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A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells *in vitro*, and testosterone decrease at lower levels

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ABSTRACT

The major herbicide used worldwide, Roundup, is a glyphosate-based pesticide with adjuvants. Glyphosate, its active ingredient in plants and its main metabolite (AMPA) are among the first contaminants of surface waters. Roundup is being used increasingly in particular on genetically modified plants grown for food and feed that contain its residues. Here we tested glyphosate and its formulation on mature rat fresh testicular cells from 1 to 10000 ppm, thus from the range in some human urine and in environment to agricultural levels. We show that from 1 to 48 h of Roundup exposure Leydig cells are damaged. Within 24–48 h this formulation is also toxic on the other cells, mainly by necrosis, by contrast to glyphosate alone which is essentially toxic on Sertoli cells. Later, it also induces apoptosis at higher doses in germ cells and in Sertoli/germ cells co-cultures. At lower non toxic concentrations of Roundup and glyphosate (1 ppm), the main endocrine disruption is a testosterone decrease by 35%. The pesticide has thus an endocrine impact at very low environmental doses, but only a high contamination appears to provoke an acute rat testicular toxicity. This does not anticipate the chronic toxicity which is insufficiently tested, and only with glyphosate in regulatory tests.

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1. Introduction

An environmentally-linked syndrome called testicular dysgenesis has emerged (Bay et al., 2006; Skakkebaek et al., 2001). It includes a decrease in sperm quantity and quality (Auger et al., 1995; Carlsen et al., 1992), an increase in congenital malformations such as cryptorchidism and hypospadias (Toppari et al., 2010), and a preoccupying increase of testicular cancer incidence (Bergstrom et al., 1996). This indicates that the testis is a sensitive target for xenobiotics. The food/water/air intake of xenobiotics in the young as well as in adults may lead to endocrine disruption at a reproductive and more specifically testicular level (Anway et al., 2006; Savitz et al., 1997). *In vitro*, *ex vivo* and *in vivo* experiments are necessary approaches to help us understand the mechanisms of xenobiotics actions at a developmental and/or adult stage.

In this work, we have chosen to test one of the most used pesticides round the world. Roundup (R) formulations are non selective herbicides composed of mixtures of glyphosate (G) and adjuvants such as polyoxyethylene tallowamine (POEA) (Benachour et al., 2007b). These compounds, with the G metabolite aminomethylphosphonic acid (AMPA), are major contaminants in surface waters with levels reaching for instance 24 ppb for G in groundwater (IFEN, 2007). Moreover, these residues also concentrate in approximately 80% genetically modified plants grown for food and feed, which are rendered R tolerant, up to 400 ppm (maximal residual levels, U.S. EPA, 1998). We tested here R from 1 ppm to agricultural working dilutions on rat testicular cells.

It is known that G is a weed killer inhibiting the shikimic acid pathway in plants, essential for aromatic amino acids synthesis, and it penetrates and is stabilized in the cells with the help of the adjuvants (Cox, 1998, 2004). R used in our study contains 360 g/l of G and various xenobiotics added as adjuvants. Therefore R is a good model to study *in vitro* combined effects, and especially synergistic ones for xenobiotics (Benachour et al., 2007a; Richard et al., 2005). In fact, G and/or R can also induce mortality in human cells, revealed by disruptions of mitochondrial succinate dehydrogenase, caspases 3/7, and adenylate kinase (Benachour and Séralini, 2009). R is even responsible for oxidative damage in human epidermal cells (Gehin et al., 2005). G and/or R also have side targets in mammals such as cytochrome P450 reductase, StAR, aromatase and sexual steroid receptors of cells involved in reproduction or in transfected human cells (Gasnier et al., 2009; Richard et al., 2005; Stocco et al., 1995; Walsh et al., 2000).

In mammals, and rats in particular, the respiratory and hepatic systems (Adam et al., 1997; Beuret et al., 2005) can be altered by this herbicide, as well as hepatobiliary and reproductive functions including sperm production or libido, and even fetal development

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(Chan and Mahler, 1992; Dallegrave et al., 2003, 2007; Yousef et al., 1995). Therefore its impact in mammalian reproduction is documented, but not its direct possible testicular impact nor the mechanism of action or the sensitivity of adult gonadal cells.

