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Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity

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ABSTRACT

Pesticides are always used in formulations as mixtures of an active principle with adjuvants. Glyphosate, the active ingredient of the major pesticide in the world, is an herbicide supposed to be specific on plant metabolism. Its adjuvants are generally considered as inert diluents. Since side effects for all these compounds have been claimed, we studied potential active principles for toxicity on human cells for 9 glyphosate-based formulations. For this we detailed their compositions and toxicities, and as controls we used a major adjuvant (the polyethoxylated tallowamine POE-15), glyphosate alone, and a total formulation without glyphosate. This was performed after 24 h exposures on hepatic (HepG2), embryonic (HEK293) and placental (JEG3) cell lines. We measured mitochondrial activities, membrane degradations, and caspases 3/7 activities. The compositions in adjuvants were analyzed by mass spectrometry. Here we demonstrate that all formulations are more toxic than glyphosate, and we separated experimentally three groups of formulations differentially toxic according to their concentrations in ethoxylated adjuvants. Among them, POE-15 clearly appears to be the most toxic principle against human cells, even if others are not excluded. It begins to be active with negative dose-dependent effects on cellular respiration and membrane integrity between 1 and 3 ppm, at environmental/occupational doses. We demonstrate in addition that POE-15 induces necrosis when its first micellization process occurs, by contrast to glyphosate which is known to promote endocrine disrupting effects after entering cells. Altogether, these results challenge the establishment of guidance values such as the acceptable daily intake of glyphosate, when these are mostly based on a long term in vivo test of glyphosate alone. Since pesticides are always used with adjuvants that could change their toxicity, the necessity to assess their whole formulations as mixtures becomes obvious. This challenges the concept of active principle of pesticides for non-target species.

1. Introduction

Pesticide formulations are mixtures of adjuvants and so-called "active principles" on plants for herbicides, and insects for insecticides, etc. The supposed specificity of active principles on their targets does not mean a priori that they are the most toxic compounds of the formulations on human cells. Numerous mammalian (Colborn et al., 1993) and other animal studies (Hawthorne and Dively, 2011) evidenced side effects for pesticides. The toxicology of mixtures cannot be fully understood without knowing the differential toxicity of the various compounds of the formulations and their combined effects. Surprisingly, to measure their side effects, the active principles of pesticides are generally tested alone at a regulatory level in long-term mammalian trials, although their adjuvants are developed at least to enhance their stability and penetration into cells. However, most of the adjuvants are classified as inert.

Here we tested the differential and combined cytotoxicity of the major pesticides in the world which are glyphosate-based herbicides (GBH), and analyzed their composition and mechanisms of action. The residues of the GBH such as Roundup (R) are also among the first contaminants of ground and surface waters (IFEN, 2006), and of some food and feed because they are present since more than 15 years in around two third of genetically modified (GM) cultivated edible plants, because they are designed at least to tolerate R (James, 2011). Glyphosate (G) is toxic in plant cells by inhibition of 5-enolpyruvylshikimate-3-phosphate synthase used as a first step in aromatic amino acid synthesis (Boocock and Coggins, 1983). Adjuvants considered as inert include, according to the formulations, surfactants like POEAs (polyethoxylated alkylamines,

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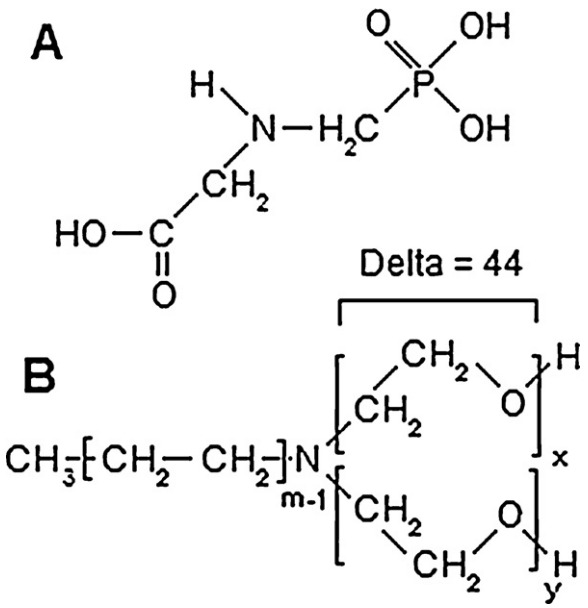

Fig. 1. Structures of glyphosate (A) and POEAs (B). Glyphosate is the N-(phosphonomethyl)glycine, C3H8NO5P). Di-ethoxylates of tallowamines adjuvants (C_mNEO_n, $n = x + y$) such as POEA are characterized by their oxide/tallowamine ratio. The delta of 44 (—CH_2—CH_2—O—) corresponded to the increment of the different peaks observed in mass spectrometry. Length of the more abundant tallowamine part in the adjuvant mixture corresponded to the maximal *m/z* of the spectrum.

Fig. 1), isobutane, light petroleum distillate, etc. that may induce among other DNA damages (Cox, 2004). However G is still generally hypothesized to be the active ingredient for non-target side effects. Unexpected side effects of G-based formulations were evidenced on non-target species, among other endocrine disruptions during spermatogenesis or pregnancy (Beuret et al., 2005; Clair et al., 2012; Dallegrave et al., 2007; Daruich et al., 2001; Oliveira et al., 2007; Romano et al., 2011; Savitz et al., 1997; Yousef et al., 1995). This may be related to adjuvants in formulation. They are indeed more and more considered as responsible for GBH toxicity (Mesnage et al., 2010; Williams et al., 2012), but the mechanistic and the nature of the cytotoxic agent(s) on human cells are still unknown. This is a general question that can arise for all pesticides.

