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Formulants of glyphosate-based herbicides have more deleterious impact than glyphosate on TM4 Sertoli cells

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ABSTRACT

Roundup and Glyphogan are glyphosate-based herbicides containing the same concentration of glyphosate and confidential formulants. Formulants are declared as inert diluents but some are more toxic than glyphosate, such as the family of polyethoxylated alkylamines (POEA). We tested glyphosate alone, glyphosate-based herbicide formulations and POEA on the immature mouse Sertoli cell line (TM4), at concentrations ranging from environmental to agricultural-use levels. Our results show that formulations of glyphosate-based herbicides induce TM4 mitochondrial dysfunction (like glyphosate, but to a lesser extent), disruption of cell detoxification systems, lipid droplet accumulation and mortality at sub-agricultural doses. Formulants, especially those present in Glyphogan, are more deleterious than glyphosate and thus should be considered as active principles of these pesticides. Lipid droplet accumulation after acute exposure to POEA suggests the rapid penetration and accumulation of formulants, leading to mortality after 24 h. As Sertoli cells are essential for testicular development and normal onset of spermatogenesis, disturbance of their function by glyphosate-based herbicides could contribute to disruption of reproductive function demonstrated in mammals exposed to these pesticides at a prepubertal stage of development.

1. Introduction

Roundup® Bioforce (R) or Glyphogan (Gan) commercial formulations are non-selective herbicides containing 360 g/L of glyphosate (G) and formulants such as polyethoxylated detersive petroleum compounds like polyethoxylated alkylamines (POEA) (Benachour et al., 2007; Mesnage et al., 2013). At certain concentrations, G inhibits the shikimic acid pathway involved in aromatic amino acid biosynthesis, and consequently induces plant death. Formulants may exert herbicidal activity in their own right, and assist with G solubilization, penetration in plants and stability (Cox, 1998, 2004; Seralini, 2015). G and its metabolite aminomethylphosphonic acid (AMPA), as well as formulants, are major contaminants of surface waters (IFEN, 2007; ANSES, 2013) and are found in air, feed and food (Takahashi et al., 2001; Acquavella et al., 2004; Cox and Surgan, 2006; Székács and Darvas, 2012). G has also been detected in the tissues, blood and urine of either humans or animals exposed directly or indirectly *via* food, water or air to herbicides (Acquavella et al., 2004; Curwin et al., 2007; Aris and Leblanc, 2011; Mesnage et al., 2012; Niemann et al., 2015).

G and/or R can induce apoptosis or necrosis in mammalian cells (Richard et al., 2005; Benachour and Seralini, 2009; Clair et al., 2012; Liz Oliveira Cavalli et al., 2013; Mesnage et al., 2013; Cattani et al., 2014). G and R are responsible for oxidative damage, enzymatic disorders and lipid peroxidation (Gehin et al., 2005; El-Shenawy, 2009; Gasnier et al., 2010; Liz Oliveira Cavalli et al., 2013). Studies have shown that formulations of glyphosate-based herbicides are more cytotoxic than G alone and suggest that formulants aggravate cell damage (Liz Oliveira Cavalli et al., 2013). Formulants of glyphosate-based herbicides are declared as inert diluents but some are more toxic than G, such as the family of POEA compounds (Adam et al., 1997; Tsui and Chu, 2003; Marc et al., 2005; Mesnage et al., 2013; Defarge et al., 2016). These formulants are ethoxylated adjuvants, which can insert

Abbreviations: AMPA, aminomethylphosphonic acid; cDH, complemented DMEM/HamF12; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, Dimethyl sulfoxide; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; G, glyphosate; Gan, glyphogan; GSH, reduced glutathione; GST, Glutathione-S-transferase; LC50, lethal concentration 50 (50 % of mortality); MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, Phosphate Buffer saline; POEA, polyethoxylated alkylamine; POE-15, POE (15) tallowamine; R, Roundup® Bioforce; SD, succinate dehydrogenase

