6. Comments by Fellous et al. dated 21 March 2005 regarding the publication by Richard S. et al.

1.4. Letter from the DGAL dated 20 May 2005 (reference 0500204): copy of the Monsanto dossier for the SSM mentioning the referral of the chairman of Com Tox in view of the examination of the publication by Richard S. et al. and of the reply from Monsanto for June 2005

1.5. Letter from the DGAL dated 27 May 2005 (reference 0500254): report of the referral examination (to be included in the agenda for December 2005)

1.6. Letter from the DGAL dated 12 October 2005 (reference 0500561): sending to the SSM of an electronic copy of two documents and the German comments on the publication by Richard S.

1.7. CD-ROM containing the electronic version of documents 1.3, 1.3.1., 1.3.2., 1.3.3., 1.3.4., 1.3.6., and the complete version of Amouroux I. et al.

2. Analysis of the publication *"Differential effects of glyphosate and Roundup on human placental cells and aromatase"* (Richard et al., Environ. Health Perspect. 2005)"

2.1. Summary of the data:

2.1.1. The toxicity of Glyphosate (Sigma-Aldrich) and of Roundup ("commercial"; 360 g/l of glyphosate), alone or combined, is tested on a line of human placental JEG3 cells by measuring cellular viability (MTT test) and *in vitro* aromatase activity (radio-immunological dosage) searching for the mechanism that modulates it (quantification of the mRNA), after exposure for 1, 18, 24 or 48 hours at concentrations that are said to be "representative" of the recommended uses (2% of Roundup or equivalent concentration of glyphosate with pH adjustment) or lower levels. In addition, the aromatase activity of microsomes from the placentas of non-smoker females and from horse testicles was measured using the same exposure parameters, and the activity of purified enzymes from horse testicles was determined (spectral studies, measurement of the reductase NADPH activity).

2.1.2. The results show:

2.1.2.1. a **considerable reduction of the viability** of JEG3 cells (few data assessed, multiple curves),

- the reduction for Roundup was twice that for glyphosate
- depending on the duration of exposure: documentable for 1 h and trebling for 18 h of exposure …
- depending on the concentration: first survival dropping point (around 20%) for 18 h of exposure with 0.2% of Roundup and 0.8% of glyphosate, and zero viability for 18 h of exposure to 0.4% of Roundup and 1.9% of glyphosate),
- cannot be explained by the acidity of the solutions (pH = 5.8)
- increased for glyphosate in the presence of 0.1% of Roundup (for all concentrations of glyphosate).

2.1.2.2. a **considerable inhibition of the aromatase activity** of the JEG3 cells (few data assessed, multiple curves)

- only for Roundup for 18 hours of exposure with a concentration of more than 0.01% (IC50 = 0.04%) while an exposure of one hour increases the activity by around 40% regardless of the concentration (tested range: 0.01% to 0.2%)
- only present for the glyphosate when adding Roundup at 0.02% (2 concentrations tested: 0.18% and 0.38% of glyphosate)
- attributed to an effect on the expressivity of the gene CYP19 (drop of the mRNA)

2.1.2.3. a **considerable inhibition of the microsomal aromatase activity** of the human (placenta) and equine (testicle) cells

- 3 times greater for Roundup (IC50 = 0.6%) than for glyphosate
- identical in the 2 cellular models tested
- attributed to direct interaction of glyphosate on the active site (spectral studies)
- associated with a smaller reduction of the reductase NADPH activity (IC50 = 5%)

2.1.3. In their comments on these results, the authors of the publication stress the following points:

2.1.3.1. The effect on the viability of JEG3 cells (ten times greater for Roundup than for glyphosate) was highlighted by means of:

- using a serum-free medium, ensuring greater bioavailability and thereby optimising the detection of the effect
- long exposure times, enabling a "*genomic action*" and a bioconcentration (with several references supporting the toxic or genotoxic effects and the concentration of Roundup: Peluso M. et al., 1998, Lioi M.B. et al., 1998, Mitchell D.G. et al., 1987, Vigfusson N.V. et al., 1980 and Yousef M.I. et al., 1995)

2.1.3.2. The aromatase inhibition in JEG3 cells is induced by exposure with sufficient duration to non-toxic concentrations of Roundup on its own (it being assumed that the stimulation observed for a short exposure time is due to increased membrane permeability and improved bioavailability in the substrate, induced by the presence of Roundup adjuvants).

2.1.3.3. This aromatase inhibition, which could result in a direct action on the enzyme, is confirmed by measurements on human and equine microsomes which also show an inhibiting potential of glyphosate (4 times less than that of Roundup).

2.1.3.4. The addition of low concentrations of Roundup to the glyphosate would grant the latter a cytotoxic and aromatase-inhibiting potential, bioavailability being enhanced by the adjuvants.

2.1.4. The authors therefore conclude that:

- there exists a potential for endocrine disorders inducible in mammals by glyphosate concentrations 100 times lower than those used for farming purposes.

- the direct action of glyphosate on the aromatase can explain certain reprotoxic effects observed *in vivo*, supported mainly by several bibliographical references that report pregnancy problems among users of glyphosate-based herbicides (Savitz D.A. et al. 2000), a disruption of the cell cycle in sea urchin eggs (Marc J. 2002) or even post-transcriptional expression of a protein regulating steroidogenesis in mouse tumoural Leydig cells (Walsch L.P. et al., 2000)

2.2. Comments:

2.2.1. In terms of its contents, this publication, which aims to put forward a suspicion of reprotoxic effects by means of a potential endocrine disorder mechanism, has several **major methodological gaps**:

2.2.1.1. A considerable number of the *in vitro* experiments were conducted on JEG3 cells, a tumoural human cell line (potential proven by grafting onto nude mouse) derived from a choriocarcinoma having a hypertriploid karyotype (70 chromosomes on average) with 5 chromosomes which are highly altered and cannot be classified and a single X chromosome. These cells are capable of transforming the steroid precursors into oestrone and into oestradiol. Such a karyotypical profile can grant the cellular model a response specificity that ensures no final conclusions can be obtained without validating the reproducibility of the observations in

other, mainly non-placental, cell lines.

2.2.1.2. Although claimed to be representative of or even 10 times lower than those used for farming applications, the concentrations of Roundup used in the various experiments of this publication must, in fact, be considered to be extremely high, since we are dealing with direct exposure of cells or sub-cellular fractions.

- From the diagrams, an alteration of cellular viability appears with concentrations of more than 0.2% and aromatase inhibition with concentrations of around 0.02% of Roundup, which respectively corresponds to 700 and 70 mg/l of glyphosate. Taking into account the limiting factors represented by oral absorption (30%), skin absorption (0.3%) and elimination kinetics (less than 1% of tissue waste after 7 days), such levels would involve considerable human exposure, or several dozen litres of Roundup diluted at 2%.

- In its comments on the publication, the reporting country (Germany) stresses that the 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.

2.2.1.3. A noteworthy fact, if the effects of Roundup on cellular viability and aromatase inhibition (measured in cells and microsomes) are greater than those of glyphosate (having no noticeable inhibiting effect on the cells), is that the addition of very low levels of Roundup would grant the glyphosate a considerable cytotoxic effect (in the presence of 1% of Roundup) and aromatase inhibition (in the presence of 2% of Roundup), the phenomenon being attributed by the authors to an enhancement of the effects of the glyphosate by the Roundup adjuvants. Such an interpretation seems contestable, all the more so since the authors mention a "*multiplying effect of Roundup on the endocrine effect of the glyphosate*" in their conclusion, knowing that:

- The experimental protocol does not make it possible to show the influence of the adjuvants or, *a fortiori*, a synergic effect with the glyphosate, since Roundup also contains glyphosate (it would be necessary to obtain independent data regarding the adjuvants, glyphosate and the connection between the two)

- The toxicity of the adjuvants is not argued, and no reference is made to the results published in this field, notably:

    - The multiple non-specific effects of surfactant agents on a broad range of cellular targets, such as attacks on membrane permeability or integrity following the induction of an ionic unbalance (work by Amouroux I et al.[i] regarding sea urchin eggs)

    - Attacks on the mitochondrial membrane induced by various surfactant agents intended for household use (Farmer D.R. et al.[ii])

- No analysis of the consistency of the various results seems to have been carried out, for example placing the effects of pH on the cellular model used in perspective (stressing that they adjusted the pH of the glyphosate solutions to that of the Roundup solutions, which is a pH of 5.8, the authors specify that this acidification can only explain a 23% reduction in cell survival after 18 hours of exposure, which is out of proportion to that induced in the experiments; the fact remains that ¼ of cell toxicity results from acidity!!!).

2.2.2. In terms of its form, this publication comprises **multiple instances of bias in its arguments and its interpretation** of the data:

2.2.2.1. To support the risk of human exposure to glyphosate, the authors refer to the works of Acquavella J.F. et al.[iii], reporting maximum levels of exposure to glyphosate of 0.004 mg/kg among farmers and their families, values with no common measurement through experimentation.

2.2.2.2. To support the effects of glyphosate on reproduction and growth, the authors refer to the works of Savitz D.A. et al.[iv], which report a moderate increase in the risk of premature births among the wives of farmers who were exposed, three months before the pregnancy began, to a large variety of pesticides including atrazine, glyphosate, organophosphates, 2-4D, etc. In a later publication (Arbuckle T.E. et al.[v]) on the same issue ("Ontario Farm Family Health Studies"), an increase on the limit of statistical significance in the risk of late miscarriages after pre-conception exposure to glyphosate is apparent in the absence of an adjustment for possible confusion factors, so that the interviewers themselves consider this result to be exploratory, mainly bearing in mind the limitations relating to the measurement of exposures, which were assessed using questionnaires, investigation complements being provided for specifying the molecules incriminated using validated biological markers of exposure.

2.2.2.3. To support their lack of knowledge regarding the glyphosate action mechanism and the possibility of multiple enzymatic effects, the authors refer mainly to the publication by Williams G.M. et al.[vi], which consists of a review of the regulatory studies published on glyphosate and its main metabolite (aminoethylphosphonic acid or AMPA) as well as on the formulations of Roundup and the main surfactant agent used in it ("polyethoxylated tallow amine or POEA"). This summary, which examines the various toxicological constituents step by step, states that "*the experimental evidence shows that neither glyphosate nor AMPA tend to concentrate in any of the tissues, that no considerable toxicity is observed in the critical, sub-critical or chronic studies, that there is no convincing proof of direct lesions of* in vitro *or* in vivo *DNA, and Roundup and its formulants do not therefore pose any risks as regards the production of hereditary/somatic mutations, …that glyphosate is not carcinogenic, that glyphosate, AMPA and POEA are not toxic for reproduction or growth… that the standardised studies of these molecules show no effect on endocrine modulation…*" and therefore concludes that "*in current and foreseeable conditions of use, Roundup does not pose any risk to humans*" …The reference to this publication therefore appears to be absurd.

2.2.2.4. In order to support their concerns, the authors refer to the publication by Marc J. et al.[vii], which reports a disruption of the cell cycle induced by Roundup in sea urchin eggs.

- This study of Roundup (170 g/l of glyphosate isopropylamine) and glyphosate (Cluzeau Info Labo, France) reports:

  - the dose-dependent induction of a delay in the first division of the sea urchin embryo, considerable after six hours of exposure to a concentration of 0.8% of Roundup, with the division stopping with concentrations of 1% (documentable effect for exposures of less than 1 hour in a window not exceeding 1 hour after fertilisation).

- no effects of pure glyphosate on the kinetics of the first mitosis in a range of concentration from 1 to 20 mM (respectively corresponding to the amount of glyphosate present in 0.1% to 2% of Roundup)

- the induction of a mitotic delay by the glyphosate (1-10 mM) in the presence of 0% - 2% of Roundup (knowing that Roundup alone has no effect on the cycle at this concentration), which leads the authors to imply a synergic effect

- the absence of any effect from Roundup on egg lethality or damage to its subsequent growth (cytological studies)

- the absence of any *in vitro* effects of Roundup and glyphosate on the activities of the CDK1/cyclin B complex (controlling the entry into phase M of the cycle) but the *in vivo* induction of a delay in the activation of this complex as shown by the complete suppression of H1 kinase activity and the considerable inhibition of protein synthesis (with no reduction of cyclin B synthesis) in the presence of 0.8% of Roundup. This observation leads the authors to establish the hypothesis that Roundup acts on a protein, unknown to date, which is required for activating the CDK1/cyclin B complex!

- These results lead the authors to conclude that an alteration of the cellular cycle can be induced by concentrations of Roundup that greatly exceed those of herbicidal uses (the residual concentrations in water and soil being of around one nanomole, while those causing a disruption of the cycle are of around one millimole), **but...**:

- that this difference of concentrations can be partially compensated by very different exposure times (?)

- that the initial target in the embryo is not yet identified and may be affected by much lower concentrations of Roundup

- that 100% of cells are attacked during the experiment, while carcinogenesis comes from several cells, resulting in the possibility of an effect with much smaller concentrations

- that the surfactant agents contained in Roundup act on the cellular cycle in synergy with glyphosate, which shows the existence of a single glyphosate (?)