The detailed known composition indicate that major adjuvants are ethoxylated, such as POEAs which are themselves mixtures of di-ethoxylates of tallowamines characterized by their oxide/tallowamine ratio. POEA commonly used in GBH is the POE (15) tallowamine (POE-15). We thus compared the toxicity and the composition of 9 formulations varying in adjuvants contents: Roundup Ultra, Roundup GT, Roundup GT+, Roundup Bioforce, Roundup 3plus, Glyphogan, Topglypho 360, Clinic E.V., and Bayer GC. For controls, we tested a formulation containing POE-15 without G (Genamin T200), and POE-15 alone. The compositional analysis of these products was performed by a non-quantitative mass spectrometry (MALDI-TOF MS/MS), considered as the best way to analyze pesticides formulations (Corbera et al., 2010; Cserháti and Forgács, 1997). Physico-chemical properties of POE-15 were approached by the measurements of its critical micelle concentration (CMC), determined by absorption changes in its presence of Coomassie blue CBB R-250.

We used HEK293, JEG3 and HepG2 cell lines, three models where unexpected effects of GBH have already been demonstrated (Benachour and Seralini, 2009; Gasnier et al., 2009). JEG3 cells are a useful model for examining placental toxicity (Letcher et al., 1999), and HepG2 for hepatic toxicity (Urani et al., 1998). HEK293 were chosen because of the sensitivity of embryonic cells, Roundup causing pregnancy outcomes (Savitz et al., 1997). Moreover, we have demonstrated that these cell lines are even less sensitive than primary cells (Benachour and Seralini, 2009; L'Azou et al., 2005), and therefore are possibly representative of a real cellular toxicity. For cytotoxicity measurements, we assayed mitochondrial succinate dehydrogenase (SD) activity (MTT assay), G and its formulations are indeed known to target mitochondria (Astiz et al., 2009; Peixoto, 2005). Cytotoxicity was also characterized by the measurement of apoptosis and necrosis, respectively by caspases 3/7 activation (Liu et al., 2005) and adenylate kinase leakage after membrane alterations (Crouch et al., 1993).

Overall, we questioned if an active toxic principle in a target species may be always generalized as such in a non target one, and thus if the regulatory toxicological tests on active principles alone are relevant.

2. Materials and methods

2.1. Chemicals

Glyphosate (N-phosphonomethyl glycine, G, CAS: 1071-83-6) was purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). GBH formulations available on the market were by alphabetical order: Bayer GC (12.5% of G, 1–5% of POE-15, homologation 05873567), Clinic EV (42% of G, 11% of POE-15, homologation 9900039), Genamin T200 (60–80% of POE-15, homologation 8500170), Glyphogan (39–43% of G, 13–18% of POE-15, homologation 9100537), Roundup Grand Travaux (400 g/L of G, R GT, homologation 8800425), Roundup Grand Travaux plus (450 g/L of G, 90 g/L of ethoxylated etheralkylamine (EtO-EA), R GT+, homologation 2020448), Roundup Ultra (41.5% of G, 16% surfactant, homologation 9700259), Roundup Bioforce (360 g/L of G, homologation 9800036), Roundup 3plus (170 g/L of G, 8% surfactant homologation 9300241), Topglypho 360 (360 g/L of G, homologation 2000254). POE-15 (CAS: 61791-26-2) was purchased from ChemService (West Chester, PA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other compounds, otherwise noticed, were obtained from Sigma–Aldrich. MTT was prepared as a 5 mg/mL stock solution in phosphate-buffered saline, filtered through a 0.22 μm filter before use, and diluted to 1 mg/mL in a serum-free medium.

2.2. Cell lines and treatments

The human embryonic kidney 293 cell line (HEK 293, ECACC 85120602), was provided by Sigma–Aldrich (Saint-Quentin Fallavier, France). The hepatoma cell line HepG2 was provided by ECACC (85011430). JEG3 cell line (ECACC 92120308) was provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM (Abcys, Paris, France) containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (a mixture of penicillin, streptomycin and fungizone) (Lonza, Saint Beauzire, France), 10 mg/mL of liquid kanamycin (Dominique Dutscher, Brumath, France) and 10% Fetal Bovine Serum (PAA, les Mureaux, France). JEG3 cells were supplemented with 1 mM sodium pyruvate. Cells were grown with this medium at 37 °C (5% CO_2, 95% air) during 48 h to 80% confluence, and then washed and exposed 24 h with serum-free EMEM to various chemicals. This model was validated (Benachour et al., 2007) since cytotoxic effects were similar in presence of serum but delayed by 48 h. The dilutions of formulated herbicides, adjuvants and G alone were prepared in serum free medium as stock solutions at a similar pH.

2.3. Cytotoxicity biomarkers

After treatments, the following tests were applied: succinate dehydrogenase (SD) activity assay (MTT) (Mosmann, 1983). Integrity of mitochondrial dehydrogenase enzymes indirectly reflects the cellular mitochondrial respiration. The optical density was measured at 570 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The bioluminescent ToxiLight bioassay (Lonza, Saint Beauzire, France) was applied for the membrane degradation assessment, by the intracellular adenylate kinase (AK) release in the medium; this is described as a necrosis marker (Crouch et al., 1993). Finally, the apoptotic cell death was evaluated with the Caspase-Glo 3/7 assay (Promega, Paris, France). Luminescence was measured using a Mithras LB 940 luminometer (Berthold, Thoiry, France). These methods were previously described (Benachour and Seralini, 2009).

2.4. Mass spectrometry (MS)

MS experiments were carried out on an AB Sciex 5800 proteomics analyzer equipped with TOF TOF ion optics and an OptiBeam™ on-axis laser irradiation with 1000 Hz repetition rate. The system was calibrated immediately before analysis with a mixture of des-Arg-Bradykinin, Angiotenin I, Glu1-Fibrinopeptide B, ACTH (18–39), ACTH (7–38) and mass precision was better than 50 ppm. A 0.8 μL volume of the GBH solution diluted 100 times in water was mixed with 1.6 μL volumes

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of solutions of α-cyano-4-hydroxycinnamic acid matrix prepared in 50% ACN with 0.1% TFA. The mixture was spotted on a stainless steel Opti-TOF™ 384 targets; the droplet was allowed to evaporate before introducing the target into the mass spectrometer. Acquisitions were taken in manual and automatic modes. A laser intensity of 3000 was typically employed for ionizing. MS spectra were acquired in the positive reflector mode by summarizing 1000 single spectra (5 × 200) in the mass range from 100 to 2000 Da. MS/MS spectra were acquired in the positive MS/MS reflector mode by summarizing a maximum of 2500 single spectra (10 × 250) with a laser intensity of 3900. For the tandem MS experiments, the acceleration voltage applied was 1 kV and air was used as the collision gas. Gas pressure medium was selected as settings.