R contamination may come from air (dermal or pulmonary during spraying), water, feed and food. At present few studies have been conducted to know the tissue concentration of G after exposure and its possible bioaccumulation. However it was reported that occupational exposure, primarily via the oral route, results in urinary concentrations of G in the order of ppm (Acquavella et al., 2004), in the range of our lower concentrations tested (1 ppm of R corresponding to 0.36 ppm of G). More recently, it was observed that after oral administration to rats of 10 ppm of G, 30% were absorbed in males and 36% in females, with a peak observed 2 h after administration. If the majority of G (90% after 72 h) appears to be excreted via the feces or urine before metabolism, this does not exclude the bioaccumulation in some tissues. One percent of G persists after 7 days, located in particular in the colon but also primarily in bone (Brewster et al., 1991). It is known that G can bind to calcium ions, this occurs also in the soil (Sprankle et al., 1975). More recently it has been shown that after oral ingestion of 10 ppm of the herbicide, it diffuses in mammalian tissues, with a half-life of 15 h in rats, and G is then found in plasma at 5 ppm (Anadon et al., 2009).

Typically these assays are performed after administration of a single or a few doses of G in a short time, and this does not quantify the bioaccumulation in the body of a long-term real environmental exposure by air, water, food or feed, like through consumption of Roundup-tolerant edible plants, such as most agricultural genetically modified organisms. In addition, only the active ingredient in plants is well studied thus there is little information available on its metabolites, and regarding R adjuvants and their toxicokinetics.

To date, very few studies have been conducted on R effects on primary cells; one study in particular was conducted by our group on human umbilical cord cells (Benachour and Séralini, 2009), where we demonstrated the necrotic and apoptotic capacities of R at environmental levels. Consequently, in this work, we measured the differential specificities of R and G actions on adult rat freshly separated testicular cells in order to know the threshold of toxicity. These are Leydig, Sertoli, Sertoli and germ cells, and germ cells alone. An increased mortality of these cells or a disruption in enzyme or hormone production could lead to a deleterious effect on reproduction. Necrosis and apoptosis were assayed at sub-agricultural dilutions of the herbicide R, and G, its compound without adjuvants, and the endocrine disruption was tested at non cytotoxic levels from 1 ppm. This work is the first study of the side effects of the main herbicide of the world on primary testicular mammalian cells.

2. Materials and methods

2.1. Animals

Healthy adult male albino Sprague–Dawley rats (70 days ± 5) were obtained from Janvier (Le Genest-Saint-Isle, France) or from the University Center of Biological Resources (Caen, France), and were maintained on a 12 h light/dark cycle at 20–22 °C. Standard food and water were provided to the animals *ad libitum*.

2.2. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) and Ham F12 were purchased from PAN (Biotech GmbH, Dutscher, Brumath, France); and collagenase/dispase from *Vibrio alginolyticus*/*Bacillus polymyxa* was from Roche (Mannheim, Germany). Soybean trypsin inhibitor (STI), deoxyribonuclease I from bovine pancreas (DNAse I), glyphosate (G) and serum replacement 3 were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). The 4′,6′-Di Amidino-2-PhenylIndole (DAPI) was from Lonza (Verviers, Belgium). Percoll was from GE Healthcare (Saclay, France). All other reagents were of analytical grade. The herbicide R Bioforce® containing 360 g/l of acid G (R, homologation 9800036 corresponding to 100%) is a commercial formulation. Solutions of G (2% or 7.2 g/l of G final) and R Bioforce® (diluted also to 2% final) were prepared in DMEM/Ham F12 medium and adjusted to pH 7.4 and serially diluted in the same medium.

