Chemical Research in Toxicology

LETTERS TO THE EDITOR

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Reply to the Letter to the Editor Regarding Our Article (Paganelli et al., 2010)

To the Editor: The letter to the editor sent by representatives of Monsanto, Syngenta, and Dow Chemicals (among others) to *Chem. Res. Toxicol.* regarding our paper (Paganelli et al., published July 23, 2010) and the tone used in their criticism about other research papers studying glyphosate effects should come as no surprise considering the obvious conflicts of interest inherent in this work when the companies selling a product are also solely responsible for testing its safety.

These multinational corporations handle virtually all of the seed and chemical products market in the world; therefore, it cannot be inferred that research performed or supported by such companies is completely objective. Their dismissal of our research and that of other researchers harkens back to the ongoing debate about bisphenol A, where no single industry funded study has ever found adverse consequences linked with BPA exposure, whereas 90% (n > 100) of nonindustry funded studies show significant adverse consequences of BPA exposure.[1,2]

Therefore, we contend that rather than pointing out shortcomings of our research, the letter illustrates the increasing difficulty in dialogues between those with a vested interest in product sales and independent researchers who wish simply to understand whether the said products are safe. It is worse yet when multinational corporations attempt to use their own flawed science to hide and defend environmental devastation suffered by less-developed countries in areas where their products are heavily used with only minimal governmental scrutiny.

POINT A

The objections to the effects of glyphosate noted by the authors clearly indicate that they did not review the complete evidence previously published by independent (i.e., non-industry funded) groups, who reported teratogenic effects equivalent to those reported in our work, Lajmanovich[3] and Dallegrove,[4] nor did they take note of the inhibitory activity produced by atrazine[5] and glyphosate[6] on CYP19 (cytochrome P450 aromatase) that affected sex steroid metabolism leading to endocrine disruption. Furthermore, they ignore evidence that Triadimefon,[7] glyphosate (our paper), and probably atrazine[8] produce teratogenic effects through the alteration of the retinoic acid pathway.

The claim that glyphosate is not teratogenic and does not produce adverse reproductive effects is based on studies generated by the industry, as the authors of the letter indeed recognize. This constitutes a strong and material conflict of interest in the outcome of said studies. Moreover, these technical reports are often adopted as criteria for use by the state control agencies, without being corroborated experimentally by independent scientists. In fact, the WHO, in the 2009 document Classification of Pesticides Recommended by the WHO by Hazard and Guidelines to Classification (CPRHGC), states the following:

> "All reasonable precautions have been taken by the World Health Organization to verify the information contained in this publication. However, the published material is being distributed without warranty of any kind, either expressed or implied. The responsibility for the interpretation and use of the material lies with the reader. In no event shall the World Health Organization be liable for damages arising from its use."

In a different paragraph of the same document, the WHO adds the following:

> "Any classification based on biological data can never be treated as final. In the assessment of biological data, honest differences of opinion are inevitable and most borderline cases can be reclassified in an adjacent class. Variability or inconsistency in toxicity data due to differences in susceptibility of test animals, or to experimental techniques and materials used can also result in differing assessments."

Moreover, in the following paragraph, the WHO rescued a text from the proposal approved by the World Health Assembly in 1975:

> "The classification criteria are guide-points intended to supplement but never to substitute for special knowledge, sound clinical judgment or experience with a compound. Reappraisal might be necessary from time to time."

In addition, the reports referenced by WHO (1994) are mainly based on technical information provided by companies interested in producing and marketing the product and its formulations. For example, 180 reports were performed and/or supplied by Monsanto. Among them, more than 150 were not published and therefore were not subjected to peer review. Other key technical reports provided as references in the same document, such as 17 reports from Agrichem (producer and marketer of pesticides based in The Netherlands), 5 from Luxan BV, (Netherlands), and 5 from Rhône Poulenc were not published either.

These reports are used in a complementary manner as the main source for the considerations used for classification. For example, the unpublished 1990 Monsanto report is quoted twice to justify the spraying of glyphosate, and the unpublished 1988 Monsanto report is quoted twice in reference to methodological aspects and metabolic transformations.