- This publication attracts various types of criticism:

- only Roundup can be considered as disrupting the kinetics of the cell cycle and the consequences of this effect remain obscure since the eggs present no anomalies that can be detected under microscopic examination and then develop normally

- in the absence of any effect of glyphosate on the cycle, no valid conclusions can be proposed regarding the existence of a possible synergetic effect with the Roundup formulants (only an experiment comprising a study of the formulants, glyphosate and the mix of formulants/glyphosate would be able to prove it)

- the sensitivity of the sea urchin embryo model to different types of stress, mainly to surfactant agents, is not questioned (Amouroux I et al., 1999)

- the conclusions drawn by the authors are based more on speculation than on hard scientific facts; no place is reserved for placing the toxicological data published regarding

glyphosate in perspective, in particular mutagenesis and carcinogenesis

- In conclusion, this study should only be considered to be capable, at most, of shedding some light on a potential action mechanism, but to be devoid of any power to predict carcinogenesis (a delay in the cycle can, according to this point of view, be considered to be more beneficial insofar as it generally allows the cell to repair lesions better). Such results cannot be used to question all the experiments that prove the non-mutagenic and non-carcinogenic nature of glyphosate.

2.2.2.5. To support their experiments on aromatase, the authors refer to the publication by Walsch L.P. et al.[viii], showing an inhibition of steroidogenesis induced by Roundup (180 g/l; source not specified) in a tumoural cell line of mouse Leydig cells.

- This study reports:

  - A dose reduction depending on the synthesis of progesterone induced only by Roundup (CI_{50} = 24.4 ± 0.67 µg/ml) with no concomitant reduction of protein synthesis; on the other hand, glyphosate (source ?) does not disrupt neither the synthesis of steroids nor that of proteins, in a range of concentrations from 0 to 100 µg/ml (results not reported in the publication), which results in the hypothesis of target/s that is/are specific to Roundup.

  - An inhibition of the enzymatic activities of steroidogenesis (segmentation – P450 of the lateral cholesterol chain, 3 beta-hydroxysterioid dehydrogenase) after 2 hours of exposure to 25 µg/ml of Roundup, is fully reversible after the end of the exposure time, but not enough to explain the drop in steroid synthesis (result taken from experiments combining stimulation by a cAMP dibutyryl, a precursor of hydroxycholesterol …). In addition, neither the mitochondrial level of these enzymes nor of the corresponding mRNA are reduced far enough to explain the inhibition of steroidogenesis.

  - On the other hand, Roundup considerably reduces the level of the StAR protein [2] (involved in cholesterol passing through the mitochondrial membrane) without reducing the corresponding mRNA rate, indicating a disruption of post-transcriptional regulation, before kinasic activity, since Roundup does not reduce phosphokinase A (PKA) activity.

- Although the mechanism used by Roundup to disrupt post-transcriptional regulation of the StAR protein remains unknown, the authors stress that this phenomenon is produced by one or several formulants, since glyphosate does not alter steroidogenesis.
- These results have been questioned by the firm (document 1.3.2.) which reports on work[ix] [x] performed in cooperation with academic research laboratories (documents not supplied), showing that the reduction of progesterone synthesis in this MA-10 line of Leydig cells would show an attack on the mitochondrial membrane; these results were recently recalled in an abstract available on the internet (Farmer D.R. et al., 2005).

3. Conclusion:

The endocrine disorder effects of Roundup or even of glyphosate put forward by Richard et al., as well as their potential for disrupting the cellular cycle and its mutagenic and carcinogenic

[2] Steroidogenic Acute Regulatory Protein

consequences put forward by Marc J. et al. do not provide any elements that are relevant for proving toxicity in humans, knowing that:

3.1. The conclusions are only based on *in vitro* experiments relating to non-validated, non-representative cellular models (tumoural lines, sea urchin eggs) which were directly exposed to supra-physiological concentrations of the substances.

3.2. A broad spectrum of regulatory studies of mutagenesis, carcinogenesis and toxicity for reproduction does not provide evidence of the effect of glyphosate with the highest concentrations tested. The EU has furthermore used a safety factor of 100 on the basis of other effects observed in the long-term study on rats, for fixing the ADA of glyphosate (0.3 mg/kg).

3.3. No epidemiological studies allow direct incrimination of glyphosate or the formulations of Roundup as regards their effects on reproduction.

3.4. Remarkably, Roundup appears to be more “active” than glyphosate in the various biological parameters measured. This phenomenon is also observed in other cellular models used to examine the cellular cycle (sea urchin eggs) or the synthesis of steroidal hormones (Leydig tumoural cells). Such observations lead, evidently, to questioning the effect of surfactant agents on cellular and/or mitochondrial membranes, while several publications prove their toxicity across a very large number of biological parameters. It is therefore highly possible, as certain authors suggest, that direct exposure of cells to these formulants can explain all the effects found in all these *in vitro* experiments.

3.5. 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.).

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[i] Amoroux I, Pesando D., Noel H., Girard J-P. Mechanisms of cytotoxicity by cosmetic ingredients in sea urchin eggs, Arch. Environ. Contam. Toxicol., 36, 28-37, 1999

[ii] Farmer D.R., Levine S.L., Heydens W.F., Garnett, R., Han Z. Papadopoulos V., Mitochondrial mediated effects of surfactant on MA-10 cells steroidogenesis, Abstracts/Toxicological Letters, 158S (2005) – S258

[iii] Acquavella J.F., Bruce H., Alexander B.H., Mandel J.S., Gustin C., Baker B., Chapman P., Bleeke M., Glyphosate biomonitoring for farmers and their families: results from the farm family exposure study. Environ. Health Perspect., 112: 321-326, 2004

[iv] Savitz D.A., Arbuckle T., Kaczor D., Curtis K.M., Male pesticides and pregnancy outcome, Am. J. Epidemiol., 146, 1025-1036, 2000.

[v] Arbuckle T., Linz M., Mery L., An explanatory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population, Environ. Health Perspect., 109: 851-857, 2001

[vi] Williams G.M., Kroes R., Munro I.C., Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate for humans, Regul. Toxicol. Pharmacol., 31: 117-165, 2000

[vii] Marc J., Mulner-Lorillon O., Boulben S., Hureau D., Durand G., Bellé R., Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation, Chem. Res. Toxicol., 15: 326-331. 2002

[viii] Walcsh L.P., McCormick C., Martin C., Stocco D.M., Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression, Environ. Health Perspect., 108: 769-776, 2000

[ix] Levine S.L., Farmer D.R., Heydens W.F., Han Z., Wall C., Papadopoulos V., Non-specific alteration of steroidogenesis in vitro by supra-physiological levels of surfactant, Society of Environmental Toxicology and Chemistry, 22nd annual meeting abstracts, 2003

[x] Heydens W.F., Levine S.L., Farmer D.R., Han Z., Wall C., Papadopoulos V., Non-specific alteration of steroidogenesis in Ma-10 Leydig cells by supra-physiological concentrations of the surfactant in Roundup herbicide, Toxicologist, 131, 2003

AGENCE FRANÇAISE
DE SÉCURITÉ SANITAIRE
DES ALIMENTS

LA DIRECTRICE GENERALE

Maisons-Alfort, le 26 mars 2009

AVIS

de l'Agence française de sécurité sanitaire des aliments relatif au glyphosate et aux préparations phytopharmaceutiques à base de cette substance active

L'Agence française de sécurité sanitaire des aliments (Afssa) a été saisie le 28 janvier 2009 par la Direction générale de la santé et la Direction générale de l'alimentation d'une demande d'avis relatif au glyphosate et aux préparations phytopharmaceutiques à base de cette substance active suite à la publication dans la revue scientifique "Chemical Research in Toxicology" d'un article intitulé "*Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic and placental cells*" de Nora Benachour et Gilles-Eric Séralini, article paru le 23 décembre 2008 sur internet.

Il est demandé à l'Afssa d'analyser ces travaux afin de déterminer s'ils sont de nature à remettre en cause les autorisations accordées pour toutes les spécialités phytopharmaceutiques à base de glyphosate ou de modifier leurs conditions d'utilisation.

Après consultation du Comité d'experts spécialisé "Produits phytosanitaires : substances et préparations chimiques", réuni les 24 et 25 mars 2009, l'Agence française de sécurité sanitaire des aliments émet l'avis suivant.

CONTEXTE

Cet article fait suite à deux autres articles parus précédemment provenant de la même équipe[1, 2] qui étudient *in vitro* les effets cytotoxiques du glyphosate et de préparations à base de cette substance active. Dans l'ensemble des articles, cette équipe traite des cellules en culture avec le glyphosate seul, son métabolite majeur l'AMPA[3] et/ou l'une ou plusieurs de ses préparations.

Méthodologie

La toxicité du Glyphosate (G) (origine Sigma-Aldrich), du métabolite AMPA (origine Sigma-Aldrich), du formulant tensioactif POEA (amine de suif poly-éthoxylée[4], fourni par le CNRS/Roscoff) et de 4 formulations de Roundup (origine Monsanto) achetées sur le marché (Roundup Express ou R7.5, Roundup Bioforce ou R360, Grands Travaux ou R400[5] et Grands Travaux plus ou R450, contenant respectivement 7,2 g/L, 360 g/L, 400 g/L et 450 g/L de glyphosate), seuls ou en association, est testée sur 3 modèles cellulaires humains (lignée de cellules tumorales placentaires JEG3, lignée de cellules rénales embryonnaires 293 et cultures primaires de cellules endothéliales de la veine ombilicale HUVEC). Les paramètres mesurés sont la viabilité cellulaire (activité mitochondriale par le dosage de l'activité de la succinate déshydrogénase (SD) à l'aide du test MTT, atteinte membranaire par le dosage de l'adénylate kinase (AK) et apoptose par le dosage des caspases 3 et 7). Ces mesures sont complétées par un examen microscopique pour une analyse morphologique de l'apoptose (marquage DAPI).

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1 Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., and Séralini, G. E. (2005) Differential effects of glyphosate and roundup on human placental cells and aromatase. *EnViron. Health Perspect. 113*, 716– 720.

2 Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., and Séralini, G. E. (2007) Time and dose-dependent effects of roundup on human embryonic and placental cells and aromatase inhibition. *Arch. EnViron. Contam. Toxicol. 53*, 126–133.

3 AMPA : acide aminométhylphosphonique, métabolite trouvé dans les études de métabolisme dans le sol, l'eau, les végétaux et les animaux.

4 Polyethoxylated tallowamine.

5 Préparation ne disposant plus actuellement d'autorisation de mise sur le marché.

Les produits testés sont mis en solution dans le milieu de culture sans sérum à la concentration de 1 ou 2 % qui sont, d'après les auteurs, les concentrations recommandées par le fabriquant pour préparer les bouillies herbicides à appliquer. Le pH de la solution de glyphosate a été ajusté dans le milieu de culture à 5,8, équivalent à celui de la préparation Roundup Bioforce (360 g/L). Les cellules sont mises en contact pendant 24 heures en continu dans le milieu de culture sans sérum à diverses dilutions des solutions initiales.

Résultats

Selon les auteurs, les résultats montrent que :

- le glyphosate et les 4 formulations de Roundup induisent une mortalité cellulaire dans les 3 types cellulaires étudiés, avec "*une toxicité comparable pour chacun, mais à des concentrations différentes*" ;
- un effet du métabolite AMPA et du tensioactif POEA sur la mortalité cellulaire, via l'atteinte mitochondriale (SD) et membranaire (AK) ;
- un effet combiné du glyphosate, du métabolite AMPA ou du tensioactif POEA sur l'intégrité membranaire (combinaisons des composés 2 à 2 en retenant pour chacun la concentration maximale sans effet sur l'activité mitochondriale) ;
- l'induction par le glyphosate et le Roundup Bioforce (R360) d'une mortalité au moins en partie liée à une apoptose médiée par la voie des caspases.

Rappelant les résultats de leurs études antérieures sur le Roundup (effets sur la viabilité cellulaire et la synthèse des stéroïdes hormonaux, potentiel de bioaccumulation), les auteurs en concluent que :

- "*le niveau seuil d'action de l'herbicide doit prendre en compte la période et la durée d'exposition, la présence d'adjuvants, notamment le POEA, le métabolisme et la bioaccumulation ou les effets retardés dans le temps*";
- "*les effets ci-dessus sont démontrés en dessous des dilutions de l'herbicide recommandées en agriculture […] les mélanges disponibles sur le marché peuvent endommager les cellules voire la mort cellulaire aux niveaux résiduels attendus, en particulier dans les denrées alimentaires*".

AVIS DE L'AFSSA

Cette publication appelle plusieurs commentaires d'une part, d'ordre méthodologique et d'autre part, en termes d'interprétation des résultats.