2.3. Isolation, purification and culture of Leydig cells

The rats were sacrificed and the testes were quickly decapsulated and placed in DMEM/Ham F12 nutrient medium (1:1, v/v). The crude interstitial cells were separated from seminiferous tubules by incubation in a medium containing collagenase/dispase (0.05%), STI (0.005%), and DNase I (0.001%) at 32 °C for 15 min in a shaking water bath, followed by several decantations and a filtration through 30-mesh nylon. The Leydig cells were purified on a discontinuous gradient of Percoll (20–80%) prepared in medium as previously described (Lefevre et al., 1983). Leydig cell fractions were collected, washed with the medium and their purity was appreciated by histochemical staining for the specific 3β-hydroxysteroid dehydrogenase activity; 85–90% of positive cells were labeled. Leydig cells viability was determined by Trypan blue exclusion test and was near 90%. After purification, Leydig cells were maintained in DMEM/Ham F12 nutrient medium (1:1, v/v) at 32 °C (5% CO_2, 95% air) with or without hCG, human homolog of LH physiologically involved in endocrine regulation of Leydig cells.

2.4. Isolation, purification and culture of Sertoli and germ cells

Sertoli and germ cells were isolated from the same rat testes by three enzymatic digestions on pellets obtained after previously described decantations. These pellets contain seminiferous tubules. Briefly, after the first enzymatic digestion described above (32 °C, 15 min), a second one was performed in the same conditions during 30 min. The third and last one was in a solution with 0.1% hyaluronidase and 0.005% STI at 37 °C for 30 min. After centrifugation (900 rpm, 2 min), pellets contained Sertoli and germ cells. A second centrifugation (2500 rpm, 10 min) was necessary to isolate germ cells. Around 10^6 germ cells/well were seeded in 96-wells plates before treatments. The Sertoli and germ cells mixture was at a density of 2×10^6 cells/well in the same plates and cultured for 48 h in Ham's F12/DMEM medium (1:1, v/v) supplemented with serum replacement 3 at 32 °C (5% CO_2 and 95% air). On day 3, to obtain purified Sertoli cells cultures when necessary, germ cells were removed with an osmotic shock using a 20 mM Tris–HCl solution (pH 7.2). Treatments with different dilutions were applied on day 5 on Sertoli cells.

2.5. Adenylate kinase measurement

The bioluminescent ToxiLight™ bioassay (Lonza, Verviers, Belgium), developed by Crouch et al. (1993), is a non-destructive enzymatic bioassay. It measures quantitatively the luminescence of adenylate kinase (AK) of mammalian injured cells in culture (Crouch et al., 1993). The AK is a membranous enzyme present in all eukaryotic cells, and is released into culture medium when cells are damaged (the membrane integrity is disrupted during necrosis or secondary necrosis that occurs as a result of apoptosis). The AK release in the medium converts ATP from ADP, which is then

measured on a luminometer (Mithras LB 940, Berthold, Thoiry, France); the bioluminescent reaction is produced by luciferase. When the cytolysis increases, AK increases in the supernatants, resulting in higher light intensity.

Before the assay, 10^5 cells per well in 96-well plates (Dutsher, Brumath, France) or 3×10^5 cells per well in 24-well plates (Dutsher, Brumath, France) were treated with different dilutions of R or G ± 1 UI/ml of hCG during 3, 6, 9, 12, 18, 24 or 48 h. The adenylate kinase detection reagent (AKDR) was prepared in a buffer (5 g/10 ml). Then 50 µl of supernatant were transferred to an opaque black 96-well plate. Fifty microliter of AKDR reagent were deposited into each well. The plates were then placed under agitation for 15 min in the dark, and light was measured using the luminometer.

2.6. Caspase 3/7 activity

The Caspase-Glo™ 3/7 assay (Promega, Paris, France) measures the activities, in 96-well white plates (GBO, Dutscher, France), of caspases 3 and 7, key-caspases of apoptosis, in cell cultures using a bioluminescence-based method. The reagent contained a proluminescent substrate of caspases 3/7, containing the tetrapeptide sequence Z-DEVD-aminoluciferin, in a buffer including a detergent for cell lysis and other components stabilizing caspases activities (Bondzio et al., 2008; Liu et al., 2005; Riss and Moravec, 2004). After cell lysis, the cleavage of the substrate by caspases released aminoluciferin, which was then able to generate a "glow-type" luminescence, produced by luciferase related to the caspase 3/7 activity in the sample. This assay was designed for automated high-throughput screening of caspases 3/7 activities, specific for apoptosis.