However, there are no reports concerning procedures for designating the teams responsible for the selection of studies used as reference, analysis, evaluation, and classification or the final considerations. In particular, there is no evidence provided that the people responsible for such reports have the required expertise or scientific oversight to make the reports credible.

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In the 2009 CPRHGC document, the WHO states the following:

> "In practice, the majority of classifications will be made on the acute oral LD50 value. However, dermal toxicity must always be considered since it has been found that, under most conditions of handling pesticides, a high proportion of the total exposure is dermal."

This implies other reasons for considering the WHO classification insufficient to protect the health of the population and the environment from damage produced by agrochemicals. For instance, the classification is based primarily on acute oral and, eventually, dermal toxicity. While these determinations are standard procedures in toxicology, they leave out chronic toxicity and sublethal toxicity assays and do not consider access through the respiratory tract.

In addition, the 2004 report from the JMPR FAO/WHO Expert Meeting, often misleadingly cited as a scientific report by advocates of glyphosate, contains no scientific references that support the conclusions drawn within its 383 pages. It is unclear as to who performed the research, what methodology was used, and what form of peer review was employed to evaluate the quality of the material contained in the report. As a matter of fact, the document acknowledges in the Introduction that

> "Most of the summaries and evaluations contained in this report are based on unpublished private property before the Committee to make assessments."

Therefore, we argue that it is high time that data used to determine whether products are safe are generated by independent entities with no ties to the manufacturers of the said products, or those who have a financial interest in the approval of the said products.

POINT B

The letter also criticizes the literature cited in our work. Benitez-Leite's paper[9] is a study that points to the correlation between malformations and exposure to pesticides in Paraguay, giving a precise idea of the outcome of heavy agrochemical use in Paraguay. The mentioned paper identifies living near treated soy fields, dwellings located less than 1 km from treated fields, storage of pesticides in the home, and contact with pesticides as significantly associated risk factors for congenital malformations. This study brings concerns about the situation created in Paraguay by the expansion of industrialized soy crops which require the intensive use of agrochemicals (which, needless to say, includes glyphosate).

Despite the dismissal by the companies, several malformations observed by Benitez-Leite (for example, anencephaly, microcephaly, facial defects, myelomeningocele, cleft palate, synotia, polydactily, and syndactily) are indeed consistent with the well-known and expected syndrome caused by misregulation of the retinoic acid (RA) pathway. RA is a well-known teratogen that causes craniofacial abnormalities (by misregulation of sonic hedgehog and otx2 expression) and posterior regression syndrome in all vertebrates tested including, unfortunately, humans.[10,11]

Notwithstanding the corporate disinformation provided by our critics, the malformations observed by Benitez-Leite et al. are indeed consistent with the well-known and expected malformations caused by increase of RA.

These conclusions should be taken into account together with studies on the incidence of malformations and cancer conducted in Chaco, an Argentine province with records in soybean harvest and use of glyphosate. These official records (often hidden by the Argentine government) reveal a 3-fold increase in developmental malformations in the province and a 4-fold increase cancer in the locality of La Leonesa.[12]

These data should be sufficient to raise the alert worldwide and lead to the commissioning of an independent study to provide an unbiased and dispassionate evaluation of the information rather than relying on studies commissioned by companies [13] or requested by the U.S. Drug Enforcement Agency in support of their efforts to eradicate coca plantations. Suggestively, an epidemiological surveillance conducted between December, 2004 and April, 2008 in Cali and Valle del Cauca in Colombia revealed that cyclopy is an endemic event with a prevalence 14- to 43-fold higher than that reported in the literature.[14]

Long before our work, reports appeared in the mainstream press about the effects of agrochemicals on human and animal health based on direct observations from physicians and health workers. This was a very important warning about the environmental consequences of using 200 million liters of GBH/year in Argentina and led to a vigorous debate about the safety of GBH and the "precautionary principle", urging to initiate epidemiological studies.[15]

Finally, we find unfortunate the expression "consistently inappropriate deemed irrelevant for human health and Risk Assessment purpose" with regard to the work of Dr. Seralini and Dr. Marc. This criticism is an unscientific value judgment that does not refute the quality of the work but rather seeks to discredit, without providing evidence to the contrary. Such comments are unwarranted in the scientific literature and should be considered together with the source: a corporate entity seeking to continue the production and use of a product that independent scientists have found to have adverse health consequences.