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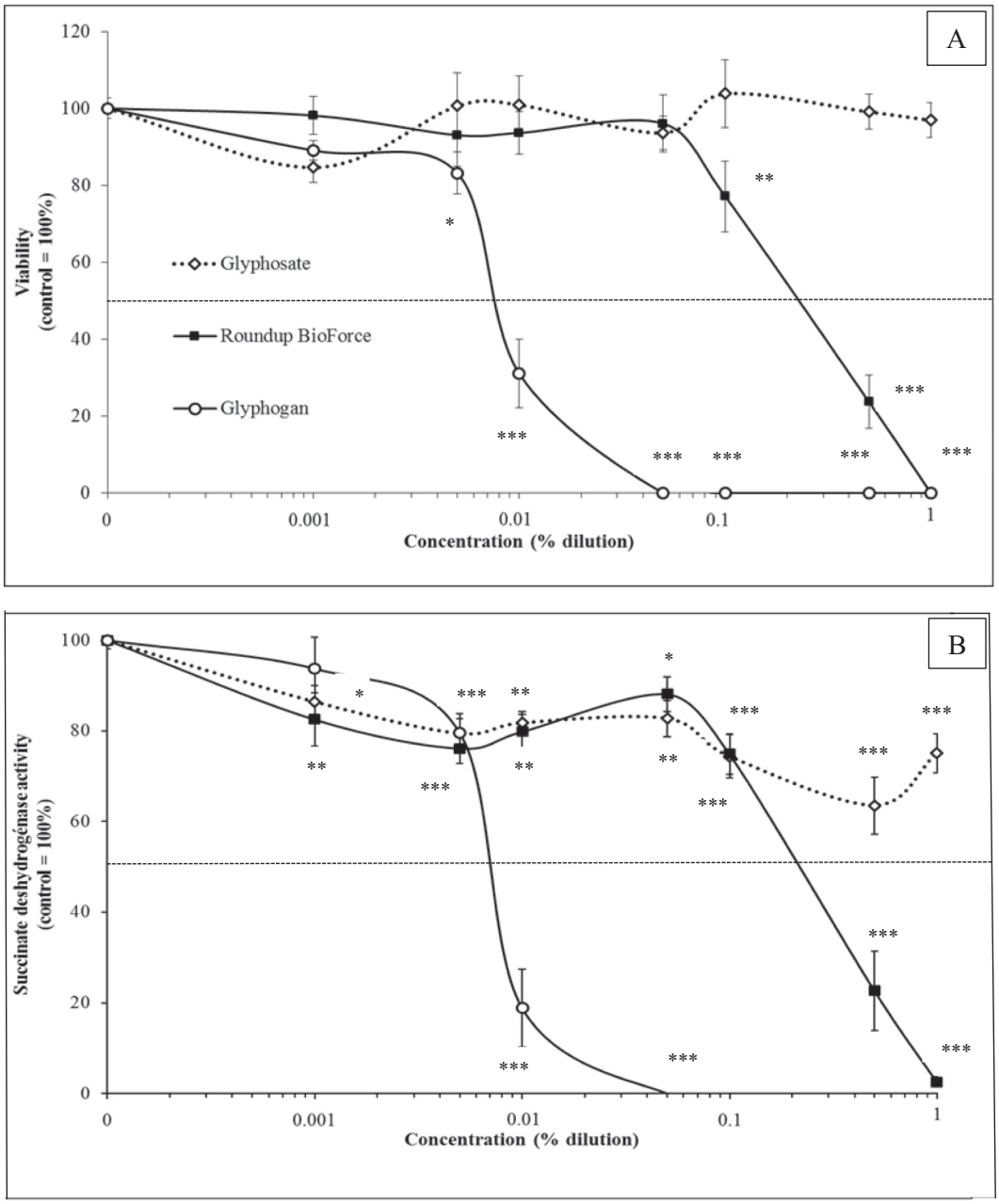

Fig. 1. Effects of Roundup, Glyphogan or Glyphosate on TM4 viability after 24 h of treatment. Cells were grown at 37 °C (% CO_2, 95% air) in complemented DMEM/Ham F12 medium (cDH) for 24 h until 80% confluent. Then, cells were exposed to different dilutions of glyphosate formulations (Roundup Bioforce® or Glyphogan) or equivalent doses of Glyphosate in cDH for 24 h. Cytotoxicity of Roundup Bioforce®, Glyphogan or Glyphosate alone were evaluated using the Crystal violet (A) and MTT (B) assays. A value of 0% of succinate dehydrogenase activity reveals total cell death (B). SEM are shown in all instances (Anova test $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$). The LC50 of Roundup Bioforce and Glyphogan are indicated by the empty square above the curves.

into cell membranes, disrupting their structure and function (Nobels et al., 2011) and have the ability to penetrate into cells (Mesnage et al., 2013).

In mammals, and rats in particular, the respiratory, hepatic, renal, cardiovascular and brain systems can be altered by R (Adam et al., 1997; Daruich et al., 2001; Beuret et al., 2005; Seralini et al., 2014; Gress et al., 2015; Larsen et al., 2016; Mesnage et al., 2017). Sperm production, sperm quality and libido (Yousef et al., 1995; Dallegrave et al., 2007; Romano et al., 2012; Abarikwu et al., 2014; Cassault-Meyer et al., 2014; Lopes et al., 2014), pregnancy (Savitz et al., 1997; Daruich et al., 2001; Beuret et al., 2005), and fetal development (Chan and Mahler, 1992; Yousef et al., 1995; Dallegrave et al., 2003) including reproductive development (Romano et al., 2012), are affected by this herbicide. Alterations of the structure of the testis and/or epididymis have also been demonstrated (Oliveira et al. 2007, Romano et al., 2010). Studies have shown that R affects reproduction in animals by endocrine disruption (Oliviera et al., 2007; Romano et al., 2010; Abarikwu et al., 2014), which is known to have an impact on survival and physiological function of testicular cells (Carreau and Hess, 2010).

Prepubertal exposure of male rats to R alters testicular morphology (reduction of seminiferous epithelium height) and serum testosterone concentration (Romano et al., 2010). Acute R administration at low doses induces oxidative stress and activates multiple stress-response pathways, leading to cell death in prepubertal rat testis including Sertoli cells (de Liz Oliveira Cavalli et al., 2013). Mature rat Sertoli cells, as Leydig and germ cells, are also sensitive to R. Their insensitivities to G

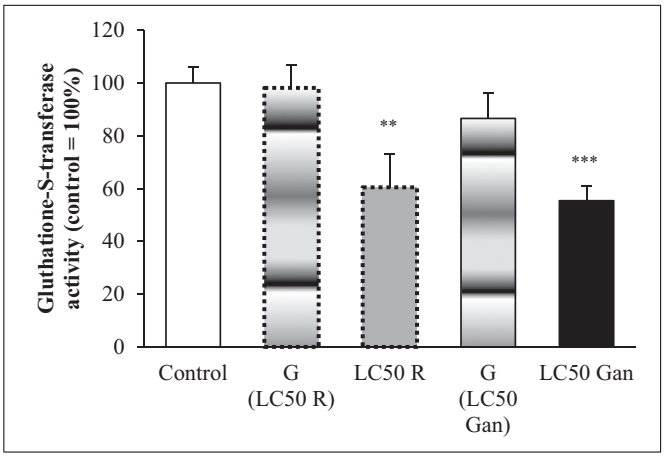

Fig. 2. Effects of Glyphosate (G) alone or in herbicide formulations (Bioforce (R) or Glyphogan (Gan)) on TM4 cell Glutathione-S-transferase activity after 24 h exposure. Cells are treated at LC50 concentrations of formulations (0.22% R, 0.0075% Gan) or equivalent doses of G. GST activity was evaluated in S9 cell fractions. SEM are shown in all instances (Anova test $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$). For more details see the caption of Fig. 1.