Before the assay, 10^5 cells per well in 96-well white transparent background plate were treated with different dilution of R or G ± 1 UI/ml of hCG during 3, 6, 9, 12, 18, 24 or 48 h. The Caspase-Glo® 3/7 reagent was prepared in the buffer provided (Promega, Paris, France). After 30 min at room temperature, 50 µl of Caspase-Glo® 3/7 reagent was added to 50 µl of culture medium containing the cells previously treated in each well. After shaking the plate during 15 min, an incubation period of 45 min at room temperature in the dark was needed to stabilize the signal before luminescence measurement with the luminometer.

2.7. Analysis of DNA in situ by DAPI

DAPI is a fluorescent stain that binds strongly to DNA after passing through cell membrane. After a 24 h incubation in presence of various dilutions of G or R, 24-wells plates were centrifuged (900 rpm, 15 min) and the medium was removed slowly. Leydig cells (3×10^5/well) were fixed for a day in absolute ethanol–chloroform–acetic acid (6:3:1, v/v/v) at −20 °C. The wells were washed with PBS (pH 7.4) and incubated with 1 µg/ml of a solution containing DAPI during 30 min (Travert et al., 2006). Each well was washed with water and then examined with a microscope using a fluorescent mode (DMLB, Leica). Labeled DNA of viable cells was scattered throughout the nucleus, and bright condensation of chromatin revealed apoptotic cells (magnification 400×).

2.8. 3β-HSD activity

Leydig cells, previously prepared and seeded in 96-well plates as described above, were exposed for 24 h to different concentrations of R Bioforce®, or equivalent non cytotoxic concentrations of G, in medium DMEM/HamF12 to 32 °C (5% CO_2, 95% air). The 3β-hydroxysteroid dehydrogenase (3β-HSD), a Leydig cell specific enzyme involved in particular in testosterone synthesis, was measured at the end of the treatment. The 3β-HSD reagent containing DHEA (substrate), NAD (cofactor), NBT and nicotinamide was added to wells containing Leydig cells pretreated and then incubated at 37 °C for 45–60 min. Once the cells are stained brown, a solution of acetic acid (10%) was added to each well to solubilize formazan crystals previously formed. The 3β-HSD enzyme activity was then evaluated by reading the optical density of each well at 560 nm (formazan) through a plate reader (Mithras LB 940, Berthold, Thoiry, France).

2.9. Radioimmunoassay (RIA) of testosterone

The radioimmunoassay was performed on the same Leydig cells by competition and stopped using the method of activated charcoal. Indeed, the steroid dose is in competition with its tritiated counterpart by incubating 200 µl of standard solution of unlabeled testosterone (7.5–800 pg of testosterone/200 µl phosphate buffer), phosphate buffer (0.1 M Na_2HPO_4, 0.9% NaCl, 0.5% BSA, 0.01% NaN_3 – pH 7.4) or culture supernatant with 100 µl of radioactive testosterone (3000 cpm/100 µl phosphate buffer) and 100 µl of rabbit anti-testosterone antibody (final concentration 1/18000). After 30 min at room temperature, the mixture is placed at 4 °C until the next day. Then 500 µl of charcoal/dextran (50%/5%) at 4 °C are added. After 10 min of incubation at 4 °C, the tubes were centrifuged 10 min at 2400 rpm at 4 °C and the radioactivity of the supernatant was then counted. The sensitivity of the assay was 12 pg of testosterone per tube.