Commentaires d'ordre méthodologique

Les lignées cellulaires employées présentent des caractéristiques qui peuvent être à l'origine d'un biais important dans l'interprétation des résultats :

- la lignée humaine JEG3 est une cellule cancéreuse provenant d'un choriocarcinome humain. Cette lignée est hypertriploïde et présente majoritairement 71 chromosomes (au lieu de 46 dans les cellules humaines). Outre les anomalies génomiques, cette lignée présente de nombreuses anomalies du génome telles que translocations, inversions et délétions : t(4;11)(p15;q13), i(13q), t(10p15q), del(18)(q21) ;
- la lignée de cellules humaines de rein 293 qui est une lignée transformée par un adénovirus 5. Il s'agit d'une cellule cancéreuse, elle est hypotriploïde et présente majoritairement 64 chromosomes (au lieu de 46 dans les cellules humaines), 4.2 % des cellules présentent une ploïdie supérieure. Outre les anomalies génomiques, cette lignée présente de nombreuses anomalies du génome : der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13) ;
- la lignée appelée HUVEC (pour Human Umbilical Vein Endothelial Cells) n'est pas référencée à l'ATCC, elle est commercialisée par une société appelée LONZA. Il s'agit de cellules endothéliales provenant de veines ombilicales humaines. On ne dispose pas d'information sur ces cellules. Les cellules sont utilisées à un passage précoce (5 ou 6).

Pendant la phase d'exposition aux différents produits, les cellules sont maintenues en culture dans un milieu sans sérum ce qui peut conduire à perturber l'état physiologique des cellules. Une telle méthodologie pourrait être acceptable pour des traitements courts (3-4 heures) mais en aucun cas pour des traitements longs de 24 heures. De plus, le glyphosate utilisé dans l'étude est du glyphosate acide alors que dans les préparations testées il est sous forme de sel

d'isopropylamine. Le pH de la solution à la plus forte concentration a été ajusté dans le milieu de culture à 5,8. Pour les dilutions de la solution de glyphosate et des autres préparations testées, aucune précision n'est donnée sur le pH.

La publication ne mentionne aucun témoin positif notamment pour le test d'apoptose.

Commentaires sur les résultats

1 La cytotoxicité du glyphosate apparaît aux concentrations supérieures ou égales à 1 %, soit 3,6 g/L. tandis que l'AMPA n'est que très légèrement plus toxique sur les 3 types cellulaires, en prenant comme marqueur l'activité succinate deshydrogénase mitochondriale.

A ces niveaux de doses, on peut s'interroger sur l'impact du pH et des variations de pression osmotique sur la survie cellulaire.

2 Le glyphosate induit à forte dose de l'apoptose déterminée par l'activité caspase 3/7.

Ces résultats pourraient n'être pas spécifiques de l'effet du glyphosate mais dus à l'effet du pH et/ou à l'osmolalité qui induisent de l'apoptose comme cela a pu être montré dans une étude sur des cellules en culture (Meintières et Marzin, 2004[6]).

3 L'apoptose apparaît plus marquée sur les cellules HUVEC.

Les auteurs ne formulent aucune hypothèse pour expliquer cette observation.

4 Les préparations sont plus toxiques que le glyphosate administré à des doses équivalentes, mais cette augmentation de toxicité peut s'expliquer par l'effet du tensioactif POAE.

En raison des propriétés du tensioactif et de l'augmentation de l'osmolalité du milieu de culture, il est possible que les membranes cellulaires et celles des organites soient désorganisées. De plus, le tensioactif possède une toxicité propre par une action sur les membranes et favorise l'augmentation de la pénétration cellulaire par les constituants du mélange. De très nombreux agents tensioactifs présentent *in vitro* des effets cytotoxiques et inducteurs d'apoptose. C'est par exemple ce qu'a démontré Debbasch *et al.* (2001)[7] avec le chlorure de benzalkonium, par ailleurs largement utilisé en usage local, cutané et oculaire pour la désinfection, sans que cela ne conduise à des effets toxiques inacceptables.

Enfin, concernant les associations AMPA+glyphosate+POEA, les résultats diffèrent d'une lignée à l'autre sans que l'on puisse en tirer des conclusions claires.

Conclusions

1 Les conclusions ne reposent que sur des expérimentations *in vitro* portant sur des modèles cellulaires non validés, non représentatifs (en particulier des lignées tumorales ou transformées) exposés directement à des concentrations de produits extrêmement élevées dans des conditions de culture ne respectant pas les conditions physiologiques cellulaires normales. Ces travaux ne mettent en lumière aucun nouveau mécanisme d'action du glyphosate et des préparations contenant du glyphosate.

2 Un large spectre d'études réglementaires de mutagenèse, de cancérogenèse, et de toxicité pour la reproduction visant à évaluer les effets du glyphosate a permis de définir une dose journalière admissible[8] (DJA). Cette valeur de référence, fixée à 0,3 mg/kg de poids corporel

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6 Apoptosis may contribute to false-positive results in the in vitro micronucleus test performed in extreme osmolality, ionic strength and pH conditions. Meintières S. et Marzin D.. Mutation research. 2004 ; 560(2) : 101-18.

7 Quaternary ammoniums and other preservatives' contribution in oxidative stress and apoptosis on Chang conjunctival cells. Debbasch C, Brignole F, Pisella PJ, Warnet JM, Rat P, Baudouin C Invest Ophthalmol Vis Sci. 2001 Mar;42(3):642-52.

8 DJA : La dose journalière admissible (DJA) d'un produit chimique est une estimation de la quantité de substance active présente dans les aliments ou l'eau de boisson qui peut être ingérée tous les jours pendant la vie entière, sans risque appréciable pour la santé du consommateur, compte tenu de tous les facteurs connus au moment de l'évaluation. Elle est exprimée en milligrammes de substance chimique par kilogramme de poids corporel (OMS, 1997).

et par jour lors de l'évaluation européenne, est fondée sur une dose sans effet observé déduite d'une étude à long terme 2 ans par voie orale chez le rat à laquelle un facteur de sécurité de 100 a été appliqué pour prendre en compte l'extrapolation de l'animal à l'homme.

Dans le cadre des demandes de mise sur le marché, les préparations font également l'objet d'études spécifiques réglementaires qui permettent d'évaluer la toxicité des formulants et les effets cumulatifs potentiels de ces derniers avec la substance active.

L'évaluation prend en compte les effets des formulants, sur la base d'une évaluation des dangers et des risques en utilisant des doses de référence comme la DJA pour le consommateur et l'AOEL[9] pour l'opérateur et l'exposition qui est estimée en se basant sur des modèles ou des données expérimentales.

3 Les formulations de Roundup apparaissent plus "actives" que le glyphosate sur les divers paramètres biologiques mais ce phénomène est également observé dans d'autres modèles cellulaires utilisés pour examiner le cycle cellulaire (œuf d'oursin) ou la synthèse des hormones stéroïdiennes (cellules tumorales de Leydig). De telles observations conduisent, à l'évidence, à mettre en cause l'effet des tensioactifs sur les membranes cellulaires et/ou mitochondriales, d'autant que plusieurs publications en démontrent les effets sur un grand nombre de paramètres biologiques. Il est donc hautement probable que l'exposition directe de cellules à ces formulants puisse expliquer l'ensemble des effets constatés dans toutes ces expérimentations *in vitro*.

4 Les auteurs sur-interprètent leurs résultats en matière de conséquences sanitaires potentielles pour l'homme, notamment fondées sur une extrapolation *in vitro-in vivo* non étayée. Compte tenu des facteurs limitants que représentent l'absorption orale (environ de 30 %), l'absorption cutanée (environ de 3 %), la cinétique d'élimination (présence de moins de 1 % de résidus tissulaires à 7 jours) mesurées dans les études de métabolisme du glyphosate, les teneurs mises en jeu dans ces expérimentations impliqueraient des expositions humaines au glyphosate considérables pour obtenir de tels effets cytotoxiques chez l'homme.

Au regard de ces éléments, l'Agence française de sécurité sanitaire des aliments estime que les effets cytotoxiques du glyphosate, de son métabolite AMPA, du tensioactif POAE et des préparations à base de glyphosate avancés dans cette publication n'apportent pas de nouveaux éléments pertinents qui soient de nature à remettre en cause les conclusions de l'évaluation européenne du glyphosate ni celles de l'évaluation nationale des préparations.

Pascale BRIAND

Mots-clés : glyphosate

[9] AOEL : (Acceptable Operator Exposure Level ou niveaux acceptables d'exposition pour l'opérateur) est la quantité maximum de substance active à laquelle l'opérateur peut être exposé quotidiennement, sans effet dangereux pour sa santé.

afssa

Maisons-Alfort, 26 March 2009

NOTICE

from the French Agency for Food Safety
regarding glyphosate and phytopharmaceutical preparations
based on this active substance

A request was made to the French Agency for Food Safety (Afssa) on 28 January 2009 by the Directorate-General for Health and the Directorate-General for Foods for advice regarding glyphosate and phytopharmaceutical preparations based on this active substance following publication in the scientific journal "Chemical Research in Toxology" of an article entitled *"Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic and placental cells"* by Nora Benachour and Gilles-Eric Séralini, an article published on the internet on 23 December 2008.

Afssa was requested to analyse these works in order to determine whether they were of a nature to call into question the permits granted for all phytopharmaceutical speciality products based on glyphosate or to alter their conditions of use.

Following consultation with the committee of experts specialising in "Phytosanitary products: chemical substances and preparations", which met on 24 and 25 March 2009, the French Agency for Food Safety issued the following notice.

CONTEXT

This article followed two other articles published previously which originated from the same team[1,2] who are studying *in vitro* the cytotoxic effects of glyphosate and preparations based on this active substance. In all of the articles, this team examined cells in culture with glyphosate alone, with its main metabolite AMPA[3] and/or with one or more of its preparations.

Methodology

The toxicity of Glyphosate (G) (obtained from Sigma-Aldrich), the metabolite AMPA (obtained from Sigma-Aldrich), the tensioactive co-formulant POEA[4] (supplied by CNRS, Roscoff) and four formulations of Roundup (obtained from Monsanto) purchased on the market (Roundup Express or R7.5, Roundup Bioforce or R360, Grands Travaux or R400[5] and Grands Travaux plus or R450, containing respectively 7.2 g/L, 360 g/L, 400 g/L and 450 g/L glyphosate), alone or in combination, was tested on three human cell models (placental tumour cell line JEG3, embryonic renal cell line 292 and primary cultures of endothelial cells from the umbilical vein HUVEC). The parameters measured were cell viability (mitochondrial activity by dosage of the succinate dehydrogenase (SD) activity by means of the MTT test, membrane attack by dosage of adenylate kinase (AK) and apoptosis by dosage of caspases 3 and 7). These measures are complemented by a microscopic examination for morphological analysis of the apoptosis (DAPI marking).

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[1] Richard, S., Moslemi, S., Sipahutar, H., Benachour, N. and Séralini, G.E. (2005) Differential effects of glyphosate and roundup on human placental cells and aromatase. *EnViron. Health Perspect. 113*, 716-720.

[2] Banachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., and Séralini, G.E. (2007) Time and dose-dependent effects of roundup on human embryonic and placental cells and aromatase inhibition. *Arch. EnViron. Contam. Toxicol. 53,* 126-133.

[3] AMPA: aminomethyl phosphonic acid, a metabolite found in metabolic studies on soil, water, vegetables and animals.

[4] Polyethoxylated tallow amine.

[5] A preparation currently no longer licensed for marketing.

The products tested were put in solution in the culture medium without serum in concentrations of 1 or 2% which are, according to the authors, the concentrations recommended by the manufacturer to prepare the herbicidal compositions for application. The pH of the glyphosate solution was adjusted in the culture medium to 5.8, equivalent to that of the preparation Roundup Bioforce (360 g/L). The cells were brought into contact for 24 hours continuously in the culture medium without serum in various dilutions of the initial solutions.

Results
According to the authors, the results show:

- That the glyphosate and the four formulations of Roundup induce cell mortality in the three cell types studied, *"each with comparable toxicity, but at different concentrations"*;
- An effect of the metabolite AMPA and the tensioactive POEA on cell mortality, through mitochondrial (SD) and membrane (AK) attack;
- A combined effect of glyphosate, the metabolite AMPA or the tensioactive POEA on membrane integrity (combinations of two compounds, using the maximum concentration for each with no effect on mitochondrial activity);
- Induction by the glyphosate and the Roundup Bioforce (R360) of mortality at least partly connected with apoptosis mediated through the caspases.

Recalling the results of their earlier studies on Roundup (effects on cell viability and hormonal steroid synthesis, bioaccumulation potential), the authors conclude from these that:

- *"the action threshold for the herbicide must take into account the period and the duration of exposure, the presence of adjuvants, in particular POEA, metabolism and bioaccumulation or delayed effects over time"*;
- *"the above effects are demonstrated below the dilutions of the herbicide recommended in agriculture […] the mixes available on the market may damage cells or even (cause) cell death at the residual levels expected, in particular in foodstuffs".*

AFSSA's advice
This publication raises several comments, on the one hand, of a methodological nature, and on the other hand, in terms of interpretation of the results.