POINT C

Glyphosate penetration through the cell membrane and subsequent intracellular action is greatly facilitated by adjuvants such as surfactants.[16,17] The authors of the letter conveniently avoid discussing this fact. Moreover, the companies they represent are not required to reveal the composition or safety of adjuvants used in the commercial formulations which are protected as trade secrets. It was for precisely this reason that we tested both the active principle, glyphosate, as well as commercial formulations for teratogenicity. This is a more realistic test of whether glyphosate or the formulations (which vary by manufacturer and intended use) is responsible for the malformations observed. The calculated intracellular concentration for glyphosate injected into embryos was 60 times lower than the glyphosate concentration present in the 1/5000 dilution of the GBH which was used to culture whole embryos. Notwithstanding this, both gave similar phenotypes and changes in gene expression, suggesting that the effects are attributable to the active principle of the herbicide.

The authors of the letter claim that the glyphosate doses used are 9−15 times greater than the acute LD_{50} value for frog embryos of the same species. In fact, the study by Edginton et al. (2004) on which they support this statement uses a different commercial formulation. Because the adjuvant composition can change the permeability of the membranes to the active component, the effective concentration in the cells cannot be compared between studies. Moreover, results from Seralini's group suggest that the

adjuvants per se may pose adverse effects in cell cultures.[18] Indeed, Edginton et al. claim that the surfactant is the major toxic component of the formulation they use. On the other hand, the LD50 of teratogens may vary between batches of embryos. The LD50 is not an accurate criterion to analyze the developmental defects that indeed occur in the survivors.

The authors representing the companies present a series of calculations according to the toxicological point of view, while cell biology and molecular and developmental biology are absent in their considerations. Moreover, they do not discuss the following key issues:

(a) In the field, usually the main route of systemic entry is the respiratory tract instead of the digestive tract.

(b) Direct blood concentration is only an average indicator of the presence of the chemical and does not provide evidence about its tissue distribution. Recently, a 2-compartment model study suggested that a considerable diffusion of the herbicide into the tissue is reached after intravenous administration.[19]

(c) They do not consider the fact that the human placenta is permeable to glyphosate; 15% of administered glyphosate by perfusion *in vitro* experiments trespasses the human placentary barrier.[20]

(d) They ignore the possibility that very low concentrations (pg/cell and not necessarily evenly distributed to all cells) may be sufficient to cause embryonic lethality (which is consistent with increased frequency of embryonic death and spontaneous abortions) or to modify normal embryonic pattern formation.

(e) They do not consider the paper of Dallegrove et al.[4] who observed craniofacial ossification defects and loss of caudal vertebrae in rats orally treated with sublethal doses of GBH. These alterations were statistically significant ($p < 0.05$, χ^2 test) in comparison with the control group and, importantly, were dose-dependent, indicating a specific effect. Although these authors do not address the molecular basis of the teratogenic effects they observe, an altered retinoid signaling pathway is a major candidate to be considered, for the following reasons: normal craniofacial morphology is the result of complex interactions between embryonic tissues and requires precise regulation of cell movement, growth, patterning, and differentiation. Mutations or misregulation of genes that influence any of these processes would cause craniofacial abnormalities, such as facial clefting and craniosynostosis. Among the critical genes involved in craniofacial development is the Msx family of homeodomain transcription factors.[21] Msx genes contribute in maintaining the balance between proliferation and differentiation during pre- and postnatal skull morphogenesis. Mutant mice for msx2 show incomplete or delayed ossification of the calvarial bones (i.e., those that constitute the upper part of the cranium and surround the cranial cavity), while the double mutants for msx1 and msx2 are deficient in calvarial ossification, thus resembling the "Skull, general incomplete ossification" observed in GBH-exposed embryos by Dallegrove et al. Regulation of the Msx genes by retinoids is supported by (a) the identification of a retinoic acid-responsive element in the 5′ flanking region of human MSX1 gene; and (b) functional *in vivo* evidence that indicates that endogenous retinoid signaling controls the spatial expression of this gene by inhibition. Therefore, it is conceivable that an increase in retinoid signaling upon exposure to GBH might inhibit msx expression, thus impairing the ossification of the cranial bones.