2.5. Critical micelle concentrations (CMC) determinations

CMC determinations were performed and adapted according to (Samsonoff et al., 1986). CMC was measured by the incorporation of Coomassie brilliant blue R-250 (CBB-R250) in micelles formed by serial dilutions of detergents. The CCB-R250 reagent was prepared as previously described (Bradford, 1976). Varying concentrations of adjuvants were added in a volume of 1 mL, 100 μL of CBB-R250 was added to make a final concentration of 80 μg/mL. Solutions were shaken and distributed in 96 well-plates in triplicate. Absorption was then measured against a water blank at 600 nm using a Mithras LB 940 luminometer (Berthold, Thoiry, France). The validation of the technique was performed with triton X-100, with a CMC of 0.15–0.20 mM (Courtney et al., 1986).

2.6. Statistical analysis

The experiments were repeated at least 3 times in different weeks on 3 independent cultures ($n = 9$). LC_{50} values were calculated by a nonlinear regression using sigmoid (5-parameters) equation with the GraphPad software. All data were presented as the means ± standard errors (SEMs). Statistical differences were determined by Student's t-test using significant levels with $p < 0.01$ (**) and $p < 0.05$ (*).

3. Results

Here we studied for the first time the precise involvement of the adjuvants and G in GBH induced toxicity, on three human cell lines from different embryonic origins (kidney, liver, and placenta) in order to test their specificities. We first compared mitochondrial respiration (SD activity) in presence of 9 formulated mixtures of G and adjuvants, G alone, formulating agents without G (Genamin), and a major adjuvant of some formulations, POE-15 (Fig. 2). All chemicals are cytotoxic, inducing similar dose-dependent patterns on HEK293, HepG2, and JEG3 in 24 h. JEG3 were up to 2-fold more sensitive to treatments than HEK293 and HepG2 in comparison to control. We observed for all cell lines different ranges of toxicities