Comments of a methodological nature
The cell lines used present characteristics which may be at the source of a significant biais in the interpretation of the results:

- The human line JEG3 is a cancerous cell originating from a human choriocarcinoma. This line is hypertriploid and in the majority of cases presents 71 chromosomes (rather than the 46 in human cells). Apart from the genomic anomalies, this line presents numerous anomalies of the genome such as translocations, inversions and deletions: t(4;11)(p15;q13), i(13q), t(10p15q), del(18)(q21);
- The line of human kidney cells 293 which is a line transformed by an adenovirus type 5. This is a cancerous cell; it is hypotriploid and in the majority of cases presents 64 chromosomes (rather than the 46 in human cells); 4.2% of the cells present higher ploidy. Apart from the genomic anomalies, this line presents numerous anomalies of the genome: der(1)t(1;15)(q42;q13), der(19)t(3;19)(q12;q13), der(12)t(8;12)(q22;p13);
- The line called HUVEC (human umbilical vein endothelial cells) is not referenced in ATCC; it is marketed by a company called LONZA. These cells are endothelial cells from human umbilical veins. No information on these cells is available. The cells are used in early passage (5 or 6).

During the stage of exposure to the various products, the cells are kept in culture in a medium without serum, which could lead to disturbance of the physiological state of the cells. A methodology such as this might be acceptable for short treatments (3-4 hours) but in no circumstances for long treatments of 24 hours. Moreover, the glyphosate used in the study is glyphosate acid, whereas in the preparations tested it is in the form of an isopropylamine salt. The pH of the strongest

concentration of the solution was adjusted in the culture medium to 5.8. For the dilutions of the glyphosate solution and the other preparations tested, no precise information is given about the pH.

The publication does not mention any positive evidence for the apoptosis test.

Comments on the results

1. The cytotoxicity of glyphosate appeared at concentrations equal to or higher than 1%, or 3.6g/L, while AMPA is only very slightly more toxic on the three cell types, taking the mitochondrial succinate dehydrogenase activity as a marker.

   At these dosage levels, the effect of the pH and the variations in osmotic pressure on cell survival may be questioned.

2. The glyphosate in strong doses induces apoptosis determined by the caspase 3/7 activity.

   These results may not be specific to the effect of the glyphosate but due to the effect of the pH and/or osmolality which induce apoptosis, as has been shown in a study on cells in culture (Meintières and Marzin, 2004[6]).

3. Apoptosis appears more marked in the HUVEC cells.

   The authors do not formulate any hypothesis to explain this observation.

4. The preparations are more toxic than glyphosate administered in equivalent doses, but this increase in toxicity can be explained by the effect of the tensioactive POAE.

   Owing to the properties of the tensioactive and the increase in the osmolality of the culture medium, it is possible that the cell membranes and those of the organelles are disorganised. Moreover, the tensioactive has its own toxicity through an action on the membranes and promotes an increase in cell penetration by the constituents of the mixture. A great many tensioactive agents present *in vitro* cytotoxic effects and induce apoptosis. This has been demonstrated for example by Debbasch *et al* (2001)[7] with benzalkonium chloride, which is moreover widely used locally, cutaneously and ocularly for disinfection, without this causing unacceptable toxic effects.

Finally, with regard to the combinations AMPA + glyphosate + POEA, the results differ from one line to the next without it being possible to draw clear conclusions from this.

Conclusions

1. The conclusions rest only on experiments *in vitro* concerning 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. These works do not bring to light any new action mechanism of glyphosate and preparations containing glyphosate.

2. A wide spectrum of statutory studies on mutagenesis, carcinogenesis and reproduction toxicity aimed at assessing the effects of glyphosate have made it possible to define an admissible daily intake (ADI)[8]. This reference value, set at 0.3 mg per kg of body weight per day at the time of the European assessment, is based on a dose with no observed effect deduced from a two-year

[6] Apoptosis may contribute to false-positive results in the in vitro micronucleus test performed in extreme osmolality, ionic strength and pH conditions. Meintières S. and Marzin D.. Mutation research. 2004; 560(2): 101-18.

[7] Quaternary ammoniums and other preservatives' contribution in oxidative stress and apoptosis on Chang conjunctival cells. Debbasch C, Brignole F, Pisella PJ, Warnet JM, Rat P, Baudouin C. Invest Ophthalmol Vis. Sci. 2001 Mar; 42(3): 642-52.

[8] ADI: the admissible daily intake (ADI) of a chemical product is an estimate of the quantity of the active substance present in foods or drinking water which can be ingested every day throughout the consumer's lifetime without appreciable risk to the health of the consumer, taking account of all factors known at the time of the assessment. It is expressed in milligrams of chemical substance per kilogram of body weight (WHO, 1997).

long-term study with oral administration in rats to which a safety factor of 100 has been applied to take account of the extrapolation from animal to human.

In the processing of requests to market a product, preparations are also subjected to specific statutory studies allowing the assessment of the toxicity of the co-formulants and the potential cumulative effects of the latter with the active substance.

The assessment takes account of the effect of the co-formulants, based on an assessment of the dangers and risks using reference doses such as the ADI for consumers and the AOEL[9] for operators and the exposure which is estimated based on models or experimental data.

3. The formulations of Roundup appear more “active” than glyphosate on various biological parameters but this phenomenon is also observed in other cell models used to examine the cell cycle (sea urchin ovum) or the synthesis of steroidal hormones (Leydig tumour cells). Such observations evidently call into question the effect of the tensioactives on the cell membranes and/or mitochondrial membranes, particularly since several publications demonstrate their effects on a large number of biological parameters. It is therefore highly probable that direct exposure of cells to these co-formulants can explain all the effects noted in all of these *in vitro* experiments.

4. 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. Taking account of the limiting factors represented by oral absorption (around 30%), cutaneous absorption (around 3%), elimination kinetics (presence of less than 1% of tissue residues at 7 days) measured in studies on the metabolism of glyphosate, the contents brought into play in these experiments would entail considerable human exposure to glyphosate to obtain such cytotoxic effects in humans.

Taking these factors into consideration, the French Agency for Food Safety judges that the cytotoxic effects of glyphosate, its metabolite AMPA, the tensioactive POAE and other glyphosate-based preparations put forward in this publication do not bring out any pertinent new facts of a nature to call into question the conclusions of the European assessment of glyphosate or those of the national assessment of the preparations.

Pascale BRIAND

Keywords: glyphosate

[9] AOEL: (Acceptable Operator Exposure Level) is the maximum quantity of active substance to which the operator may be exposed daily without danger to his health.

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ORIGINAL ARTICLE

Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis

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Abstract It is well established that surfactants can elicit cytotoxic effects at threshold concentrations by changing the permeability and solubilizing components of cell membranes. The purpose of this study was to characterize the relationship between perturbation of the mitochondrial membrane resulting from treatment with representative cationic, nonionic, and anionic surfactants and the extent to which this perturbation affects steroid formation and StAR protein expression and activity in MA-10 Leydig cells. The StAR protein is synthesized as an active 37 kDa extramitochondrial form, which is processed into a 30 kDa intramitochondrial form after cholesterol transfer and mitochondrial import and processing. It has been shown in several *in vitro* studies that the mitochondrial electrochemical gradient is required for the StAR protein to transfer cholesterol to the inner mitochondrial membrane. Each substance that was tested produced a concentration-dependent decrease in steroid formation in hCG-stimulated MA-10 cells. Decreases in progesterone production were accompanied by loss of mitochondrial membrane potential and by a decrease in the levels of the 30 kDa form of the StAR protein. However, levels of the 37 kDa form of the StAR protein did not decrease, indicating no effect on StAR protein expression. These results demonstrate how perturbation of the mitochondrial membrane by surfactants inhibits import, processing, and cholesterol transfer activity and underscore the importance of including sensitive assays that evaluate mitochondrial function when screening for potential effects on steroidogenesis with *in vitro* test systems.

Keywords Surfactant · Steroidogenesis · Steroidogenic acute regulatory protein · Mitochondria · Leydig cells · Testis

Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
DMEM	Dulbecco's modified Eagle's medium
G3PDH	glyceraldehyde–3–phosphate dehydrogenase
hCG	human chorionic gonadotropin
HPLC	high-performance liquid chromatography
IC_{50}	median inhibitory concentration
LDH	lactate dehydrogenase
MTT	3–(4,5–dimethylthiazol–2–yl)–2, 5–diphenyltetrazolium bromide
P450scc	cytochrome P450 side-chain cleavage
PBS	phosphate buffered saline

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POEA	polyoxyethylenealkylamine
RIA	radioimmunoassay
StAR	steroidogenic acute regulatory

Introduction

It is well established in the scientific literature that surfactants can elicit cytotoxic effects at threshold concentrations. The disturbance of normal cellular function results from changes in the permeability of cell membranes, solubilizing components of cell membranes, and possibly via fusion of cell membranes (Lucy 1970; Dimitrijevic et al. 2000). Disruption of membrane integrity is at the center of many of the observed biological effects of surfactants. Consequently, effects observed when surfactants are applied directly to cells in culture, without considering barriers such as cell membranes and metabolism that may prevent the surfactant from reaching the site of action in the whole animal, must be interpreted with extreme caution. Therefore, when examining the potential for toxicity of surfactants on specific cellular functions cytotoxicity measurements must be included in parallel assays and evaluated carefully.

The primary objective of this study was to understand the relationship between disruption of mitochondrial membrane function caused by commonly used cationic, anionic, and nonionic surfactants and its subsequent effect on steroidogenesis in an *in vitro* test system following acute exposure. For this purpose, cultured MA-10 Leydig cells were chosen because they are frequently used as a model system to understand biochemical regulation of steroidogenesis, usually with an endpoint of progesterone production. The biosynthesis of all steroid hormones begins with the transfer of cholesterol across the inner mitochondrial space from the outer mitochondrial membrane to the inner mitochondrial membrane, a process that depends on the action of the StAR (steroidogenic acute regulatory) protein (Christenson et al. 2001; Stocco 2001). The conversion of cholesterol to pregnenolone is catalyzed by the P450scc (cytochrome P450 side-chain cleavage) component of the cholesterol side-chain cleavage enzyme system located on the matrix side of the inner mitochondrial membrane (Simpson and Boyd 1966). Pregnenolone is then converted to progesterone by the 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme system in the cytosol. The biologically active form of the StAR protein is synthesized as a 37 kDa extramitochondrial protein, which is then inactivated after cholesterol transfer by processing into the 30 kDa intramitochondrial form (Artemenko et al. 2001; Bose et al. 2002). It has been shown in several *in vitro* studies, including MA-10 Leydig cells, that the mitochondrial electrochemical gradient is required for the StAR protein to facilitate cholesterol transfer to the inner membrane of mitochondria (King and Stocco 1996; King et al. 1999, 2000; Diemer et al. 2003; Granot et al. 2003; Hales et al. 2005). Additionally, dissipation of the mitochondrial electrochemical gradient has been shown to block import of the StAR protein into the mitochondria (King et al. 2000; Diemer et al. 2003; Granot et al. 2003; Hales et al. 2005).

The secondary objective of this study was to broaden an earlier investigation of the effect of direct exposure to a concentrated Lawn and Garden Roundup branded formulation on cAMP-induced progesterone production in MA-10 Leydig cells (Walsh et al. 2000). Walsh et al. (2000) demonstrated that inhibition of progesterone production and decreased intramitochondrial levels of the StAR protein in MA-10 Leydig cells was not caused by the active ingredient (glyphosate) in the Roundup branded product, but by an unknown component in the formulation. This unknown component is, in fact, identified by Monsanto code as MON 0818, a surfactant blend predominantly comprised of nonionic POEA (polyoxyethylenealkylamine) surfactant. Nonionic surfactants are amphipathic molecules consisting of a hydrophobic fatty acid-derived alkyl chain and a hydrophilic ethylene oxide chain made of polymerized ethylene oxide, and are an integral part of many pesticide formulations (Seaman 1990). Surfactants increase the leaf retention of spray solutions (De Ruiter et al. 1990) and generally improve the effectiveness of active ingredients by increasing their availability at the target site (Nalewaja et al. 1990, 1991). A surfactant is added to Roundup branded herbicides for this purpose, and results in greater penetration of glyphosate into the plant (Sherrick et al. 1986; Kirkwood 1993; Stock and Holloway 1993). In addition to testing the concentrated Lawn and Garden Roundup branded formulation, we also tested a formulation blank that contained the identical concentration of MON 0818 but not the active ingredient. The formulation blank was included to

characterize the effect of only the surfactant system on progesterone production in MA-10 cells and to characterize the mechanism by which direct exposure to the surfactant system in the formulation can inhibit progesterone production in MA-10 cells.

