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LETTER TO THE EDITOR: DEVELOPMENTAL AND REPRODUCTIVE OUTCOMES OF ROUNDUP AND GLYPHOSATE IN HUMANS AND ANIMALS

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To the Editor:

The authors of the bibliographic review "Developmental and Reproductive Outcomes in Humans and Animals After Glyphosate Exposure: A Critical Analysis" (Williams et al., 2012) tentatively analyzed four of our articles concerning the cellular toxicity and endocrine-disrupting effects of glyphosate (G)-based herbicides (GBH) including Roundup (R) (Gasnier et al., 2009; Richard et al., 2005; Benachour et al., 2007; Benachour and Seralini, 2009). GBH are the most used pesticides worldwide and major pollutants of rivers and surface waters; moreover, they have become common food and feed contaminants since the development of agricultural edible genetically modified (GM) plants, most of which are tolerant to R (James, 2011). Therefore a review on the developmental and reproductive outcomes induced by these formulations is of great interest. This is also why we have analyzed numerous endpoints of cellular toxicities induced by GBH on more than 10 cell types, and for the first time on cells crucially involved in the development. These include a human embryonic kidney-derived cell line, HEK293; fresh umbilical cord cells; and placental tissue. Of course, any sign of toxicity on these models may also concern developmental effects, in contrast to the assertion of Williams et al. (2012). We have also studied GBH-induced endocrine disruption in other publications that were not reviewed by Williams et al. (2012). These reports focus on the hormonal effects of this pesticide on rat testicular cells (Clair et al. 2012) and the disruptions on human mitochondrial activity, caspases 3/7, cytochromes P-450, and glutathione *S*-transferase (Gasnier et al., 2010, 2011); in addition, they address membrane integrity and the study of combined effects with a modified insecticide produced by GM plants on a human embryonic cell line (Mesnage et al., 2012a), but also the possible effects on the family of an agricultural worker and his children (Mesnage et al., 2010a). We also synthesized these data (Mesnage et al., 2010b).

Williams et al. (2012) admit that we have demonstrated that GBH have more cytotoxic effects than G on human cells, that there is a specific toxicity for adjuvants, and that G alone and R provoke human endocrine effects. However, they underestimate our experimental evidence and discredit our findings. There are according to them two major reasons for that, with which we disagree. Their review also contains several mistakes about our original experiments that we shall not detail, available for readers. For instance, the unfounded belief that we had residual detergents in our placental microsomes that could impact on GBH-induced endocrine disruption does not take into account the very high aromatase activity obtained in our controls, which was considerably reduced by GBH. We also measured a disruption of aromatase activity in in-cell aromatase assays without detergents.

Altogether, our results demonstrate two major findings that are closely linked to the

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reasons that were not taken into account by Williams et al. (2012). First, because of the differential effects between GBH and G, a review making conclusions about G alone is not relevant for the study of an environmental exposure to GBH, whereas GBH are in fact the relevant mixtures to be assessed as food/feed and major environmental pollutants. Indeed, G is never used alone in agriculture or gardening; in fact, at recommended dilutions it does not have any herbicidal properties alone, and it is quite easy to demonstrate this fact in trials. It is known that the adjuvants help G penetration into cells but also enter themselves, are stable (Nobels et al., 2011; Banduhn and Frazier, 1978), and may change G metabolism. We have shown that the mixtures of G and adjuvants are far more toxic even at 5–10 ppm than G alone, and that they are endocrine disruptors. G in R causes dose-dependent inhibitions of transcriptional activities of androgen receptors from 0.2 ppm, and then similarly with both estrogen receptors alpha and beta below toxic levels (Gasnier et al., 2009), by contrast to the interpretation of Williams et al. (2012). This is possibly due not only to adjuvants, but rather to the mixture effects of the GBH. We have also demonstrated that G inhibits by itself the aromatase catalytic site on the purified enzyme (Richard et al., 2005) or once entered into cells, and therefore inhibits estrogen synthesis, which is highly involved in developmental and reproductive physiology. This is why the regulatory tests performed by manufacturers on G alone in vivo, which are cited by Williams et al. (2012) to conclude that G is safe, are not at all relevant for the assessment of GBH toxicity. In addition, unlike in the Williams et al. redundant remarks, including some on the studies from other groups, the membrane-damaging effects of surfactants are obviously not a confounding factor in an in vitro system since these effects are designed to be necessary for G penetration through plant membranes, for instance, in vivo, and were also detailed in rat liver mitochondria (Peixoto, 2005). Thereby, adjuvants contained in the pesticides formulations are an aggravating factor of the GBH and G real toxicities. This is the reason why we suggested long-term in vivo toxicity tests on mammals with formulated pesticides containing adjuvants.