The other significant, dose-dependent effect of GBH exposure in rodent embryos described by Dallegrove et al. is "Caudal vertebrae: absent". It is well known to embryologists that exposure of mouse embryos to RA at a similar period of development produces agenesis of caudal vertebrae, which is caused by the down-regulation of posterior Hox genes.[22]

The arguments espoused by our critics do not and cannot rule out the possibility (which would be rather easy to check) that people exposed to GBH spraying accumulate glyphosate in their blood that can circulate and expose multiple tissues in the body to different concentrations of the chemical, producing different consequences. The vertebrate embryo is far from a black box that responds uniformly and monotonically to chemical insult. One possible example of the effects of spraying is the genotoxic effects reported in people exposed to agrochemicals and the effects in cultured cells exposed to dilutions of GBH that have been extensively studied by different laboratories in Argentina and Paraguay, Colombia, Ecuador, and France.[23−25] These studies raise important questions regarding the safety of GBH and have never been adequately addressed.

It is an indisputable fact that our work to date shows a direct association between the abnormal expression of key molecular markers (*shh*, *otx2*, *pax6*, etc.) and altered morphogenesis caused by increased retinoic acid signaling. Most notably, effects of the alleged toxic doses of glyphosate that we have used in our experiments are rescued by the addition of a retinoic acid antagonist that blocks the activity of the retinoic acid receptor. This evidence that links GBH (and potentially other chemicals) to increased activity of the retinoic acid signaling pathway (a very well-known teratogen, even to industrial scientists) highlights the increased number of embryonic malformations and spontaneous abortions in populations subjected to spraying with GBH and other cocktails.

In addition, it should be noted that microinjection of pure glyphosate and incubation of embryos with dilutions of the GBH produced the same type of phenotypic changes. These can and must be addressed in the context of embryological strategies and the potential molecular mechanisms suggested by the effects. It is obvious that providing proof-of-principle is an important epistemological way to verify that ideas are plausible and feasible as a necessary precursor to future studies.

We contend that it is an epistemological error and a lack of scientific rigor to reject strategies and, more generally, to ignore alternative views about scientific evidence and developmental mechanisms that are key factors in assessing chemical safety for convenience, indolence, or profit. Speeches and position papers reassuring the public instead of scientific debate are not helpful. Worse yet is the propagation of misinformation. Sadly, such strategies are not new in the modern world and harken back to the debates about tobacco safety in the 1970s.

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NOTE ADDED IN PROOF

In the course of correcting the proofs for this reply, two new letters to the editor were submitted by Dr. Mulet (Universitat Politecnica de Valencia) and Dr. Palma (Asociación Argentina de Productores en Siembra Directa, AAPRESID). The response to representatives of Monsanto, The Dow Chemical Company, United Phosphorus Inc., Nufarm Americas Inc., and Syngenta Ltd. is clear enough to avoid infinitely extending this exchange of letters that transcends the purely scientific interest. Despite this, it seems appropriate to make some comments:

(1) The present note is added in order to avoid falling into the habit of repetition.

(2) It seems pertinent to remark that one of the new letters was sent by a representative of the business association APRESSID, pretending to assume the role that would be expected by a peer review panel in the evaluation process of publication. This is achieved through disqualification in order to defend positions that have nothing to do with independent scientific activity. The doubts and skepticism expressed in the letters about dilution factors of the injected material, effect of pH, injection of substances in embryos, its meaning, its assessment, and interpretation are issues in Molecular Embryology and Developmental Biology that have been largely tested and accepted in these disciplines. Space and time excuse me for not being involved in discussions with interlocutors who are not familiar with this experimental discipline. These critics, besides impoverishing independent scientific discussion, show a light and inconsistent reading of our article.

(3) Finally, this is not the place for discussing epistemological issues about the value of different sources of knowledge.