Materials and methods

Materials

MA-10 mouse Leydig tumor cells were a gift from Dr. Mario Ascoli (Department of Pharmacology, University of Iowa, Iowa City, IA, USA). Purified human chorionic gonadotropin (hCG) (batch CR-125 of biological potency 11 900 IU/mg) was a gift from NIDDK, NIH. [1,2,6,7–*N*–^{3}H]progesterone (specific activity 94.1 Ci/mmol) was obtained from DuPont-New England Nuclear (Wilmington, DE, USA). Progesterone and Waymouth's medium were purchased from Gibco (Grand Island, NY, USA), horse serum from Biofluids Inc. (Rockville, MA, USA), and cell culture plastic ware from Corning (Corning, NY, USA). Antibodies to progesterone were from ICN (Costa Mesa, CA, USA). Electrophoresis reagents and materials were supplied from Bio-Rad (Richmond, CA, USA). All other chemicals used were of analytical grade and were obtained from commercial sources.

Test substances

Lauryl sulfate sodium salt (sodium dodecyl sulfate, anionic, 99.5% purity) and benzalkonium chloride (cationic, 99.5% purity) were purchased from Sigma (St. Louis, MO, USA). Bio-soft N25-9, an alcohol ethoxylate that is based on a synthetic C_{12}–C_{15} alcohol base with an average number of 9 moles of ethoxylation (nonionic, 99.9% purity), and Bio-soft D-40, a linear alkylbenzenesulfonate (anioinic, purity 38.8% with the remainder as water) were purchased from Stepan (Northfield, IL, USA). Since the linear alkylbenzenesulfonate had a composition of 38.8% active ingredient, test concentrations were purity-corrected. The Roundup branded formulation used in this study and by Walsh et al. (2000), nominally contains 16.5% glyphosate-isopropylamine salt (which corresponds to approximately 12.2% glyphosate acid) and 6.1% MON 0818. The formulation blank nominally contained the same concentration of MON 0818 as the Roundup formulation that was tested. Both the Roundup formulation and formulation blank were supplied by the Monsanto Company (St. Louis, MO, USA).

The concentration of glyphosate-isopropylamine in the Roundup branded formulation was determined in advance of the study by HPLC using refractive index detection (Varian series RI-3; Varian; Palo Alto, CA, USA). A 50 µl sample was injected onto a pre-equilibrated MAX-1 SAX column (250 mm×4.6 mm, 5 µm particle size, 60 Å pore; Whatman, Anaheim, CA, USA) and eluted isocratically with a mobile phase of 12% methanol and 88% water containing 18 mmol/L potassium phosphate monobasic that had been adjusted to pH 2.1 with phosphoric acid. The column was eluted over 6 min at ambient temperature with a flow rate of 1.5 ml/min. Data were collected and analyzed on a LabSystems Atlas microcomputer-based chromatography station (Thermo Fisher Scientific, Inc.; Waltham, MA, USA). Glyphosate was identified and concentrations were calculated by comparison with retention times and responses of external glyphosate analytical standards. The level of glyphosate-isopropylamine was determined to be 96% of the nominal level.

The concentration of MON 0818 in the Roundup branded formulation and the formulation blank was determined in advance of the study by normal-phase HPLC using refractive index detection (Varian series RI-3). A 20 µl sample was injected onto a pre-equilibrated Chromegabond Diamine column (250 mm×4.6 mm, 5 µm particle size, 60 Å pore; ES industries, West Berlin, NJ, USA) and eluted isocratically with a mobile phase containing 99.8% ethyl acetate, 0.15% glycerin, and 0.05% triethanolamine. The column was eluted over 20 min at ambient temperature with a flow rate of 1.5 ml/min. Data were collected and analyzed on a LabSystems Atlas microcomputer-based chromatography station (Thermo Fisher Scientific). The components of MON 0818 were identified and concentrations were calculated by comparison with retention times and responses of external standards. The MON 0818 level was determined to be 99.9% of the nominal level.

MA-10 cell culture for concentration response experiments

MA-10 cells were grown in modified Waymouth's MB 752/1 medium containing 20 mmol/L Hepes, 1.2 g/L $NaHCO_3$ supplemented with 15% horse

serum and gentamicin as previously described (Ascoli 1981; Papadopoulos et al. 1990). Cells with a starting density of 500 cells per well were plated onto 96-well plates and grown to 80–90% confluence over a period of approximately 24 h. The media was aspirated and the cells were exposed in serum-free media in the presence or absence of 1 nmol/L hCG and increasing concentrations of one of the test substances for 2 h. After the 2 h incubation time, media were collected for progesterone measurement and cells were saved for protein determination.

Progesterone radioimmunoassays

Progesterone production, used as the steroidogenic index of MA-10 Leydig cells, was measured by radioimmunoassay (RIA) as previously described (Papadopoulos et al. 1990). The limit of detection for this method is 10 picograms of progesterone per ml of culture medium.

Assessment of cell toxicity

Cell toxicity was evaluated using the 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide (MTT) assay developed by Mosmann (1983). This assay measures the reduction of MTT (a yellow tetrazolium salt) to blue formazan in living cells. For the MTT assay, cells were treated as described in "MA-10 cell culture for concentration response experiments" in the presence and absence of hCG stimulation. MTT assays were performed using the TACS MTT proliferation assay (Trevigen, Inc., Gaithersburg, MD, USA) and read using the Victor quantitative detection fluorometer (EGG-Wallac, Gaithersburg, MD, USA). Cell toxicity was also determined by measuring the amount of LDH released into the culture medium using the *in vitro* toxicology assay kit from Sigma, based on the enzymatic assay described by Legrand et al. (1992) and read using the Victor quantitative detection fluorometer (EGG-Wallac).

Determination of cellular ATP levels

Cellular ATP concentrations were measured using the ATPLite-M luminescence assay (Packard BioSciences Co., Meriden, CT, USA). For this assay, cells were cultured on black 96-well ViewPlate and the ATP concentrations were measured on a TopCount NXT counter (Packard BioSciences) following the recommendations of the manufacturer.

Assessment of mitochondrial membrane potential ($\Delta\Psi_m$)

The lipophilic cationic JC-1 dye (5,5′,6,6′–tetrachloro–1,1′,3,3′–tetraethylbenzimidazolyl carbocyanine iodide) was used as a sensitive and specific marker of mitochondrial activity (Smiley et al. 1991). JC-1 dye has the property of aggregating in mitochondria upon membrane polarization, forming an orange-red fluorescent compound. If the mitochondrial membrane potential is disturbed, the dye cannot accumulate in the transmembrane and there is loss of red emission. Assessment of the effect of test substances on mitochondrial membrane potential in MA-10 cells was visualized using the DePsipher assay (Trevigen) optimized for cell culture conditions. MA-10 cells were maintained in DMEM/F12 with 5% FBS, 2.5% horse serum and 1% penicillin/streptomycin at 37°C and 3.8% CO_2. When the cells reached 80% confluence, they were plated onto plastic chamber slides at low density. After a 48 h incubation, MA-10 cells were treated with each test substance over a range of concentrations for 2 h and then incubated for 30 min with the JC-1 dye, washed twice with culture medium, mounted with anti-fade medium, and observed under the microscope at ×400 magnification. Fluorescent images were obtained at 500 ms exposure time, using an Olympus inverted microscope equipped for fluorescent microscopy and with a PM20 camera system (Olympus Corp., Melville, NY, USA).

MA-10 cell culture and treatments for measuring StAR protein expression

For the evaluation of StAR protein expression, MA-10 cells were grown in 6-well plates for approximately 48 h until reaching 80–90% confluence in DMEM/F12 medium (Cellgro, Kansas City, MO, USA) with 5% FBS and 2.5% horse serum and 1% penicillin/streptomycin at 37°C and 3.8% CO_2. The medium was aspirated and the cells were washed with serum-free medium and then treated for 2 h in serum-free medium containing 50 ng/ml hCG at levels of 5 μg/ml and 2 μg/ml for the formulation blank and alcohol ethoxylate, respectively. Cells were then washed twice with PBS and harvested in 1× cell lysis buffer

following the manufacturer's instructions (Cell Signaling, Danver, MA, USA). Cell lysates were centrifuged at 14000 g for 10 min in a cold microfuge to remove cell debris and lysates were used for western blot analysis as described below.

Western blot analysis

Lysates were loaded onto 4–20% Tris-glycine gels (Invitogen, Carlsbad, CA, USA) and run at 110 V. The proteins were then transferred to pure nitrocellulose membrane (Biorad, Hercules, CA, USA). The membranes was blocked with 5% fat-free milk and then incubated with rabbit anti-StAR (1:2000) overnight at 4°C in TBS–0.2% Tween 20 buffer. After there washes ×5 min, the membrane was then blotted with goat anti-rabbit IgG-HRP (1:5000, Transduction Laboratories, Lexington, KY, USA) for 1 h and detected with ECL (Amersham Biosciences, Piscataway, NJ, USA). The membrane was stripped with stripping buffer (100 mmol/L 2-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.7) at 50°C for 30 min and blotted with rabbit anti-G3PDH. Images were analyzed by scanning densitometry with a Kodak image station 2000MM (Kodak, Rochester, NY, USA).

Protein measurement

Cells were solubilized in 0.1 mol/L NaOH and protein levels were determined according to the method of Bradford (1976), using bovine serum albumin as a standard.

Statistical analysis

IC_{20} and IC_{50} values along with their 95% confidence intervals were calculated using a 3-parameter logistic model described by Van Ewijk and Hoekstra (1993) and the PROC NLIN procedure in SAS version 9.1 (SAS Institute Inc., Cary, NC). The IC_{20} and IC_{50} are defined as the concentrations that result in 20% and 50% inhibition relative to the control, respectively. The IC_{20} value was calculated as a surrogate for a no-observed-effect-concentration. If hormesis was observed, the logistic model described by Van Ewijk and Hoekstra (1993) was used to determine IC_{20} and IC_{50} values along with their 95% confidence intervals.

Concentration–response curves were compared using the procedure described by De Lean et al. (1978) at the 95% confidence level using the PROC NLIN procedure in SAS. Comparisons of StAR protein levels on immunoblots were conducted with Tukey's multiple comparisons test at the 95% confidence level using the PROC GLM procedure in SAS.

Results

Effect of surfactants on hCG-stimulated progesterone production in MA-10 cells

Stimulation of MA-10 Leydig cells with hCG for 2 h resulted in a significant increase in progesterone production, typically a 200-fold increase, when compared to the basal production rate in unstimulated cells (Fig. 1). Each test substance produced a concentration-dependent decrease in progesterone production in hCG-stimulated MA-10 cells (Fig. 1) and the inhibition rate for progesterone production (slope) for all of the concentration–response curves for hCG-stimulated progesterone production were comparable, with the exception of a lower rate for linear alkylbenzenesulfonate (Table 1). Indistinguishable concentration–response curves for progesterone production were observed for the Roundup branded formulation and the formulation blank ($p>0.05$), indicating that the surfactant in the formulation was responsible for the concentration-dependent decrease in progesterone production. The mean IC_{20} and IC_{50} values for the Roundup branded formulation and the formulation blank were 3.4 and 5.0 μg/ml, respectively (Table 1). The tested Roundup branded formulation and the formulation blank both contained 6.1% MON 0818, which corresponds to mean IC_{20} and IC_{50} values based on MON 0818 content for the commercial formulation and the formulation blank of 0.20 and 0.31 μg MON 0818/ml cell culture medium, respectively (Table 1). The mean IC_{20} and IC_{50} values for the formulation and the formulation blank, based on MON 0818 content, were comparable with the IC_{20} and IC_{50} value for the structurally similar alcohol ethoxylate that was tested.