Second, a major criticism of our work is that the concentrations of R and G we used are not environmentally relevant. This is probably due to the fact that our data were overlooked, or due to a lack of knowledge. The highest residues of G (and its metabolite AMPA) authorized in GM feed are around 400–500 ppm (maximum residue limits or MRL; FAO 2012), and, for instance, 2 ppm in animal kidneys (EFSA, 2009). The levels in human urine may reach 0.2 ppm for occupational exposures (Acquavella et al., 2004); this elimination indicates in this case the regular kidney cells exposure. When pigs or dairy cows received, during 28 d only, 40 ppm of G (with a 9:1 ratio of its main metabolite AMPA), 0.32 ppm was recovered in the kidney (FAO 2005). One of our recent publications (Clair et al., 2012) demonstrates that at 1 ppm, the testosterone synthesis in rat Leydig cells is inhibited by R but also with G alone. Even if we consider a 100–1000 dilution of the residues present in feed or environment to calculate the environmental human or animal cells exposure in the body, without taking into account the potential bioaccumulation in the long term (Brewster et al., 1991; Monosson, 2005), our results on endocrine-disrupting effects (starting at 0.2 ppm of G in R) settle within this range and thus provide highly relevant information. We have even shown bioamplified cytotoxic effects of GBH between 24 and 72 h in cells (Benachour et al., 2007), and this suggests, together with our experiments on radiolabeled G, a bioaccumulation (Gasnier et al., 2011) that may increase the long-term effects of. G, effects that are not ignored by the U.S. Environmental Protection Agency (U.S. EPA, 2012). We are now listing several other specific examples where the interpretations by Williams et al. (2012) are inappropriate, although our findings have already been extensively described in our papers.

These authors do not describe accurately all the models we used to demonstrate a cellular endocrine disruption of R on aromatase activity and gene expression: that is, JEG3 placental cells, fresh placental extracts, testicular

microsomes, and finally purified aromatase to prove direct enzymatic interactions with G by spectral analyses (Richard et al., 2005). G is always used as a control in the study, in contrast to the false interpretations that were raised. Moreover, they assert that the JEG3 cell cultures and fresh human placental extracts are not validated in vitro systems, thus mixing up the regulatory norms of the Ministry of Agriculture with research science. Any model may highlight some new molecular mechanisms that are not yet studied in regulatory files for pesticide agreements according to Organization for Economic Cooperation and Development (OECD) guidelines. Also, science has to take into account up-to-date knowledge to evolve; this is often not the case with regulatory norms, which need time to integrate novelty. Indeed, regulatory tests and their guidelines (i.e., good laboratory practices) have been proven in some cases to be aging models, using inappropriate controls and insensitive tests and models, and may thus cause false negative results (Myers et al., 2009). Furthermore, a large number of authors consider JEG3 cells useful for assessing placental toxicity (Letcher et al., 1999). This cell line may be even less sensitive to xenobiotics than primary cultures (L'Azou et al., 2005). Since the toxicity phenomena and at lower levels the endocrine disruptions observed (Richard et al., 2005; Benachour et al., 2007) are amplified with time by several orders of magnitude, this could well be an indication of toxicity if bioaccumulation occurred. In the following studies, we further explored the range of toxic levels as previously described. We should add that the concentrations of G required to inhibit aromatase activity in combination with R (360 g/L of G as clearly indicated in our paper) are also really indicated in Figure 4 from Richard et al. (2005), in contradiction to what these authors indicate.

Moreover, some technical points underlined by Williams et al. (2012) are precisely answered in our studies. For example, the pH effects on cells of GBH were extensively studied: in Benachour et al. (2007), Figures 5A and B show no significant difference between adjusted pH (7.4) and nonadjusted pH (5.8), and aromatase activities disruptions were quite comparable in both cases. Thus, the effects observed are not due to changes in pH, in contrast to the inaccurate reading of Williams et al. (2012). Then the authors incriminate the absence of serum in some of our experiments to explain the cellular toxicity of GBH. However, we have extensively compared (Benachour et al., 2007) cultures with and without serum showing good cell viability at all the times that we studied, even at least up to 72 h. A quicker impact of xenobiotics was also observed in serum-free cultures, but it was similarly visible 1–2 d later in the presence of serum, which buffered the effects, at least because of the albumin content. Anyway, each effect measured was compared to its appropriate control showing no toxicity (in serum-free cultures, with GBH or not, at similar times). Once again, Williams et al. made comments on our work that were too superficial.

Last but not least, one criterion to judge results from others as not related to treatments for Williams et al. (2012) is the nonlinearity of the observed effects. It is, however, very well known today that endocrine-disrupting (Vandenberg et al., 2012), or carcinogenic, nervous, or immune effects are often nonlinear. For instance, some xenobiotics present effects that are not proportional to the dose in short- or mid-term studies (including U or J curves in cell cultures). This should not be neglected (Vandenberg et al., 2012; Benachour et al., 2011), and these harmful effects may be revealed by systematic chronic tests in mammals (Seralini et al., 2009). As commercial authorizations are generally given without testing chronic effects of the whole pesticide formulation, acceptable daily intakes of pesticides are generally established neglecting adjuvants due to reasoning comparable to that of Williams et al. This is a crucial gap. Another one is that these authors fall into the trap of comparing adjuvants (supposedly inert ingredients) to inactive ingredients, which is a mistake (U.S. EPA 1997). Some so-called "inert" adjuvants of R acted as active principles for human cell toxicity, challenging the relevance of testing G as the active principle in R (Mesnage et al., 2012b).

Unfortunately for the relevance of their interpretations, we underline again that G is never used without adjuvants. Since their review on developmental and reproductive toxicity of GBH is funded by the manufacturer of the major GBH (R), and since this appears to be an influential factor in the ability (or inability) to detect and interpret biologically significant effects in toxicity studies (Vandenberg et al., 2012), we therefore question the value of their conclusions as a whole.

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