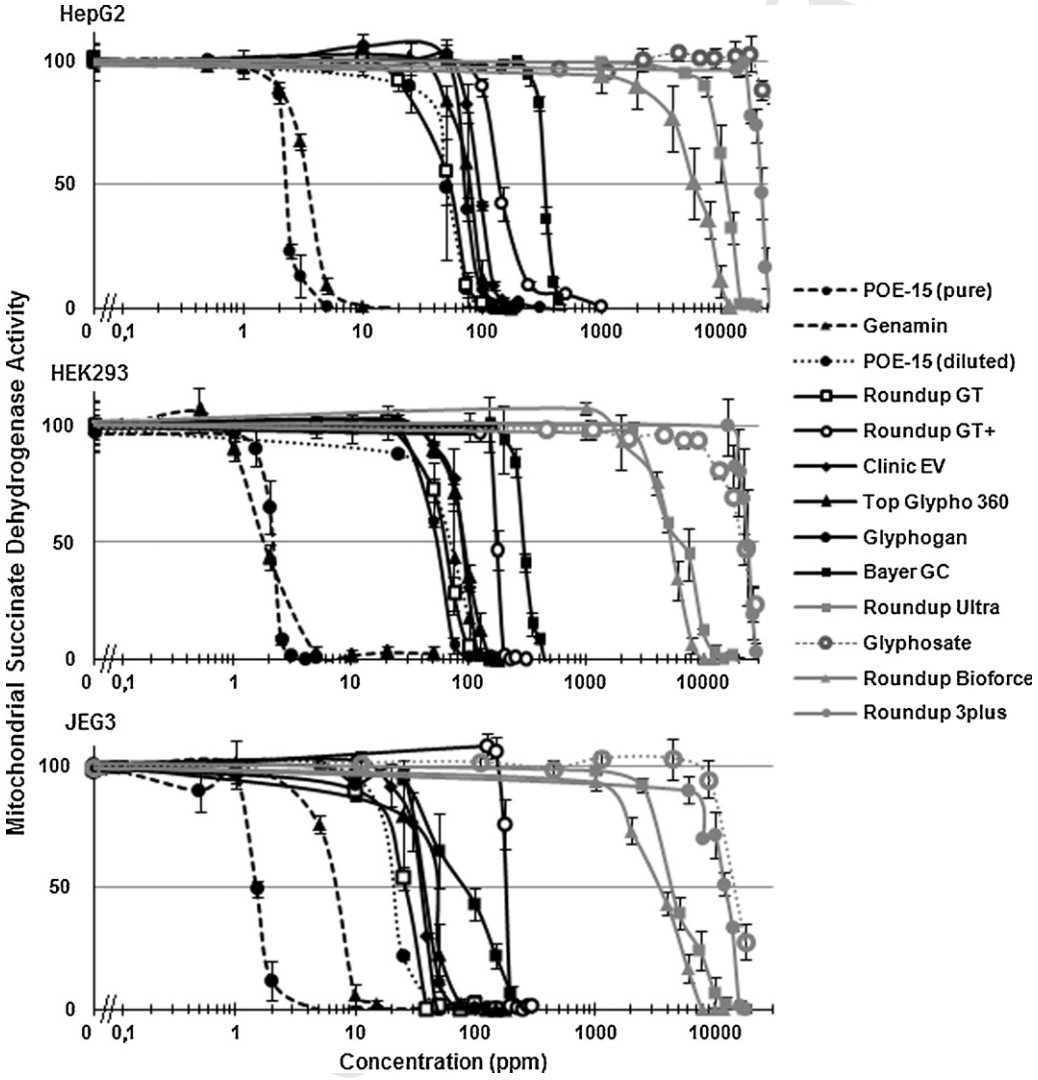

Fig. 2. Dose-dependent cytotoxic effects of glyphosate-based herbicides (GBH) or glyphosate (G) and adjuvants alone (POE-15 and Genamin) on HepG2, HEK293 and JEG3 human cell lines. Effects on the mitochondrial succinate dehydrogenase (SD) activity, reflecting cell respiration inhibition, were measured in % of control in serum-free medium after 24 h of exposure. The concentrations in ppm are dilutions of each mixture in the commercial formulation (considered as 100%). The adjuvants POE-15 and Genamin alone (a mixture containing 785 g/L of POE-15, no G) were the most toxic. The middle group approximately 100-fold less toxic was composed by GBH: Roundup GT, Roundup GT+, and Clinic EV, Top Glypho 360, Glyphogan, Bayer GC. The less toxic group was formed by Roundup Ultra, Bioforce and 3plus. SEMs are shown in all instances ($n = 9$).

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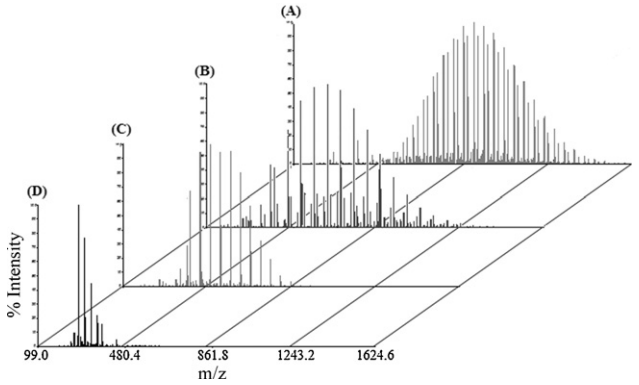

Fig. 3. MALDI-TOF analysis of glyphosate-based herbicides (GBH) main adjuvants. (A) POE-15 spectrum was centered on 900*m/z* (increment delta 44, Fig. 1), all other herbicides (group A, see Table 1) declaring a POEA adjuvant had the same spectrum. In addition, identification was confirmed by MS/MS fragmentation. (B) The 3 Roundup Ultra, Bioforce and 3plus contained another common adjuvant (600*m/z*, delta 58). (C) Adjuvants of Roundup GT+ (500*m/z*, delta 44, Fig. 1) were declared as ethoxylated etheralkylamines (EtO-EA). (D) Adjuvants of Roundup GT (300*m/z*, delta 44, Fig. 1) were identified as POE-2.

allowing the classification of the products tested as follows. The most toxic were the adjuvants alone POE-15 ($LC_{50} \sim 1$–2 ppm; agricultural dilutions: 1–2% of the herbicide formulation containing adjuvants) and Genamin, themselves around 100-fold more toxic than a middle group with the majority of formulations (6, with among them R GT and GT+). This middle group is again 100-fold more toxic than the third one which includes R Ultra, R Bioforce, R 3plus and finally G alone. Moreover, POE-15 diluted to the concentration at which it is present in Clinic E.V. (a formulation from the middle group) presented a similar toxicity than this GBH and to the middle group in general. It thus appears to be the toxic principle in human cells. In addition, we also demonstrate that two formulations claiming a similar concentration of G (360 g/L) and different adjuvants (16% of POEA or other adjuvants), Glyphogan and R Ultra respectively, exhibited very different toxicities, 150-fold stronger on average for Glyphogan on the 3 cell lines (Fig. 2). Thus some other adjuvants appear also to have some toxicity.

To check the composition in adjuvants we studied all the formulations by MALDI-TOF MS/MS (Fig. 3). Knowing that the specificities of MALDI-TOF ionization did not detect G but adjuvants, we separated 4 groups of adjuvants: (A) with a spectrum centered on 900*m/z*, POE-15 and Genamin, and those present in 4 formulations of the middle group thus containing also POE-15, (B) those contained in the third less toxic group with a spectrum centered on 600*m/z* corresponding to another common adjuvant, and (C) and (D), two other adjuvants in the formulations of the middle group, respectively in (C) R GT+ (500*m/z*) and (D) R GT (300*m/z*). The belonging of each product to each group was further confirmed by analysis of fragmentation spectra, giving for instance for ions of group A: 840.6, 858.7, 884.7, 902.8*m/z*. All these spectra corresponded to the family of alkylamines. The POE-15 had a peak increment of 44 (delta) like all group A (Table 1). The same delta in C and D were characteristic of an ethoxylated chain. C was an ethoxylated etheralkylamine, D was confirmed by fragmentation to be identical to POE-2; and a delta of 58 corresponded to another non ethoxylated adjuvant in group B. We summarized these findings with LC_{50} values (Table 1).

We then tested the linearity of the toxicity in function of G or ethoxylated adjuvants concentrations (Fig. 4). The cytotoxicity induced by GBH is not linear to G concentrations ($R^2 \sim 0.3$, Fig. 4A), but only to the 3 ethoxylated adjuvants ($R^2 > 0.93$, Fig. 4B), and not to the non-ethoxylated one, and this is obtained with all cell lines. Ethoxylated adjuvants can thus be considered as the active principle of the toxicity of GBH in human cells.

In order to understand the mechanism of action of adjuvants, three other experiments were performed. First, the critical micelle concentration (CMC) of POE-15 was determined by absorption changes of CBB R-250 (Fig. 5). The method was validated by the measurement of the CMC of the triton X-100 (0.15–0.20 mM (Courtney et al., 1986)). We evidenced a micellization of POE-15 beginning at 3 ppm, similarly to toxicity thresholds (Fig. 2). POE-15 thus appears to be able to disrupt the cellular membranes by micellization with the lipid bilayer around the CMC. This was even better understood by the differential measurement of the cytotoxicity through membrane disruption or caspases activation (Fig. 6). For the three cell lines, results are almost comparable: POE-15 and R GT+ (containing also an ethoxylated adjuvant) induced more necrosis (Fig. 6A) by membrane alterations rather than apoptosis (Fig. 6B), even if present. By contrast, G induced only apoptosis at higher levels. Ethoxylated adjuvants are thus not inert at all but cell membrane disruptors, and then induce severe mitochondrial alterations.