In contrast to the other test substances, the lowest lauryl sulfate treatment level of 1 μg/ml cell culture media resulted in a statistically significant 4-fold increase ($p<0.05$) in progesterone production in the

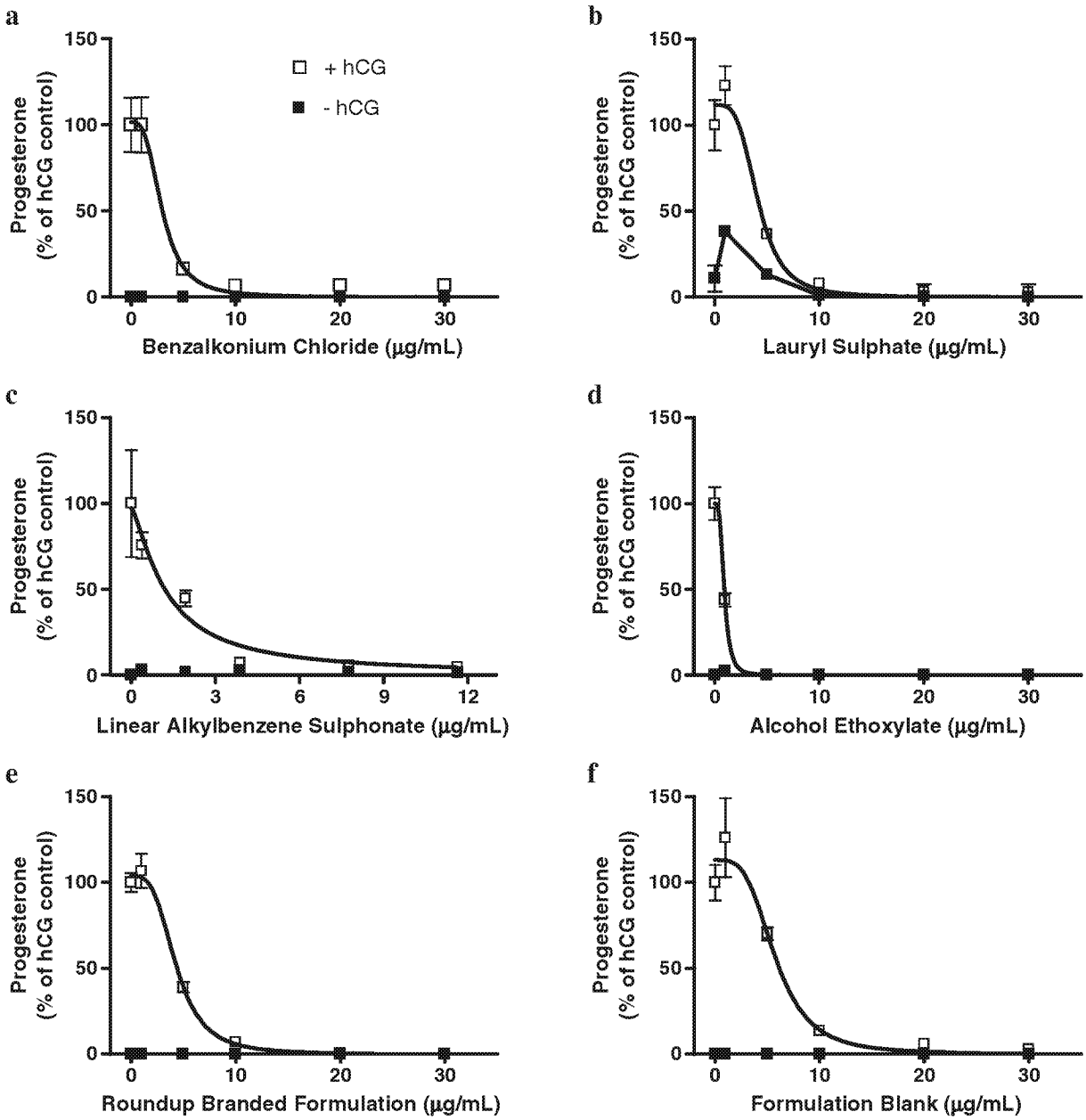

Fig. 1 Concentration-dependent reduction in hCG-stimulated progesterone production in MA-10 Leydig cells treated with surfactants. MA-10 cells were treated for 2 h in serum-free media in the presence or absence of hCG and a range of increasing concentrations of (**a**) benzalkonium chloride, (**b**) lauryl sulfate, (**c**) linear alkylbenzenesulfonate, (**d**) alcohol ethoxylate, (**e**) Roundup branded formulation, and (**f**) formulation blank containing an equivalent amount of surfactant as the Roundup branded formulation. Progesterone levels were normalized to protein concentration and are represented as mean percentage of hCG-stimulated control±standard error (n=4). Basal values for progesterone were 1.5 ng/mg protein

absence of hCG stimulation (Fig. 1b). Concentrations above 1 µg/ml had similar progesterone production values to the controls that did not have hCG stimulation.

Effect of surfactants on cytotoxicity

Evaluation of cytotoxicity using the MTT assay is based on the ability of living cells to reduce a yellow

Table 1 IC_{20} and IC_{50} values, inhibition rates for progesterone production (slopes) in hCG-stimulated MA-10 Leydig cells after 2 h of treatment, and 95% confidence intervals (CI)

Test substance	IC_{20} (μg/ml)	95% CI (μg/ml)	IC_{50} (μg/ml)	95% CI (μg/ml)	Slope of concentration–response curve	95% CI
Benzalkonium chloride	1.9	1.2–2.6	3.0	0.90–5.1	3.0	0.38–5.6
Alcohol ethoxylate	0.59	0.58–0.60	0.93	0.92–0.94	3.5	3.4–3.6
Linear alkylbenzenesulfonate	0.49	0.15–0.83	1.2	0.1–2.3	1.4	0.17–2.6
Lauryl sulfate	2.8	2.1–3.5	4.1	1.1–7.1	3.6	1.0–6.2
Roundup branded formulation[a]	2.9[b]	2.7–3.1	4.3[c]	3.5–5.1	3.3	1.7–5.0
Formulation blank	3.8[b]	2.8–4.8	5.7[c]	3.6–7.8	3.5	0.72–6.3

[a] The formulation tested was a concentrated Lawn and Garden Roundup branded formulation.

[b] The mean IC_{20} value for the Roundup branded formulation and for the formulation blank based on MON 0818 content was derived as follows: nominally 6.1% MON 0818 content in the formulation and the blank * (2.9 μg/ml+3.8 μg/ml)/2=0.20 μg MON 0818/ml.

[c] The mean IC_{50} value for the Roundup branded formulation and for the formulation blank based on MON 0818 content was derived as follows: nominally 6.1% MON 0818 content in the formulation and the blank * (4.3 μg/ml+5.7 μg/ml)/2=0.31 μg MON 0818/ml.

tetrazolium salt to blue formazan crystals. Cytotoxicity is measured as a decrease in the amount of formazan produced by treated cells compared to control cells. Each test substance in the presence and absence of hCG stimulation produced nearly identical concentration-dependent decreases in MTT activity in MA-10 cells (Fig. 2). A statistical comparison of the concentration–response curves within a test substance showed no difference in the concentration–response profiles with or without hCG-stimulation ($p>0.05$). Lack of a difference in concentration–response curves with or without hCG stimulation indicated no interaction between hCG and the ability of MA-10 cells to reduce the yellow tetrazolium salt. IC_{50} values for the MTT assay ranged from approximately 5 to 50 μg/ml cell culture medium, with the lowest values measured for benzalkonium chloride (Table 2). Nearly identical concentration–response profiles for the MTT assay were measured with the Roundup branded formulation and the formulation blank, which indicates that the surfactant component in the formulation was responsible for the observed cytotoxicity and the effect on mitochondrial function. For all test substances, the concentration–response curves for the MTT assay were far to the right of the concentration–response curves for inhibition of progesterone synthesis, with the exception of benzalkonium chloride (Fig. 2). Additionally, a comparison of the concentration responses for the progesterone and the MTT assays can be made by comparing IC_{20} and IC_{50} values (Tables 1 and 2). With the exception of benzalkonium chloride, IC_{20} levels from the MTT assay corresponded with concentrations that resulted in high levels of inhibition of progesterone synthesis ($>>IC_{50}$ values). This indicates the relatively low sensitivity of the MTT assay as a robust biomarker for effects on progesterone synthesis following only 2 h treatments (Fig. 2).

In contrast to the other test substances, concentrations of lauryl sulfate between 5 and 40 μg/ml produced a concentration-dependent increase in the ability of MA-10 cells to reduce the yellow tetrazolium salt (Fig. 2b). However, concentrations of lauryl sulfate that exceeded 40 μg/ml resulted in a rapid concentration-dependent decrease in the ability of MA-10 cells to reduce the yellow tetrazolium salt.

In addition to the MTT assay, two other cytotoxicity endpoints were evaluated in the initial phase of this study with the nonionic surfactants: measurement of cellular ATP levels and lactate dehydrogenase (LDH) levels in media collected from treated cells. Concentration–response data from these assays were comparable to those from the MTT assay (data not shown).

Effect of surfactants on mitochondrial membrane potential ($\Delta\Psi_m$)

To further assess the effects of the test substances on mitochondrial function, changes in mitochondrial membrane potential were evaluated with JC-1 dye. The level of JC-1 dye accumulation in the mitochondrial matrix is driven by the mitochondrial membrane potential, and the level of accumulation is a functional measure of the electrochemical gradient. To assess mitochondrial membrane potential, MA-10 cells were grown on cover slips and incubated over a concen-

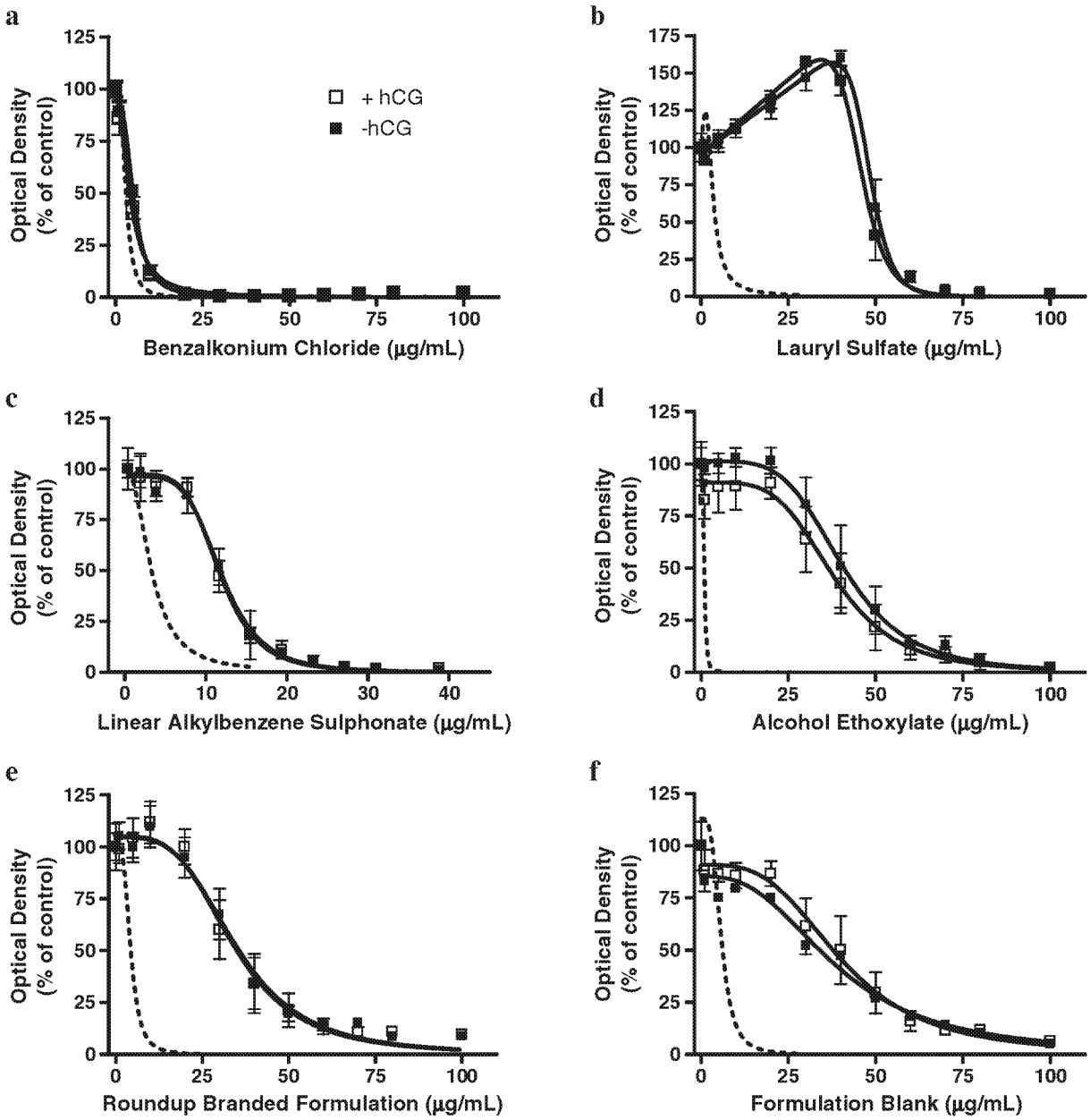

Fig. 2 Concentration-related cytotoxic effects of surfactants in MA-10 Leydig cells. Cytoxicity was assessed with the MTT assay in MA-10 cells immediately after a 2 h exposure in serum-free medium in the presence or absence of hCG and a range of increasing concentrations of (**a**) benzalkonium chloride, (**b**) lauryl sulfate, (**c**) linear alkylbenzenesulfonate, (**d**) alcohol ethoxylate, (**e**) a concentrated Lawn and Garden Roundup branded formulation, and **f** formulation blank containing an equivalent amount of surfactant as the concentrated Lawn and Garden Roundup branded formulation. In order to compare the concentration responses for the MTT assay and inhibition of progesterone production, the concentration–response curve for inhibition of progesterone production has been included as a dashed line. Optical densities were normalized to protein concentration and are represented as mean percentage of control±standard error (n=4)

tration range with the different test substances for 2 h. The results from concentration–response assays with each test substance demonstrated a concentration-dependent loss of the accumulation of JC-1 dye and the disappearance of dense aggregation of the JC-1 dyes in the transmembrane space of the mitochondria.