alone imply toxicity arising from formulants on mature testicular cells (Clair et al., 2012). These observations suggest that G alone or in R formulations may induce differential effects in prepubertal Sertoli cells essential for the onset of spermatogenesis. Consequently, the objective of the present study was to evaluate the effect on TM4 cells (a cell line derived from immature testicular Sertoli cells) of sub-agricultural dilutions of G alone, commercial G herbicide formulations (R and Gan containing the same G concentration) and sub-agricultural dilutions of POEA (one of the major formulants present in Gan, alone or in formulations). Measures assessed cell viability and effects on the detoxifying system.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM)/HamF12 (1:1; v:v) were purchased from Dutscher and Glyphosate (N-phosphonométhylglycine, G, CAS: 1071-83-6) from Sigma-Aldrich. Commercially available glyphosate formulations and formulants studied were, in alphabetical order: Genamin T200 (732 g/L polyethoxylated tallowamine, 60–80% POE (15) tallowamine (POE-15), homologation 8,500,170), Glyphogan (360 g/L of Glyphosate, homologation 9,100,537, corresponding to 100%, 13–18% of POE-15) and Roundup Bioforce® (360 g/L of Glyphosate, homologation 9800036, corresponding to 100%). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other compounds, as stated, were obtained from Sigma-Aldrich.

2.2. Culture of TM4 Sertoli cells and treatments

The murine TM4 Sertoli cell line was obtained from the American Type Culture Collection (ATCC Manassas, USA). Cells were maintained in DMEM/HamF12 medium containing 0.2% glutamine, 1.2 g/L $NaHCO_3$, 15 mM Hepes, 5% horse serum and 2.5% fetal calf serum, 100 U/mL of antibiotics and fungizone (complemented DMEM/Ham F12 medium) at 37 °C (5% CO_2, 95% air) during a period of 24 h to 80% confluence in 24-well plates or in 6-well plates (for measurement of GST activity). Cells were then exposed to various concentrations of Glyphosate alone (G) or in the commercial formulations Roundup Bioforce® (R) or Glyphogan (Gan). Cultures were also treated with various concentrations of POE-15 (Genamin), for 2 min or 24 h. The dilutions of G, R, Gan or Genamin were prepared in complemented DMEM/Ham F12 medium and adjusted to pH 7. 2.

2.3. Crystal violet cell viability assay

After incubation under the various treatment regimens, culture supernatants were discarded and cells incubated in medium containing crystal violet solution (0.1% w/v in PBS 0.01 M, pH 7.4) for 30 min at 20 °C with gentle rocking. The crystal violet entered all cells during this incubation. Excess dye and non-adherent dead cells were removed through 3 washing steps with PBS. Diluted acetic acid solution (10%) was then added to release the crystal violet taken up by cells, and the optical density reflecting living adherent cells (Feoktistova et al., 2016) was determined by absorption at 600 nm (Saotome et al., 1989) through a plate reader (Metertech, Σ960 ELISA READER).

2.4. Succinate dehydrogenase activity (MTT) assay as a viability/cytotoxicity biomarker

This enzymatic test is based on the cleavage of MTT (tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a blue-coloured product (formazan) by the mitochondrial enzyme succinate dehydrogenase (Mossman, 1983). Activity of mitochondrial dehydrogenase enzymes indirectly measures activity of the mitochondrial respiration and antioxidant defense systems and thus overall health status of cells. Culture medium was removed, and cells were washed once with PBS and then incubated with 500 µL MTT solution per well after each treatment. The plates were incubated for 3 h at 37 °C. The reaction was stopped by placing the plates for 10 min at 4 °C and then 500 µL of 0.04 N hydrochloric acid-containing isopropanol solution was added to each well. The plates were then vigorously shaken for 40 min in order to solubilize the blue formazan crystals formed. The optical density was measured using a spectrophotometer (KONTRON Instruments, UVIKON 930) at 570 nm.

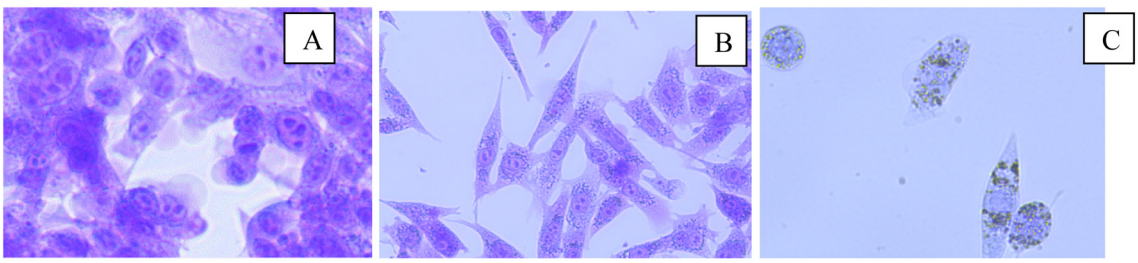

Fig. 3. Study of cytoplasmic lipid droplet accumulation within TM4 cells after 24 h of exposure to 0.5% Roundup Bioforce. At 80% confluence (see Fig. 1), cells were exposed to medium (control, A, 200 ×) or to 0.5% of Roundup Bioforce® (B, magnification 200 × and C, magnification 400 ×) for 24 h. After crystal violet staining, cytoplasmic lipid droplets (violet or brown) were assessed in viable cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