4. Discussion

This study unravels the differential nature and cytotoxicity of the main compounds from the major herbicide formulations in the world. These formulations are conceived to enhance the pesticide activity through mixtures of adjuvants and G. The latter is the active principle toxic in plants; in this study we checked how this

Table 1
Main spectral and toxicological characteristics of the herbicides (GBH) and adjuvants tested. Groups corresponded to spectra of adjuvants contained in products according to Fig. 3. Contents in glyphosate and adjuvants were indicated by manufacturers (except for POE-2) and identified by MS/MS as revealed by *m/z* and delta measurements. LC50 (ppm) are calculated from Fig. 2. nd: non detected.

Group	Products tested	Glyphosate (g/L)	Adjuvants	*m/z* (MS)	Delta (MS)	LC50 HepG2 (ppm)	LC50 HEK293 (ppm)	LC50 JEG3 (ppm)
A	Topglypho 360	360	15% POE-15	900	44	79	89	37
	Glyphogan	360	13–18% POE-15	900	44	59	54	30
	Clinic E.V	360	11% POE-15	900	44	94	89	34
	Bayer GC	96	1–5% POE-15	900	44	333	290	84
	Genamin	0	60–80% POE-15	900	44	4	2	7
	POE-15	0	POE-15	900	44	2	2	1
B	R Ultra	360	16% nc	600	58	11,000	6395	4477
	R Bioforce	360	nc	600	58	6106	5043	3560
	R 3plus	170	nc	600	58	22,000	24,000	1200
C	Roundup GT+	450	7.5% Eto-EA	500	44	145	170	115
D	Roundup GT	400	POE-2	300	44	53	62	32
	Glyphosate	>95%	nd	nd	nd	nd	19,323	1192

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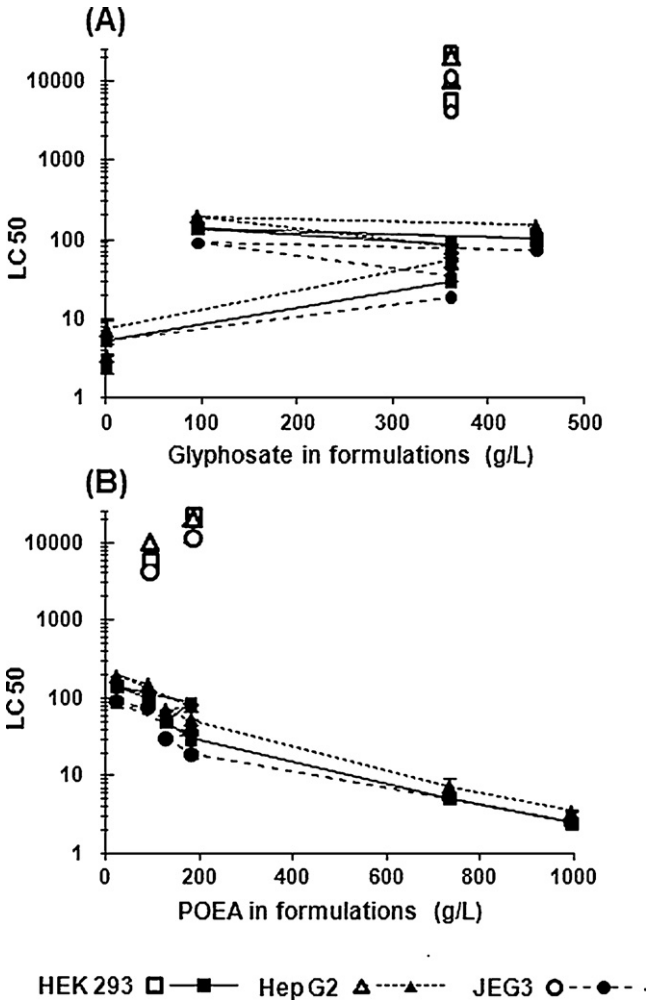

Fig. 4. Toxicity of glyphosate in formulations (A) measured by LC50, and of adjuvants in glyphosate-based herbicides (B) on the three human cell lines described. The effects on the mitochondrial succinate dehydrogenase (SD) activity were measured to calculate the LC50s (ppm) and compiled to be compared in relation to glyphosate or adjuvants concentrations. The form of the symbols is related to the cell lines (squares for HEK293, triangles for HepG2 and circles for JEG3). For colors, black dots are ethoxylated adjuvants, white dots are others. The three described human cell lines were used in the conditions of Fig. 2 and the results were almost identical. The linear correlation was not obtained (A) between glyphosate concentration and toxicity (coefficient of determination is 0.36 for HEK293, 0.35 for HepG2 and 0.29 for JEG3), but was demonstrated between the concentrations in the formulations of ethoxylated adjuvants (B) and toxicity (coefficient of determination is 0.94 for HEK293, 0.97 for HepG2 and 0.93 for JEG3). SEMs are represented in all instances ($n = 9$).

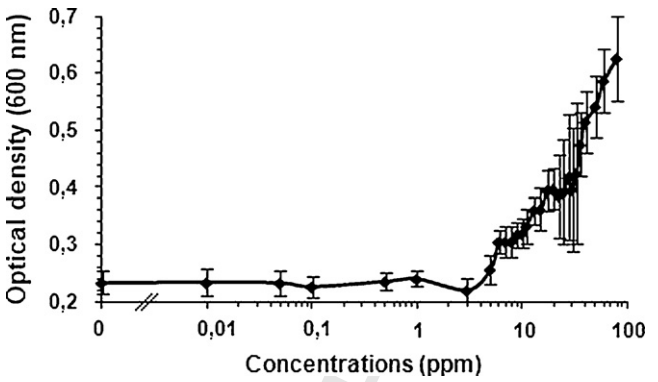

Fig. 5. Critical Micelle Concentration (CMC) of the POE-15 determined by absorption changes of Coomassie Brilliant Blue R-250. CBB R-250 was added to serial dilutions of POE-15 in serum-free medium. D.O. at 600 nm was measured with a spectrophotometer. A major breakpoint was evidenced in the curve around 3 ppm, at the CMC. SEMs are shown in all instances ($n = 9$).

active principle is differentially toxic on non-target organisms in comparison to the so-called inert adjuvants in numerous formulations.