Table 2 IC_{20} and IC_{50} values and 95% confidence intervals (CI) for the MTT assay in MA-10 Leydig cells after 2 h of treatment

Test substance	−hCG		+hCG	
	IC_{20} (μg/ml)	95% CI (μg/ml)	IC_{20} (μg/ml)	95% CI (μg/ml)
Benzalkonium chloride	2.3	1.8–3.1	3.2	2.9–3.5
Alcohol ethoxylate	30	27–32	27	24–30
Linear alkylbenzenesulfonate	5.2	4.6–5.8	5.1	4.5–5.6
Lauryl sulfate	48	47–49	46	43–49
Roundup branded formulation[a]	23	21–26	23	21–26
Formulation blank	23	18–28	26	23–29
	IC_{50} (μg/ml)	95% CI (μg/ml)	IC_{50} (μg/ml)	95% CI (μg/ml)
Benzalkonium chloride	5.2	4.7–5.7	4.2	3.5–5.0
Alcohol ethoxylate	41	39–42	38	35–41
Linear alkylbenzenesulfonate	12	11–13	12	11–13
Lauryl sulfate	51	50–52	49	48–50
Roundup branded formulation[a]	35	32–38	34	30–38
Formulation blank	39	32–45	40	36–44

[a] The formulation tested was a concentrated Lawn and Garden Roundup branded formulation.

Representative images for the JC-1 assay using benzalkonium chloride, an alcohol ethoxylate, and the concentrated Lawn and Garden Roundup branded formulation are shown in Fig. 3a–c. Control cells show clear aggregation of the dye in MA-10 cells, which is illustrated by the vivid orange-red bright aggregates throughout the cells. With each test substance there was a significant disappearance of the distinct orange-red bright spots and a loss of red fluorescence for test concentrations approximately at and below the IC_{50} value for progesterone production. Results with the formulation blank were comparable to the results obtained with the Roundup branded formulation (data not shown).

Effect of surfactant exposure on StAR protein levels

To investigate the effect of direct exposure to surfactants on StAR protein expression in MA-10 cells, changes in StAR protein levels were analyzed. Stimulation with hCG for 2 h resulted in a significant increase in measured levels of the 30 kDa mature form of the StAR protein compared to the unstimulated cells (Fig. 4a and b). Stimulation with hCG and co-treatment with either the formulation blank or the alcohol ethoxylate for 2 h, at approximately the IC_{50} value for the formulation blank and approximately twice the IC_{50} value for the alcohol ethoxylate, significantly decreased levels of the 30 kDa form of the protein when compared to the untreated hCG cells (Fig. 4a and b). Although treatment with both nonionic surfactants caused a significant decrease in levels of the 30 kDa StAR protein in hCG-stimulated cells, levels of the 37 kDa StAR protein were not significantly different in treated and untreated hCG-stimulated cells (Fig. 4c and d).

Discussion

In this study, we examined the effect of representative cationic, nonionic, and anionic surfactants on steroidogenesis in MA-10 Leydig cells. Each test substance produced a concentration-dependent decrease in steroid formation in hCG-stimulated MA-10 cells and the rates for inhibition of progesterone production were comparable, with the exception of a lower inhibition rate with linear alkylbenzenesulfonate. Comparable rates for inhibition of progesterone production are consistent with these substances having the same mode of action (Eaton and Klaasen 1996). An evaluation of mitochondrial function with the JC-1 assay showed that decreased progesterone production in MA-10 cells is accompanied by loss of mitochondrial membrane potential. Additional assays were performed with two nonionic surfactants using the same exposure regime to assess the effect of surfactant exposure on StAR protein expression in

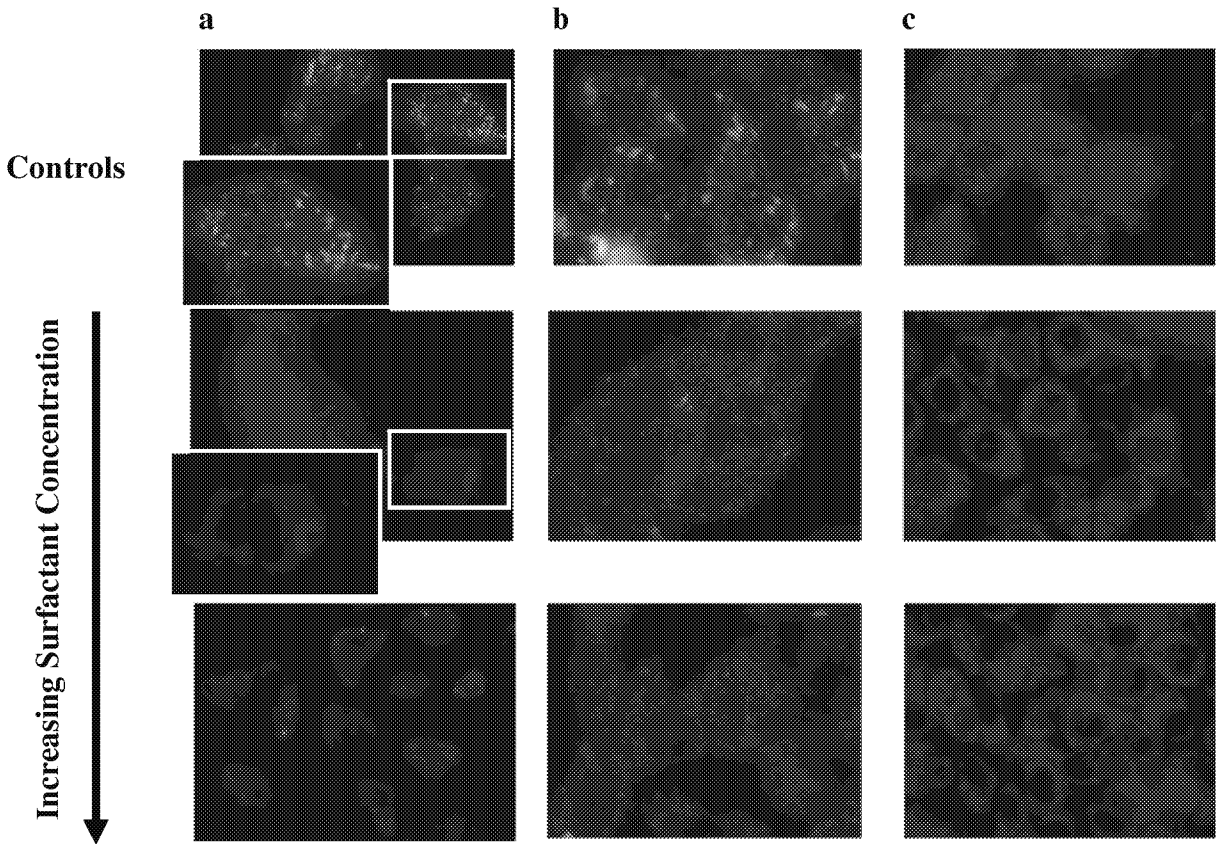

Fig. 3 Exposure to surfactants causes dissipation of mitochondrial membrane potential. MA-10 Leydig cells were treated for 2 h before vital staining of the cells with JC-1 was performed. Note that the control cells have intact mitochondrial membrane potential as indicated by the orange-red aggregates. (**a**) Cells were treated with concentrations of 0 (control), 1, and 2.5 μg benzalkonium chloride/ml medium. For the control treatment and the low dose level, a cell has been enlarged to highlight the treatment effect on mitochondrial JC-1 aggregation. (**b**) Cells were treated with concentrations of 0 (control), 0.1, and 1 μg alcohol ethoxylate/ml medium. (**c**) Cells were treated with concentrations of 0 (control), 1, and 5 μg of the Roundup branded formulation/ml media. Treatment caused a concentration-dependent loss of the mitochondrial aggregation and fluorescence. Pictures shown are representative of at least four independent experiments

hCG-stimulated MA-10 cells. In these assays, treatment did not affect levels of the active 37 kDa form of the StAR protein but did result in decreased levels of the 30 kDa form. Collectively, these results are consistent with previous studies that demonstrated that StAR protein function and steroidogenesis requires an intact mitochondrial membrane potential (King and Stocco 1996; King et al. 1999, 2000; Diemer et al. 2003; Granot et al. 2003; Hales et al. 2005).

In addition to using the JC-1 assay to evaluate the effect of each test substance on mitochondrial function, the widely used MTT assay was included to evaluate cytotoxicity and effects on mitochondrial function in MA-10 cells. The MTT assay evaluates the ability of viable cells to reduce a yellow tetrazolium salt to blue formazan crystals. Cytotoxicity was indexed as a decrease in the amount of formazan produced in treated cells compared to control cells. In most cases, the MTT assay showed relatively low sensitivity for detecting mitochondrial effects compared to the JC-1 assay. The concentration–response curves for the MTT assay were far to the right of the curves for inhibition of progesterone synthesis and the effect levels observed with JC-1 dye, with the exception of the MTT concentration–response curves for benzalkonium chloride. The similarity of concentration–response profiles for progesterone production and for the MTT assay is apparently related to benzalkonium chloride being a cationic surfactant. The positive charge of benzalkonium chloride likely allowed it to accumulate in the mitochondria by membrane-driven potential, in

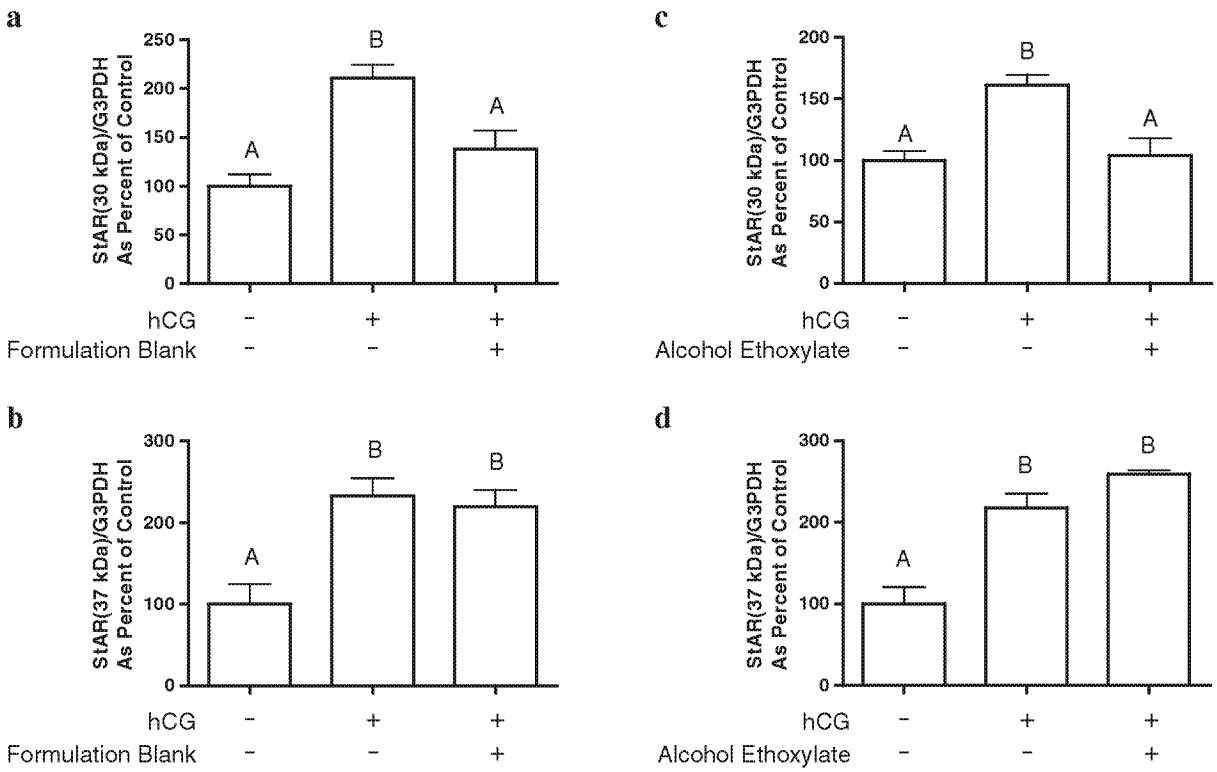

Fig. 4 Quantitation of StAR protein levels in MA-10 Leydig cells treated either with the formulation blank at a concentration of 5 μg/ml (graphs **a** and **c**) or with an alcohol ethoxylate at a concentration of 2 μg/ml (graphs **b** and **d**). MA-10 cells were treated for 2 h in serum-free medium and cell lysates were evaluated by western blot analysis as described in the Materials and Methods section. Optical densities for the 30 and 37 kDa proteins were normalized to G3PDH optical densities and are represented as mean percentage of control±standard error of the mean (n=4)

the same manner by which the cationic JC-1 dye accumulated in mitochondria. In fact, the cationic surfactants cetyltrimethylammonium bromide (CTAB) and nonyltrimethylammonium bromide (NTAB) have been shown to accumulate in the mitochondrial matrix by a membrane-driven uptake mechanism (Bragadin and Dell'Antone 1996). The accumulation of CTAB and NTAB by mitochondria disrupted the mitochondrial membrane potential, which was concluded to result from enhanced mitochondrial membrane permeability caused by membrane solubilization.