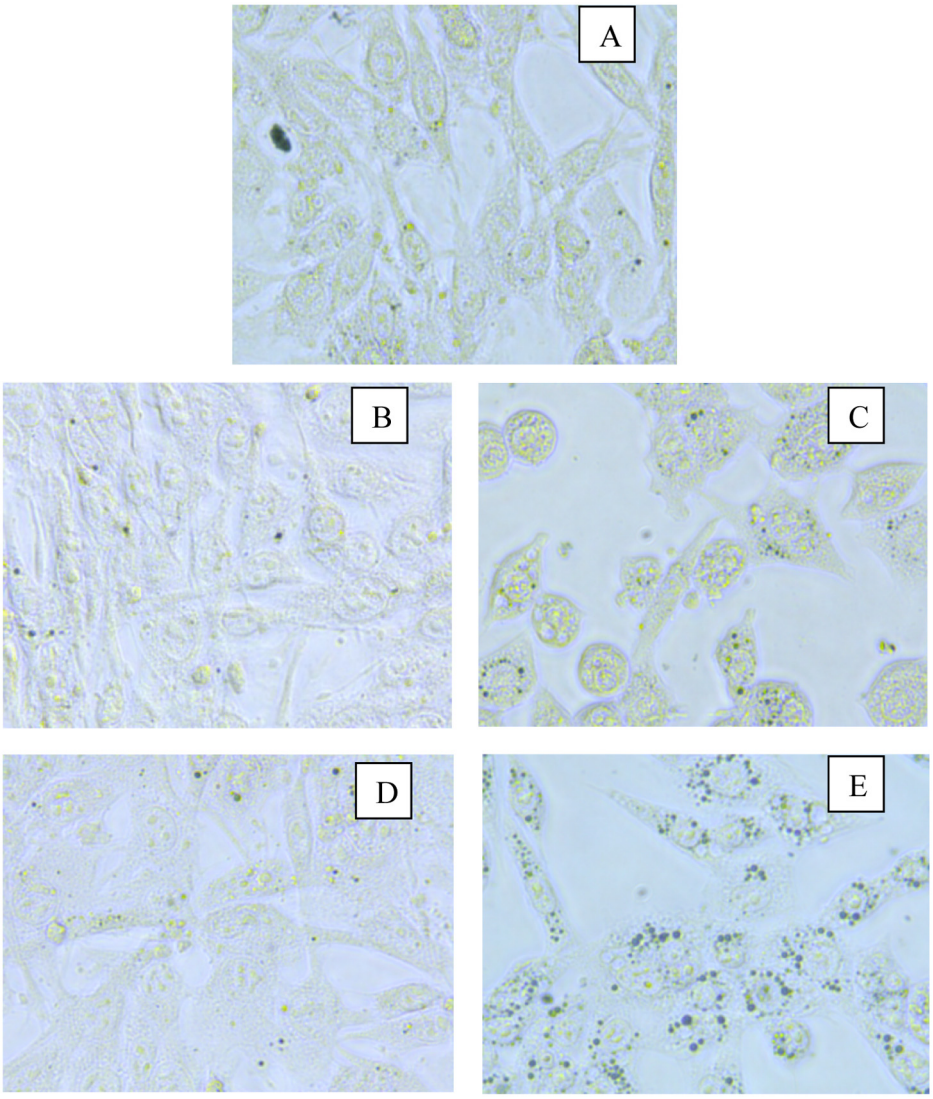

Fig. 4. TM4 cell lipid droplet assessment after 24 h treatment with Roundup Bioforce or Glyphosate alone. At 80% confluence, cells were exposed to various concentrations of Roundup Bioforce® or equivalent doses of Glyphosate for 24 h (see Fig. 1.). After Sudan Black B staining, lipid droplets appear black (200 ×) in control cells (A); cells treated with glyphosate 0.25% (B) or 0.5% (D), with Roundup Bioforce 0.25% (C) or 0.5% (E).

2.5. Measurement of glutathione-S-transferase (GST) activity, an anti-xenobiotic defense system

For preparation of S9 fractions enriched in GST, the medium was removed, and cells were detached by treatment with trypsin-EDTA and washed twice with PBS by centrifugations (600*g*, 5 min) at room temperature. Cells were then resuspended in 500 µL of 50 mM phosphate buffer pH 7.2 containing 0.25 M sucrose and 1 mM DTT, then were homogenized and centrifuged at 9000*g*, 4 °C for 30 min to pellet cell debris. Supernatants corresponding to the S9 fraction (membrane and cytosolic enzymes) were collected and stored at −80 °C until further evaluation for enzyme activities. Protein concentration of each S9 fraction was determined using the Bradford assay.

The protocol of GST activity measurement was adapted from Habig et al. (1979). Briefly 250 µg of S9 cell fraction was mixed with 10 µL of 100 mM reduced L-glutathione (GSH, dissolved in deionized water) and 990 µL phosphate buffer in duplicate. Phosphate buffer pH 6.5 was prepared by mixing 0.7 volume of 0.1 M KH_2PO_4 and 0.3 volume of 0.1 M Na_2HPO_4. The reaction was initiated by the addition of 10 µL of 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) substrate stock solution dissolved in 95% ethanol. After incubation for 90 s at 37 °C, the optical density was measured at 340 nm every 60 s for 3 min using the spectrophotometer.

2.6. Lipid Sudan Black B staining for lipid droplet detection

Sudan Black B stains lipids, including phospholipids and sterols. The protocol was adapted from Sheehan and Storey (1947). Culture medium was removed and cells were washed once with PBS. Cells were then incubated for 5 min at room temperature with 500 µL of Sudan Black solution (0.18% w/v in 70% ethanol) with gentle shaking. This solution was removed, and cells were washed 3 times or more with 70% ethanol to remove excess stain and then at least 3 times with PBS. Following this staining procedure, lipid droplets appear black within cells. Then, intracellular Sudan Black B was extracted by incubation with DMSO for 30 min with gentle rocking and measured quantitatively at 600 nm using a microplate reader.