Here we demonstrate that all formulations are more toxic than G alone on three human cell lines as previously underlined (Benachour and Seralini, 2009; Richard et al., 2005). Then for the first time we separated experimentally three groups of formulations differentially toxic according to the amount of ethoxylated adjuvants. The 3 less toxic formulations (like G alone) were demonstrated to contain no ethoxylated adjuvants by mass spectrometry, and are around 10,000 times less toxic on mitochondrial activity than POE-15 alone, the major adjuvant. All the other formulations were toxic proportionally to the dilutions of POE-15 or other ethoxylated adjuvants in the formulations, in a linear manner to some extent; in fact G does not buffer or amplify direct POE-15 toxicity.

Thus POE-15 appears to be clearly the toxic principle in human cells. It begins to be active with negative effects on cellular respiration and membrane integrity between 1 and 3 ppm, when its first micellization process occurs in this work. This membrane disruption then lead to the necrotic adjuvant-linked effects observed, amplifying the necrosis/apoptosis ratio by contrast to G at higher levels as shown. Accordingly, it was found (Chamel and Gambonnet, 1997) that a CMC of the $C_{18}NEO_{20}$ congener of a POEA is around 2 ppm. Its partition coefficient measured at around 1.7 confirmed its lipophilic character and its ability to penetrate the cells. It is known that ethoxylated adjuvants can insert in cells membranes, disrupting their structure and functions as previously shown in bacteria (Nobels et al., 2011). This is a general property of surfactants (Boeije et al., 2006). We notice that among different class of surfactants, ethoxylated adjuvants are of the more toxic, even potentially genotoxic (Nobels et al., 2011). Importantly, this is not only observed in vitro because when rats are treated with G, R and POEA, the latter was also found to be the most toxic compound (Adam et al., 1997), even in other animal models (Marc et al., 2005). This was demonstrated for other pesticides (Eddleston et al., 2012). Generally, the question of the toxicity of adjuvants in pesticides is more and more recognized (Brausch and Smith, 2007; Krogh et al., 2003; Tsui and Chu, 2003).

This does not exclude cellular endocrine disruptions below these levels that may not be due to POE-15 alone (or other ethoxylated adjuvants), but that occur through glyphosate entering in aromatase active site for instance (Richard et al., 2005) or in androgen receptor which is inhibited from 0.2 ppm of G in adjuvants (Gasnier et al., 2009). It should not be forgotten that G has its own toxicity and may also exert long term or chronic toxicity. The active principle G alone has been evidenced to cause oxidative stress (Astiz et al., 2009; Cavusoglu et al., 2011), endocrine disruption (Clair et al., 2012), or developmental effects (Marc et al., 2005). G was even recently described as a teratogen (Paganelli et al., 2010). In this case we have a model of multiple combined negative effects (through different cellular metabolic endpoints) caused by the main pesticide mixtures, which are the formulations themselves. This is true even if the activities of ethoxylated adjuvants on endocrine disruption must be still detailed in the future.

These results were obtained in vitro; cellular cultures replace whenever it is possible animal experimentation (Hartung, 2009). Our study was performed during 24 h and does not anticipate the elimination or the possible bioaccumulation and long term combined effects with other xenobiotics. R human cellular effects indeed increased according to time (Benachour et al., 2007) and radiolabeled G accumulated in cells within 48 h, suggesting a

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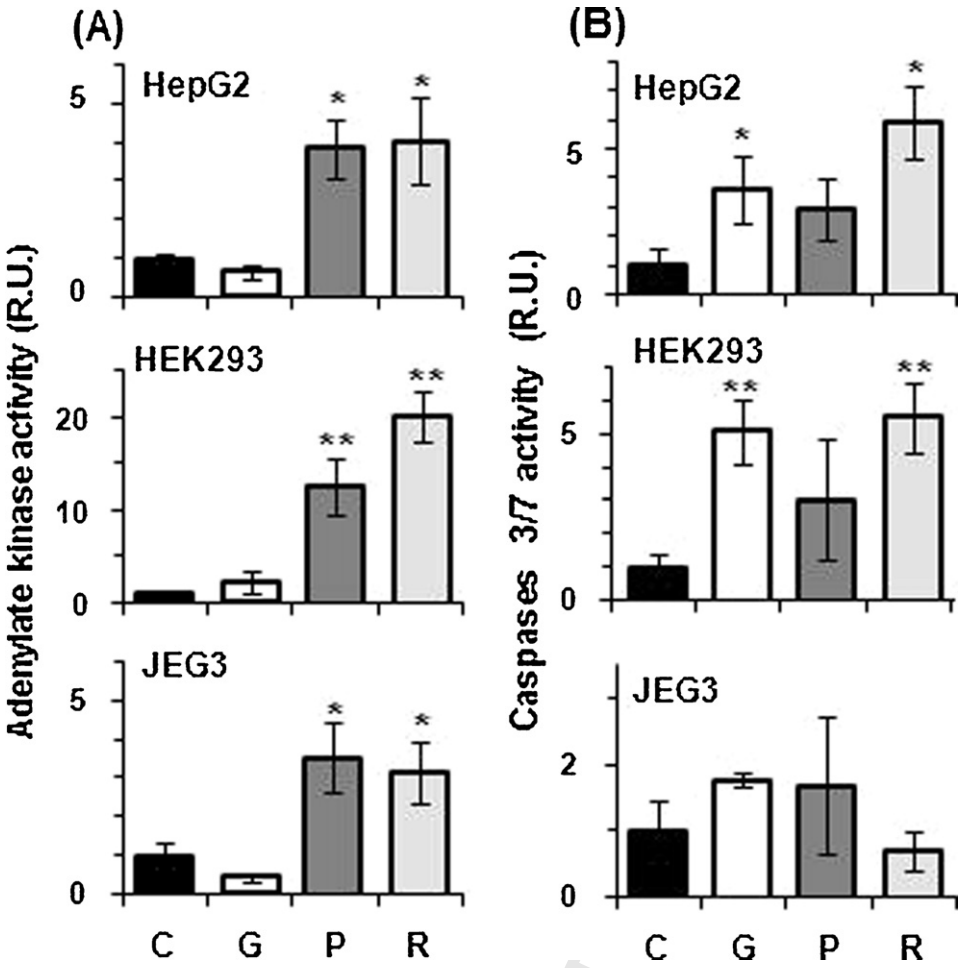