Previously, the performance of the MTT assay as an indicator of cytotoxicity during short-term exposures with Leydig cells was rigorously assessed in an evaluation of the effect of ethane dimethylsulfonate on Leydig cell function (Kelce 1994). This study demonstrated that the MTT assay is not a sensitive indicator of early events of cytotoxicity for ethane dimethylsulfonate. Although ethane dimethylsulfonate produced a concentration-dependent decrease in luteinizing hormone-stimulated testosterone production after 3 h of exposure, Leydig cell death did not become apparent for another 18–24 h when co-evaluated with the MTT assay. Based on the results from the Kelce study, the MTT assay is not recommended as a sensitive indicator of cytotoxicity and mitochondrial function unless the duration of exposure is at least 18–24 h.

As noted in the results section, cellular ATP levels and LDH release were evaluated in the initial phase of this study with the nonionic surfactants. The results from these cytotoxicity assays were comparable to the results for the MTT assays. Similarly, no significant effect on either total cellular ATP levels or the integrity of the plasma membrane was observed in assays with MA-10 Leydig cells that were exposed for 3 h to levels of hydrogen peroxide that significantly decreased progesterone production (Diemer et al. 2003).

In contrast to the general lack of response of unstimulated MA-10 cells treated with a test substance,

the lowest tested concentration of lauryl sulfate increased progesterone production. Similarly, the lower concentration levels of lauryl sulfate also stimulated activity in the MTT assay. The hormetic pattern observed with lauryl sulfate in MA-10 cells is likely related to a change in the arrangement of lipoprotein complexes in the membrane. An exact interpretation of the mechanism resulting in the hormetic response is complicated by the complex environment and the multistep nature of the process. Activation of enzyme activity following treatment with surfactants has been reported previously, particularly with microsomal proteins. For example, mammalian liver UDP-glucuronyl transferase is firmly bound to microsomal membranes and its activity has been shown to be strongly dependent on the presence of compounds that perturb membranes (Chan 1967; Graham and Wood 1973).

Further to testing the four representative surfactants, two additional test substances were investigated: a branded Roundup formulation and a formulation blank that was identical in composition but did not include the active ingredient. Both of these substances produced nearly identical concentration-dependent responses for inhibition of progesterone production, indicating that decreased progesterone production is directly related to the surfactant used in the Roundup branded formulation. In a previous study, probably using the same Roundup branded formulation as the present study, the same treatment regime produced a similar concentration-dependent decrease in $(Bu)_2$cAMP stimulated progesterone production in MA-10 Leydig cells (Walsh et al. 2000). The 2 h IC_{50} value of the commercial herbicide formulation from the Walsh et al. (2000) study was 22 μg/ml cell culture medium, compared to a 2 h IC_{50} value of 4.3 μg/ml cell culture medium in the current study. While the IC_{50} value in the present study is lower, it is considered to be within an acceptable range of interlaboratory variability taking into account the differences in cell culture media, the substance chosen to stimulate the MA-10 cells, MA-10 cell passage number, and RIA methodology. Walsh et al. (2000) also demonstrated that a 2 h treatment with 25 μg/ml of the Roundup branded formulation did not decrease *de novo* rates of StAR mRNA synthesis or have a significant impact on P450scc and 3β-HSD mRNA and protein levels. Additionally, no effect on 3β-HSD catalytic activity was observed. However, there was a modest reduction in P450scc catalytic activity. The effect on P450scc catalytic activity could have resulted from damage to the intramitochondrial membrane where the P450scc protein is found; however, an assessment of mitochondrial integrity and function was not performed. The lack of an observed effect on P450scc protein levels after a 2 h treatment is not surprising because the reported half-life for P450scc is considerably greater than 2 h and has been estimated to be greater than 24 h in pulse-chase studies (Boggaram et al. 1984).

In addition to inhibition of progesterone production, there was an indication of overt toxicity to the MA-10 cells from treatment with the Roundup branded formulation in the former study (Walsh et al. 2000). In some assays, a 39% decrease in [^{35}S] methionine incorporation was observed after 2 h of exposure to the concentrated Lawn and Garden Roundup branded formulation at the IC_{50} level for progesterone production. At the IC_{50} treatment level, these investigators also observed a 90% decrease in levels of the 30 kDa mature form of the StAR protein in mitochondrial extract. However, the level of the biologically active 37 kDa form of StAR pre-protein in whole cell or cytosolic extracts was not evaluated. Therefore, it was not possible to conclude that exposure to the Roundup branded formulation at the tested concentration resulted in decreased StAR protein expression. In the present study, treatment with the formulation blank for the same period of time did not reduce the levels of the active 37 kDa form of the StAR protein. In the Walsh et al. (2000) study, separate experiments performed with glyphosate acid demonstrated no effect on progesterone production at the highest tested concentration of 100 μg/ml, which greatly exceeds the IC_{50} value for the tested Roundup branded formulation. In an additional experiment, these investigators demonstrated that inhibition of MA-10 cell steroidogenesis after 2 h of treatment with the Roundup branded formulation at the IC_{50} level for progesterone production was transient. Full recovery of progesterone production was observed after a period of 24 h in untreated media. Similarly, full recovery has been observed in *in vitro* studies that examined the effect of polymeric surfactants on mitochondrial electron transport in HL-60 cells in culture (Rapoport et al. 2000).

Diemer et al. (2003) evaluated the effect of reactive oxygen (H_2O_2) on StAR protein levels in MA-10

Leydig cells and reported similar findings to those reported in this study. These investigators demonstrated that treating cAMP-stimulated MA-10 cells with H_2O_2 for 3 h produced a concentration-dependent decrease in progesterone production. A concentration of 250 μmol/L H_2O_2 disrupted the mitochondrial electrochemical gradient along with >50% decrease in progesterone production and levels of the 30 kDa form of the StAR protein in whole-cell extracts. However, after treatment with 250 μmol/L H_2O_2, levels of the 37 kDa StAR form of the protein in whole-cell extracts did not decrease. Additionally, no change was detected in StAR mRNA levels, P450scc protein levels, cellular ATP levels, and cell viability with the trypan blue dye exclusion assay. Diemer et al. (2003) also demonstrated that decreases in levels of the 30 kDa StAR protein after treatment were rapidly reversible, indicating rapid repair of the mitochondrial membrane and recovery of the ability of the mitochondria to import and process the StAR protein.

Several studies have evaluated the effect of direct treatment of herbicide formulations on mitochondrial function (Oakes and Pollack 1999, 2000). Oakes and Pollak (1999) investigated the effect of another commercially available herbicide formulation on mitochondrial function using submitochondrial particles. The herbicide formulation Tordon 75D was found to inhibit electron transport with an IC_{50} value in the low micromolar range. By testing individual components of the herbicide formulation, it was shown that the proprietary surfactant polyglycol 26–2, when tested alone, uncoupled mitochondrial respiration to an equal level as the commercial formulation. None of the other components of the herbicide formulation had an inhibitory effect. This is consistent with the findings in the present study which showed that the Roundup branded formulation and the formulation blank containing the surfactant produced an equivalent effect on mitochondrial function.

Perturbation of the mitochondrial membrane following surfactant exposure has also been observed in whole-animal studies. A study characterizing the effects on *Xenopus* embryos and tadpoles, following waterborne exposure to an alcohol ethoxylate, showed that mortality occurred after mitochondrial damage (Cardellini and Ometto 2001). The authors concluded that death was caused by disruption of the mitochondrial respiratory chain, which was preceded by extensive injury to the lipid and protein composition of exposed membranes. However, recovery of mitochondrial effects was rapidly observed after the treatment ended. Comparable observations of effects on mitochondrial membranes and function have been made with freshwater mollusks treated with linear alkylbenzenesulfonate (Ceron 1993). Following waterborne exposure to linear alkylbenzenesulfonate, mitochondria were observed to have gross malformations, no cristae, reduced ATP synthesis, and reduced NADH oxidase activity in tissues with direct contact with the environment.

To adequately evaluate the significance of *in vitro* studies that assess the potential for surfactant toxicity to humans following oral and dermal exposure, realistic exposure estimates are required. To date, few studies have estimated systemic surfactant exposure in humans because the study of the absorption, distribution, and metabolism of surfactant molecules is not well developed. However, this information is essential to develop a further understanding of the magnitude and duration of exposure and the likelihood of toxic effects occurring at cellular and molecular levels. Estimates of human systemic exposure for some common surfactants from direct and indirect skin contact as well as from the oral route via dishware residues have been comprehensively evaluated. Aggregate exposures for commonly used alcohol ethoxysulfates and linear alkylbenzenesulfonates have been estimated to be 29 μg/kg bw per day and 4 μg/kg bw per day, respectively (HERA 2003, 2004). These aggregate exposure estimates are consistent with earlier estimates for human systemic exposure from residues on dishes and utensils, which ranged from 0.3 to 1 mg surfactant/day (Swisher 1968). Additionally, oral intake of 100 mg on an annual basis has been reported (Moncrieff 1969) and this estimate was confirmed by studies using radiolabeled surfactants (Schmitz 1973). It has been shown in safety assessments with a variety of surfactants that exposure levels are far below levels that represent a hazard to humans with regard to their use in consumer products (Charlesworth 1976; Black and Howes 1979; HERA 2003, 2004).

In vivo, Leydig cells can only be exposed to xenobiotics via the blood. Therefore, it is most appropriate to compare treatment responses measured with MA-10 cells with estimated systemic doses. A recently published biomonitoring study, with farmers and their families, reported measured systemic doses

for glyphosate (Acquavella et al. 2004). In that study, 40% of the farmers did not have detectable levels in their urine samples despite some having made applications to over 40 ha. Additionally, 90% of the applicators had systemic exposures below 0.001 mg/kg, which corresponds approximately to a 90th centile of 0.07 mg of glyphosate excreted over the period after application for a 70 kg farmer-applicator. When a body fluid volume of 45.5 L is considered for a 70 kg farmer-applicator (0.65 L body fluid/kg×70 kg), wherein glyphosate is equally distributed, the body fluid concentration of glyphosate is estimated to be 1.5 μg/L. To derive an exposure estimate for the surfactant in the tested Lawn and Garden Roundup branded formulation, it is reasonable to make the assumption that the surfactant is absorbed through the skin to the same extent as glyphosate because skin penetration for structurally related alcohol ethoxylates through human skin has been shown to be very low (Black and Howes 1979). Consistent with this finding, when predicted systemic levels from dermal exposure to alcohol ethoxylates are compared with no-effect levels from 90-day rat feeding studies with alcohol ethoxylates, the likelihood of systemic toxic effects has been shown to be extremely low (Black and Howes 1979). For the glyphosate formulation tested in the current study, the ratio of glyphosate acid to surfactant is approximately 2:1. Therefore, the systemic concentration of the surfactant from this formulation can be estimated to be about 50% of the systemic concentration of the glyphosate (i.e., 0.50× 1.5 μg/L=0.75 μg/L body fluid). Consequently, the estimated systemic concentration for the surfactant is more than 250 times less than the mean IC_{20} value for progesterone production with the tested Roundup formulation and the formulation blank when adjusted for the level of surfactant in the formulation (mean IC_{20} of 200 μg surfactant system/L cell culture media/ 0.75 μg surfactant system/L body fluid=267). Similarly, the estimated systemic concentration for the surfactant is approximately 400 times less than the mean IC_{50} value for progesterone production with the tested Roundup formulation and the formulation blank when adjusted for the level of surfactant in the formulation (mean IC_{50} of 310 μg surfactant system/L cell culture medium/0.75 μg surfactant system/L body fluid= 413). Although several assumptions were made to generate this exposure estimate, based on the large safety factor between exposure and the IC_{20} and IC_{50} values observed in MA-10 cells, it is apparent that the POEA used in Roundup branded formulations will not disrupt steroid production in Leydig cells *in vivo*.

Conclusions

The results of this study demonstrate the nonspecific action of a variety of surfactants on cellular function in an *in vitro* test system and demonstrate how this activity can confound the conclusions regarding outcomes that are dependent upon intact cellular energetic mechanisms, such as progesterone production, when surface-active agents are tested in cultured cell systems. Additionally, the results of this study underscore the importance of including a rigorous assessment of mitochondrial function and cytotoxicity when evaluating potential effects on steroidogenesis in an *in vitro* test system. When using cellular *in vitro* systems, it is essential to consider whether the observed responses occur at levels that the cellular systems may encounter when exposure occurs to the intact animal.

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