2.7. Statistical analysis

The experiments were repeated at least in triplicate in different weeks on three independent cultures on each occasion ($n = 9$). All data are presented as the mean ± standard error (SEM). Statistical differences from controls were determined by an Anova test with $p < 0.001$

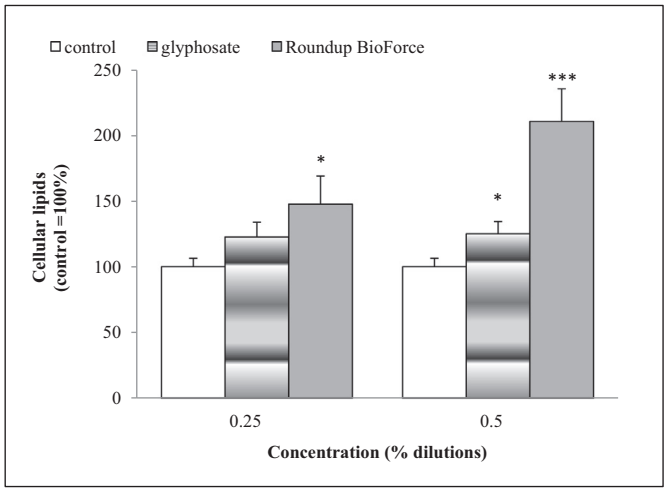

Fig. 5. Effects of Glyphosate alone or in Roundup Bioforce formulation on TM4 cell lipid droplet accumulation after 24 h exposure. Cell cultures, treatments and lipid staining were performed as described in Figs. 1 and 5. After lipid Sudan Black B extraction, quantity of stain was evaluated by spectrophotometric analysis at 600 nm. SEM are shown in all instances (Anova test $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$).

(***), $p < 0.01$ (**) and $p < 0.05$ (*).

3. Results

3.1. Viability of TM4 cells following 24 h exposure to glyphosate alone or to glyphosate-based herbicides

TM4 viability was evaluated to compare the cytotoxic potential of G alone and with formulants (R or Gan), at concentrations ranging from 10 ppm to a 1% agricultural dilution (1000 times more). G alone has no impact on cell viability during the 24 h of treatment. In contrast, during this same period, G-based formulations induced dose-dependent cell death. Significant mortality is provoked by R from 0.1% (1000 ppm), which is 10 times below the agricultural-use dilution. TM4 cells are more sensitive to Gan (from 0.005% (50 ppm)) than to R. Moreover, Gan lethal dose is 20 times lower than that of R (Fig. 1A). Therefore herbicide-induced mortality is not dependent on G. Moreover, Gan is more deleterious to TM4 cells than R.

3.2. Decreased mitochondrial succinate dehydrogenase activity induced by glyphosate alone and glyphosate-based herbicides

We compared Succinate dehydrogenase (SD) activity as a measure of mitochondrial function and viability after 24 h exposure to G alone and to equivalent doses of G with formulants (R and Gan). All chemicals tested reduced SD activity, from 0.001% (3.6 mg G/L alone and in R) or 0.005% (Gan) (Fig. 1B). Formulations are more cytotoxic than G alone.