Fig. 6. Cytotoxic effects of control (C), glyphosate (G), POE-15 (P) and Roundup GT+ (R). Cell membrane integrity reflecting necrosis (A) was measured by adenylate kinase leakage (active in the medium), and apoptosis (B) by caspases 3/7 activities, both expressed in relative units (RU) after 24 h of treatments like in Fig. 2. To understand the mechanism of cytotoxicity, the concentrations in products were those inducing 80% of the general cytotoxicity in MTT assay. SEMs are shown in all instances ($n = 12$, $^{*}p < 0.05$; $^{**}p < 0.01$).

bioaccumulation of low concentrations of G (Gasnier et al., 2011). R adjuvants may also form adducts and link to DNA avoiding a direct elimination (Peluso et al., 1998).

However, our lowest thresholds of toxicities and endocrine disruptions may be comparable to the range of environmental/occupational exposures. A farmer or a gardener spraying a GBH may be punctually exposed to 5000 ppm, and even regularly by occupational exposure. As a matter of fact G varied from 3 to 233 ppb in farmers urine (Acquavella et al., 2004), this may be in addition to a chronic dietary/drink exposure of G found around 70 ppb (Aris and Leblanc, 2011).

In conclusion, pesticide formulations should be studied as mixtures for toxic effects. The multiple combined effects could induce pathologies on a long term. Here we can question the use of ethoxylated adjuvants in herbicide formulations, since they appear as principles for human cell toxicity. This leads also to challenge guidance values such as the acceptable daily intake (ADI) of G, which is calculated with pure G in long term toxicological tests in vivo (German Federal Agency CPFS, 1998), while G is always used with adjuvants that are not immediately biodegradable (Banduhn and Frazier, 1978) and could change its toxicity. This will be also important for other active principles of pesticides, and thus their ADI can be overestimated. The necessity of studying formulations as mixtures is common to all pesticides. The pathological consequences of exposure to chronic toxicities of whole formulations could be tested with mammals over a 2-year period. This implies a complete shift in the concepts underlying chemical toxicology, which could come from mixtures studies.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Acquavella, J.F., Alexander, B.H., Mandel, J.S., Gustin, C., Baker, B., Chapman, P., Bleeke, M., 2004. Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. Environ. Health Perspect. 112, 321–326.

Adam, A., Marzuki, A., Abdul Rahman, H., Abdul Aziz, M., 1997. The oral and intratracheal toxicities of ROUNDUP and its components to rats. Vet. Hum. Toxicol. 39, 147–151.

Aris, A., Leblanc, S., 2011. Maternal and fetal exposure to pesticides associated to genetically modified foods in Eastern Townships of Quebec, Canada. Reprod. Toxicol. 31, 528–533.

Astiz, M., de Alaniz, M.J., Marra, C.A., 2009. Effect of pesticides on cell survival in liver and brain rat tissues. Ecotoxicol. Environ. Saf. 72, 2025–2032.