2.10. Measurement of mRNA expression of aromatase, androgen receptor and estrogen receptor α and β by real-time PCR

After exposure of Leydig cells for 24 h at different non cytotoxic concentrations of R or G in 6-well plates cell pellets were recovered and placed in the presence of Trizol to degrade the cells. Then chloroform was added to recover the aqueous phase containing the RNA. Precipitation of RNA is done by adding isopropanol and washing by adding ethanol (70%). After a denaturation step (10 min at 55–60 °C), the integrity of total RNA was controlled by dosing (260–280 nm) and by electrophoresis on agarose gel (1.5%) labeled by bromide ethidium. To achieve the reverse transcription (RT) 250 ng of RNA were used and placed in the presence of 200 U of MMLV-RT (Moloney murine leukemia virus reverse transcriptase), 0.2 g of random primers, 500 mM of each dNTP and 20 U of recombinant RNasin®. The samples were then placed 90 min at 37 °C to obtain the cDNA, the reaction was stopped by 5 min at 75 °C. The polymerase chain reaction was performed on cDNA using the method GoTaq® qPCR Master Mix (Promega). The PCR primers used are: L19 5′-GGA ATC TAA GAA GAT TGA CCG TC-3′ and 3′-GCC TTG TCT GCC TTC AGT TT-5′; aromatase 5′- CGT CAT GTT GCT TCT CAT CG-3′ and 3′-TAC CGC AGG CTC TCG TTA AT-5′; estrogen α receptor 5′-AAT TCT GAC AAT CGA CGC CAG-3′ and 3′-GTG CTT CAA CAT TCT CCC TCC TC-5′; estrogen β receptor 5′-CTT GCC CAC TTG GAA ACA TC-3′ and 3′-CCA AAG GTT GAT TTT ATG GCC-5′; androgen receptor 5′-TGG GGA CAT GCG TTT GGA CAG T-3′ and 3′-GCT GCC ACA AGT GAG AGC TCC G-5′. The PCR conditions were an initial step at 95 °C for 3 min, then 40 cycles of 30 s at 95 °C and 60 °C for 60 s. mRNA levels of aromatase, estrogen receptor α and β and androgen receptor were normalized using the L19 control gene.

2.11. Statistical analysis

All data are presented as the means ± Standard Errors (SEM). The experiments were repeated in triplicates on different months from three independent cultures each time ($n = 9$) unless otherwise specified. To compare the results, statistically significant differences from controls were determined by an Anova test followed

by Bonferroni post-test with $p < 0.001$ (****), $p < 0.005$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

3. Results

Cytotoxicities of G alone and with adjuvants (R), from 50 ppm to agricultural working dilutions (200 times more), were simultaneously measured on 70-day isolated adult rat testicular cells: Leydig alone, Sertoli with germ cells, purified Sertoli, and finally germ cells alone. Significant membrane degradations were provoked by R in Leydig cells within 24 h from 0.1% (1000 ppm), thus at relatively high levels, however 10 times below the lowest agricultural dilutions (Fig. 1a). This tendency was visible very rapidly from 1 h of treatment and was persistent (Fig. 2). This phenomenon increases up to five times more than in controls at higher doses; only at these doses Leydig cells reacted as though they were around 50% more resistant after 48 h of treatment (Fig. 1b). Moreover, isolated Sertoli cells were also sensitive (but maximally two times over controls) from 0.05% (500 ppm), in 24 h (Fig. 1e). By contrast Sertoli cells were almost insensitive to herbicide-induced mortality in the presence of germ cells, like germ cells alone (Fig. 1c, d, g, h). This does not exclude other limited light phenomena at higher doses (Fig. 1g). The kinetics of membrane degradation was studied in Leydig cells, which are the most sensitive to R (Fig. 2). R cytotoxicity was observed already after 1 h, and was maximal from then on and up to 24 h. We confirmed that G alone had no action at any time over 24 h in Leydig cells.