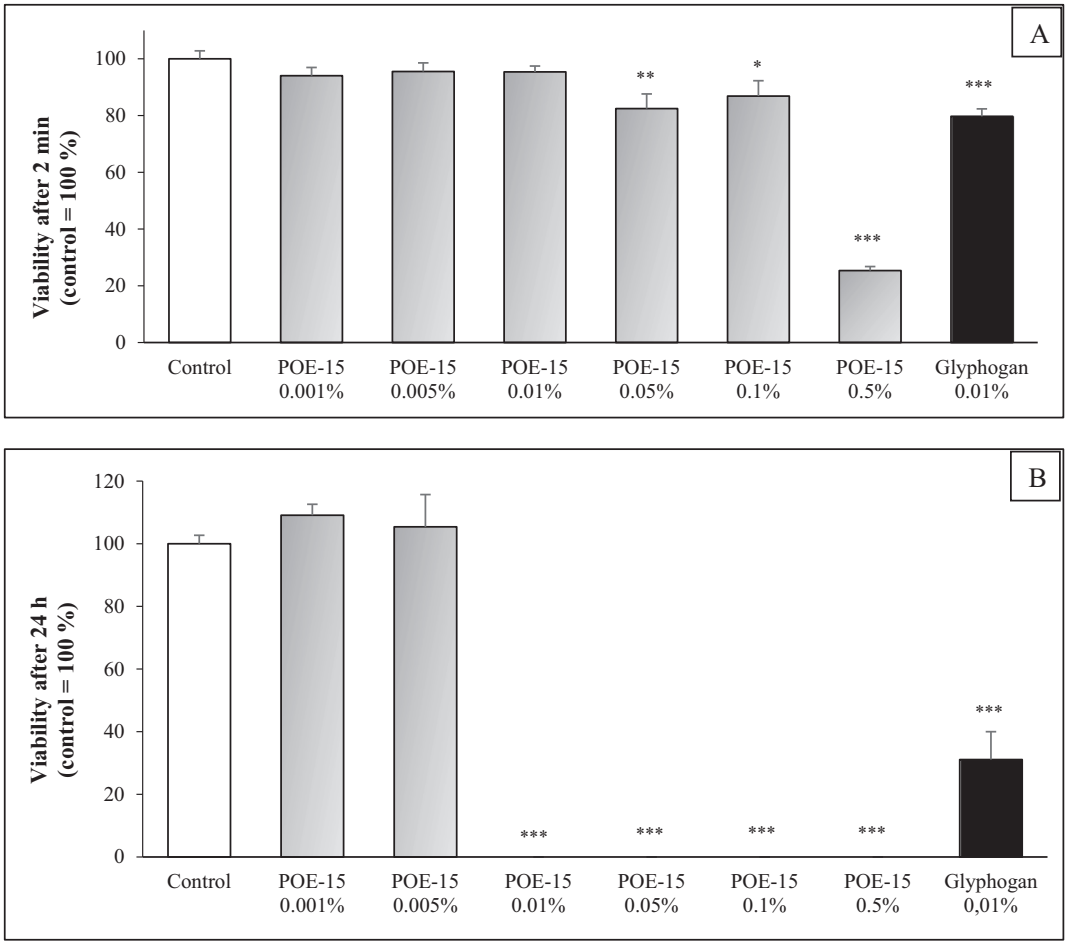

Fig. 6. Impact of similar doses of POE-15 contained in Genamin (POEA) or in Glyphogan on TM4 cell viability after either 2 min (A) or 24 h (B) of treatment. At 80% confluence, cells were exposed to different doses of POE-15 (Genamin) and compared to equivalent doses in dilutions of Glyphogan (%). Cytotoxicity was evaluated by Crystal violet staining. SEM are shown in all instances (Anova test $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$). For more details, see Fig. 1.

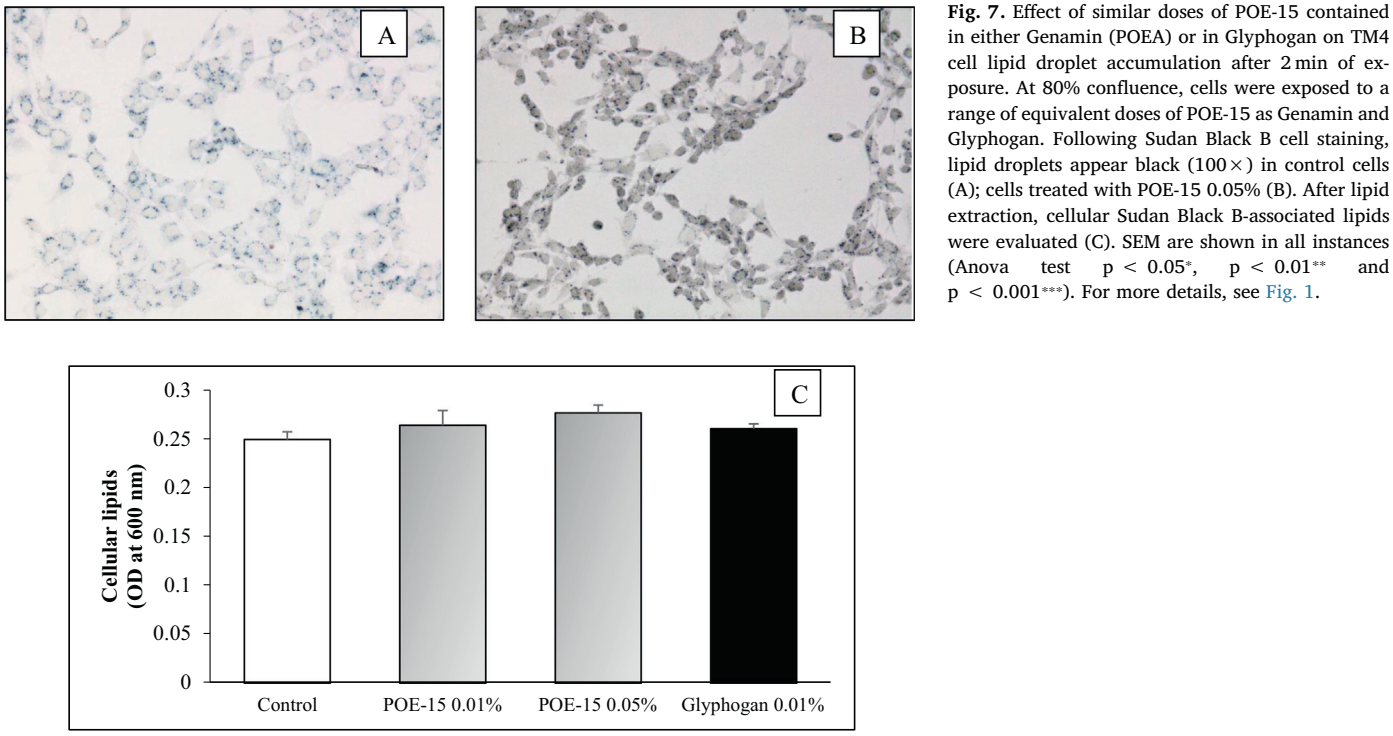

Fig. 7. Effect of similar doses of POE-15 contained in either Genamin (POEA) or in Glyphogan on TM4 cell lipid droplet accumulation after 2 min of exposure. At 80% confluence, cells were exposed to a range of equivalent doses of POE-15 as Genamin and Glyphogan. Following Sudan Black B cell staining, lipid droplets appear black (100×) in control cells (A); cells treated with POE-15 0.05% (B). After lipid extraction, cellular Sudan Black B-associated lipids were evaluated (C). SEM are shown in all instances (Anova test $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$). For more details, see Fig. 1.

Gan showed higher toxicity and killed all cells at 0.05% (20 times less than lowest agricultural use dilution) (Fig. 1B). Therefore, herbicide-induced mitochondrial function alterations are not proportional to G concentration but are formulation-dependent.

3.3. Inhibition of glutathione-S-tranferase (GST) activity by formulations but not by glyphosate

We examined the impact of G alone or in herbicide formulations on GST activity involved in the anti-xenobiotic defense system. At an LC50 concentration (see Fig. 1, 0.22% R and 0.0075% Gan), glyphosate-based formulations inhibited GST activity (40%). In contrast, equivalent doses of G alone had no impact (Fig. 2). These results imply that under these conditions, disruption of GST detoxifying activity is induced by formulants but not by G. These results show the participation of oxidative events in the mechanism of R/Gan toxicity on TM4 cells.