Banduhn, M., Frazier, H., 1978. G 3780A surfactant: biodegradation in nature waters. Report No. MSL-0488. Monsanto Agricultural Research Department, St. Louis.
Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Seralini, G.E., 2007. Time- and dose-dependent effects of roundup on human embryonic and placental cells. Arch. Environ. Contam. Toxicol. 53, 126–133.
Benachour, N., Seralini, G.E., 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. Chem. Res. Toxicol. 22, 97–105.
Beuret, C.J., Zirulnik, F., Gimenez, M.S., 2005. Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. Reprod. Toxicol. 19, 501–504.
Boeije, G.M., Cano, M.L., Marshall, S.J., Belanger, S.E., Van Compernolle, R., Dorn, P.B., Gumbel, H., Toy, R., Wind, T., 2006. Ecotoxicity quantitative structure–activity relationships for alcohol ethoxylate mixtures based on substance-specific toxicity predictions. Ecotoxicol. Environ. Saf. 64, 75–84.
Boocock, M.R., Coggins, J.R., 1983. Kinetics of 5-enolpyruvylshikimate-3-phosphate synthase inhibition by glyphosate. FEBS Lett. 154, 127–133.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.
Brausch, J.M., Smith, P.N., 2007. Toxicity of three polyethoxylated tallowamine surfactant formulations to laboratory and field collected fairy shrimp, *Thamnocephalus platyurus*. Arch. Environ. Contam. Toxicol. 52, 217–221.
Cavusoglu, K., Yapar, K., Oruc, E., Yalcin, E., 2011. Protective effect of *Ginkgo biloba* L. leaf extract against glyphosate toxicity in Swiss albino mice. J. Med. Food 14, 1263–1272.
Chamel, A., Gambonnet, B., 1997. Sorption and diffusion of an ethoxylated stearic alcohol and an ethoxylated stearic amine into and through isolated plant cuticles. Chemosphere 34, 1777–1786.
Clair, E., Mesnage, R., Travert, C., Seralini, G.E., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. In Vitro 26, 269–279.
Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384.
Corbera, M., Simonet, B.M., Salvado, V., Hidalgo, M., 2010. Characterisation of alkylamine ethoxylates (ANEOs) in commercial herbicide formulations using liquid chromatography/electrospray ionisation mass spectrometry. Rapid Commun. Mass Spectrom. 24, 2931–2937.
Courtney, H.S., Simpson, W.A., Beachey, E.H., 1986. Relationship of critical micelle concentrations of bacterial lipoteichoic acids to biological activities. Infect. Immun. 51, 414–418.
Cox, C., 2004. Herbicide factsheet—glyphosate. J. Pesticide Reform 24, 10–15.
Crouch, S.P., Kozlowski, R., Slater, K.J., Fletcher, J., 1993. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J. Immunol. Methods 160, 81–88.
Cserháti, T., Forgács, E., 1997. Separation and quantitative determination of non-ionic surfactants used as pesticide additives. J. Chromatogr. A 774, 265–279.
Dallegrave, E., Mantese, F.D., Oliveira, R.T., Andrade, A.J., Dalsenter, P.R., Langeloh, A., 2007. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch. Toxicol. 81, 665–673.
Daruich, J., Zirulnik, F., Gimenez, M.S., 2001. Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses. Environ. Res. 85, 226–231.
Eddleston, M., Street, J.M., Self, I., Thompson, A., King, T., Williams, N., Naredo, G., Dissanayake, K., Yu, L.M., Worek, F., John, H., Smith, S., Thiermann, H., Harris, J.B., Eddie Clutton, R., 2012. A role for solvents in the toxicity of agricultural organophosphorus pesticides. Toxicology 294, 94–103.
Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.C., Seralini, G.E., 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 262, 184–191.
Gasnier, C., Laurant, C., Decroix-Laporte, C., Mesnage, R., Clair, E., Travert, C., Seralini, G.E., 2011. Defined plant extracts can protect human cells against combined xenobiotic effects. J. Occup. Med. Toxicol. 6, 3.
German Federal Agency CPFS, 1998. Monograph on Glyphosate. Released by the German Federal Agency for Consumer Protection and Food Safety. Annex B-5: Toxicology and Metabolism, p. 136.
Hartung, T., 2009. Toxicology for the twenty-first century. Nature 460, 208–212.
Hawthorne, D.J., Dively, G.P., 2011. Killing them with kindness? In-hive medications may inhibit xenobiotic efflux transporters and endanger honey bees. PLoS ONE 6, e26796.
IFEN, 2006. Report on Pesticides in Waters. Data 2003–2004.
James, C., 2011. Global Status of Commercialized Biotech/GM Crops: 2009. ISAAA Brief 43.
Krogh, K.A., Halling-Sorensen, B., Mogensen, B.B., Vejrup, K.V., 2003. Environmental properties and effects of nonionic surfactant adjuvants in pesticides: a review. Chemosphere 50, 871–901.
L'Azou, B., Fernandez, P., Bareille, R., Beneteau, M., Bourget, C., Cambar, J., Bordenave, L., 2005. In vitro endothelial cell susceptibility to xenobiotics: comparison of three cell types. Cell Biol. Toxicol. 21, 127–137.
Letcher, R.J., van Holsteijn, I., Drenth, H.J., Norstrom, R.J., Bergman, A., Safe, S., Pieters, R., van den Berg, M., 1999. Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. Toxicol. Appl. Pharmacol. 160, 10–20.
Liu, J.J., Wang, W., Dicker, D.T., El-Deiry, W.S., 2005. Bioluminescent imaging of TRAIL-induced apoptosis through detection of caspase activation following cleavage of DEVD-aminoluciferin. Cancer Biol. Ther. 4, 885–892.
Marc, J., Le Breton, M., Cormier, P., Morales, J., Belle, R., Mulner-Lorillon, O., 2005. A glyphosate-based pesticide impinges on transcription. Toxicol. Appl. Pharmacol. 203, 1–8.
Mesnage, R., Clair, E., Séralini, G.E., 2010. Roundup in genetically modified plants: regulation and toxicity in mammals. Theor. Ökol. 16, 31–33.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Nobels, I., Spanoghe, P., Haesaert, G., Robbens, J., Blust, R., 2011. Toxicity ranking and toxic mode of action evaluation of commonly used agricultural adjuvants on the basis of bacterial gene expression profiles. PLoS ONE 6, e24139.
Oliveira, A.G., Telles, L.F., Hess, R.A., Mahecha, G.A., Oliveira, C.A., 2007. Effects of the herbicide Roundup on the epididymal region of drakes *Anas platyrhynchos*. Reprod. Toxicol. 23, 182–191.
Paganelli, A., Gnazzo, V., Acosta, H., Lopez, S.L., Carrasco, A.E., 2010. Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling. Chem. Res. Toxicol. 23, 1586–1595.
Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61, 1115–1122.
Peluso, M., Munnia, A., Bolognesi, C., Parodi, S., 1998. 32P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. Environ. Mol. Mutagen. 31, 55–59.
Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G.E., 2005. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716–720.
Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., de Souza, P.B., Viau, P., Bernardi, M.M., Nunes, M.T., de Oliveira, C.A., 2011. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Arch. Toxicol. 86, 663–673.
Samsonoff, C., Daily, J., Almog, R., Berns, D.S., 1986. The use of coomassie brilliant blue for critical micelle concentration determination of detergents. J. Colloid Interface Sci. 109, 325–329.
Savitz, D.A., Arbuckle, T., Kaczor, D., Curtis, K.M., 1997. Male pesticide exposure and pregnancy outcome. Am. J. Epidemiol. 146, 1025–1036.
Tsui, M.T., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52, 1189–1197.
Urani, C., Doldi, M., Crippa, S., Camatini, M., 1998. Human-derived cell lines to study xenobiotic metabolism. Chemosphere 37, 2785–2795.
Williams, A.L., Watson, R.E., Desesso, J.M., 2012. Developmental and reproductive outcomes in humans and animals after glyphosate exposure: a critical analysis. J. Toxicol. Environ. Health B: Crit. Rev. 15, 39–96.
Yousef, M.I., Salem, M.H., Ibrahim, H.Z., Helmi, S., Seehy, M.A., Bertheussen, K., 1995. Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. J. Environ. Sci. Health B 30, 513–534.

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