3.4. Lipid droplet accumulation within TM4 cells after 24 h exposure to glyphosate and roundup

After 24 h of treatment and staining with crystal violet, microscopic observation revealed more cytoplasmic lipid droplets in TM4 cells treated by 0.5% R (Fig. 3B and C) than in control cultures (Fig. 3A).

Cytoplasmic localization, colour and form of the droplets suggest a lipid nature (Fig. 3C). Thus, we stained TM4 cells with Sudan Black B, which is known to stain lipid droplets. After staining, we observed that exposure to either G alone or to 0.5% R induces an increase in cytoplasmic lipid droplets. This accumulation was higher after R treatment. Our results also indicate that 0.25% R enhances the quantity of cytoplasmic lipid droplets (Fig. 4C).

After Sudan Black stain extraction, lipid droplets were quantified, with results confirming that R as well as G (albeit to a lesser extent) induced accumulation of intracellular lipids (Fig. 5).

3.5. TM4 cell viability after 2 min or 24 h exposure to Glyphogan formulants (POEA) or Glyphogan

In this phase of our investigation, we evaluated TM4 cell viability (by crystal violet assay) following acute or 24 h exposure to similar doses of POE-15 (Genamin) or when present in Gan, the most toxic G formulation (Fig. 1). This herbicide contains formulants, such as POEA and especially POE-15 (Mesnage et al., 2013). Ethoxylated formulants of the POEA class of compounds have been demonstrated to be toxic to human cell lines (Mesnage et al., 2013; Defarge et al., 2016).

Acute treatment is not toxic until the POE-15 concentration reaches 0.01%, corresponding to 14.64 mg POEA/L. In contrast, Glyphogan at 0.01%, containing the same quantity of POE-15 (0.01%), reduced cell viability after just 2 min of exposure (Fig. 6A). Thus mortality appears not to be induced by the family of POEA molecules but by other formulants contained in Glyphogan. Acute POEA treatment decreases cell viability from 0.05% POE-15, corresponding to 73.2 mg POEA/L (Fig. 6A). At 24 h of POEA treatment, toxicity is not observed until a POE-15 concentration of 0.005% corresponding to 7.32 mg POEA/L. Total cell death is observed from 0.01% POE-15. In contrast, 0.01% Glyphogan was less able to reduce TM4 cell viability (Fig. 6B). This suggests that TM4 cells are not sensitive to the POE-15 at the dose contained in 0.01% Gan, but to other formulants. Our results indicate a very rapid induction of cell mortality by this mixture of compounds (Fig. 6A). A comparison of effects of 24 h- and acute-POEA treatments shows cytotoxic effect, increasing over time.

3.6. Accumulation of lipid and/or formulants within TM4 cells after 2 min exposure to POEA or Glyphogan

POE-15, like other ethoxylated formulants, can insert into cell membranes, disrupting their structure (Nobels et al., 2011) and have the ability to enter cells (Mesnage et al., 2013). Thus we tested the capacity of sub-agricultural doses of POE-15 (lipophilic formulants) to penetrate into cells and accumulate in the cytoplasm after acute (2 min) exposure and staining with Sudan Black B. After exposure to 0.05% POE-15 (Fig. 7B), more lipid droplets were observed in the cytoplasm of TM4 cells than in the control culture (Fig. 7A). In addition, the colour of cells was more yellow, as is POEA, suggesting the presence of this compound. Quantification of the Sudan Black B stain suggested the rapid penetration and accumulation of POEA (Fig. 7C).

4. Discussion

In this study we report for the first time differential effects of G alone or in two herbicide formulations (containing similar G concentration and different detersive petroleum formulants) or of formulants alone (POEA) on immature murine TM4 Sertoli cell line (TM4). Measurements of cell viability, respiratory chain activity, detoxification system and lipid accumulation were undertaken following 24 h of exposure to these chemicals at concentrations ranging from environmental levels to agricultural use doses (1%, 10^4 ppm). Immature rat Sertoli cells have already proven to be a sensitive model to highlight effects arising from R exposure, with possible implications to the male reproductive system arising from exposure to these xenobiotics (de Liz Oliveira Cavalli et al., 2013).

We demonstrate in this study that, at sub-agricultural use levels (10 to 10^4 ppm), TM4 cell viability is affected by glyphosate-based formulations but not by G alone. Furthermore, we found that Gan formulation was more deleterious to TM4 cells than R. Total cell death is observed at 0.05% Gan (500 ppm) *vs* 1% R (10^4 ppm). Formulants of commercial glyphosate-based herbicide formulations can thus be considered as the active principle for toxicity. Differences in the nature and concentration of R and Gan formulants at which they exert their effects may explain the differences observed in cell sensitivity to herbicide formulations (Mesnage et al., 2013, 2014; Defarge et al., 2016). We have reported the death of mature rat Sertoli, Leydig and germ cells induced by R but not by G (Clair et al., 2012). We also demonstrated G and R toxicity in several human cell lines, which are more sensitive to R than to the same doses of G alone (Richard et al., 2005; Gasnier et al., 2009, 2010, 2011; Benachour and Seralini, 2009; Mesnage et al., 2013; Defarge et al., 2016). Other *in vitro* studies have revealed R toxicity (Gehin et al., 2005; Liz Oliveira Cavalli et al., 2013). Moreover, we demonstrate that Gan is more toxic than R, which is similar to what is observed in some human cell lines (Mesnage et al., 2013, Defarge et al., 2016).

Exposure of TM4 cells to G alone, R or Gan reduces mitochondrial succinate dehydrogenase (SD) activity, with a greater cytotoxic impact seen with formulations as described for human cell lines (Benachour and Seralini, 2009; Mesnage et al., 2013; Defarge et al., 2016). However, interestingly, G alone reduces mitochondrial activity at the lowest doses tested in TM4 cells, as compared with other cell types. Olorunsogo (1990) demonstrated that G increases mitochondrial permeability to protons and Ca^{2+}, probably after cell penetration by this compound, as demonstrated in animal cells (Gasnier et al., 2011).

Moreover, our results show that inhibition of mitochondrial SD activity corresponds with cell viability inhibition from 0.005% Gan and 0.1% R. Thus in contrast to G alone, the disruption of SD activity, which is linked with respiratory chain and antioxidant defense systems (*via* ubiquinone) as well as Krebs cycle function (Rustin et al., 2002; Huang and Millar, 2013), leads to TM4 cell death at concentrations of these formulations well below what is used in an agricultural setting. Previous studies have reported that the lowering or removal of SD function in either plant or animal cells induces differential changes in organic acid levels, altered respiration and changes in the generation of mitochondrial reactive oxygen species (ROS) (Huang and Millar, 2013). Accumulation of ROS leads to oxidative stress and damage, which can culminate in cell death (de Liz Oliveira Cavalli et al., 2013). Mitochondrial dysfunction induced after 24 h exposure to G was also observed in fish sperm with a decrease in mitochondrial function (Lopes et al., 2014). The formulants present in commercial herbicides are known to increase G penetration into cells by membrane disruption (Nobels et al., 2011; Mesnage et al., 2013), and thus probably potentiate perturbation of mitochondrial permeability induced by G. This mechanism may explain the higher toxicity of formulations on mitochondrial activity.

Our results are very consistent with those reported in a study where immature Sertoli cells were shown to be targets of R toxicity within rat testis (Liz Oliveira Cavalli et al., 2013). In fact, acute exposure to relatively low doses of either G alone (36 mg/L) or in R enhances Ca^{2+} uptake in freshly isolated Sertoli cells. These authors proposed that formulants present in R, such as POEA, potentiate Ca^{2+} influx, leading to Ca^{2+} overload, which sets off oxidative stress (due to mitochondrial dysfunction) and cell necrosis. At higher concentrations, more complex mechanisms leading to cell death seem to be elicited (Liz Oliveira Cavalli et al., 2013). Indeed, a previous study has shown that the mechanisms of formulation toxicity were modulated by the critical micellar concentrations of surfactants like POEA (Mesnage et al., 2013).

Several authors have shown that G alone or in formulations could alter antioxidant defense in different organisms, eventually leading to oxidative stress (de Liz Oliveira Cavalli et al., 2013). Previously, we proposed that G alone and in formulations could affect the mitochondrial ubiquinone antioxidant defense system. We also demonstrated disruption of GST detoxifying activity by formulations in contrast to G alone. Thus the disruption of xenobiotic conjugation is induced by formulants and probably implicated in the mechanism of R and Gan toxicity on TM4 cells. GST activity was also reduced by 24 h sub-agricultural use doses of R GT + administration in human cell lines (Gasnier et al., 2010) but increased by R Original in immature Sertoli cells at similar concentrations (De Liz Oliveira Cavalli et al., 2013). In these cells, R Original exposure also induced activities of several antioxidant enzymes. These differential effects probably depend on exposure type and on the nature of the mixture of formulants.

In this study, we also demonstrated that 24 h exposure to G or R induced lipid droplet accumulation in the TM4 cell cytoplasm. This result is consistent with the presence of larger and more numerous cytoplasmic lipid droplets reported in non-ciliated cells of proximal efferent ductules of drakes treated by R, compared to non-treated animals (Oliveira et al. 2007). Recently, other studies have shown lipid droplet accumulation in Sertoli cells following mouse transient scrotal hyperthermia (Liu et al., 2012), rat testis exposure to moderate hyperthermia (Vallés et al., 2014) or rat treatment by anti-schizophrenic drugs (Soliman et al., 2014). Formation of lipid droplets in Sertoli cells was associated with cell phagocytosis of residual bodies and apoptotic germ cells (Wang et al., 2006). In our study, after G or R exposure, degenerating TM4 cells could be phagocytosed by viable cells and give rise to an accumulation of lipid droplets. Acute or 24 h treatment of TM4 cells with POE-15 induces toxic effects already been demonstrated in human cell lines (Mesnage et al., 2013) and also lipid droplet accumulation. POE-15, contained in the most toxic glyphosate-based herbicide formulation (Gan) tested in our study, can insert into and disrupt the structure and function of cell membranes (Nobels et al., 2011) and thus also potentially enter cells (Mesnage et al., 2013), as is the case with other ethoxylated formulants. Knowing that lipid droplets allow cells to safely sequester toxic lipids (Vallés et al., 2014; Welte, 2015), we hypothesize that, after G or R acute and probably 24 h exposure, storage of potentially deleterious lipophilic formulants in the cytoplasm of TM4 cells could contribute to the observed increase of lipid droplet accumulation.

5. Conclusion

Our results show for the first time that formulants of glyphosate-based herbicides induce TM4 cell mitochondrial dysfunction (like glyphosate, but to a lesser extent), disruption of cell detoxification systems, lipid droplet accumulation and increased mortality at sub-agricultural-use doses. Formulants are more deleterious than glyphosate and so should also be considered as active principles of herbicides. Our results suggest that the accumulation of ethoxylated adjuvants, such as POE-15, in cell lipid droplets leads to cell mortality. As Sertoli cells are essential for testicular development and normal onset of spermatogenesis, disturbances of their function by glyphosate-based herbicides could contribute to the disruption of reproductive functions, as demonstrated in mammals exposed to these pesticides at prepubertal stages of

development. Obviously, effects induced by glyphosate and/or formulants should be lower *in vivo* than in culture. Indeed the bioavailability of these substances for Sertoli cells when the substances are used for agricultural purposes must necessarily be several orders of magnitude lower. Demonstration of cytotoxic effects of formulants in TM4 cells, as observed in other cell lines, demonstrates that toxicity of all compounds of glyphosate-based herbicides should be studied to conclude on the safety of glyphosate-based solutions, not of glyphosate alone.

Conflict of interest statement

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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