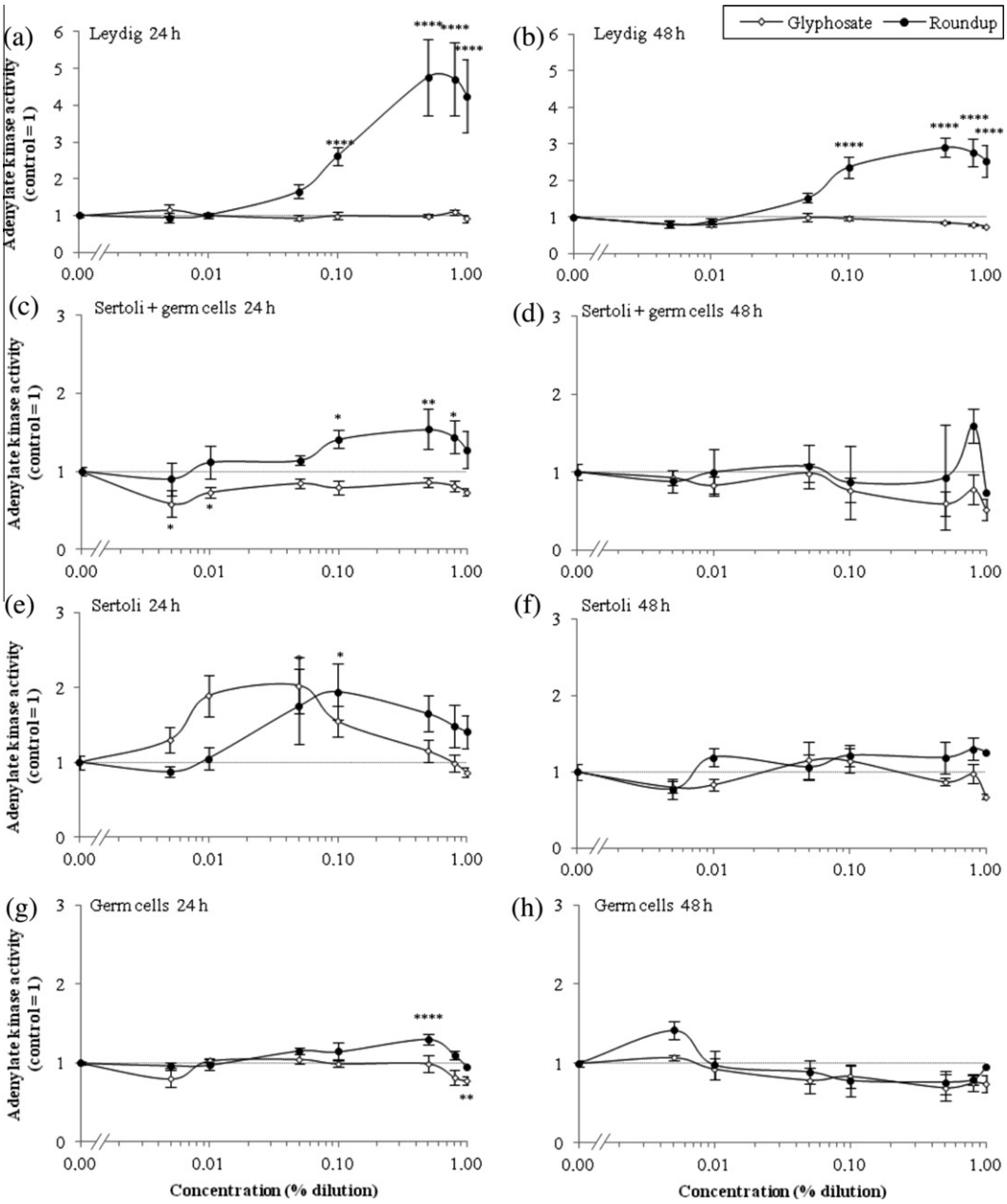


Fig. 1. Effects of Roundup or Glyphosate (empty diamonds) in DMEM/Ham F12 medium after 24 h (left column) or 48 h (right) on adenylate kinase activities of Leydig (a and b), Sertoli with germ cells (c and d), purified Sertoli (e and f) and germ (g and h) cells. Cytotoxicity of Roundup Bioforce® and glyphosate alone were measured through adenylate kinase activities indicating membrane degradations in primary cultures of testicular cells. The cells were exposed to different doses of Roundup Bioforce® or equivalent doses of glyphosate in DMEM/HamF12 medium in 96-wells plate at 32 °C (5% CO_2, 95% air) during 24 h or 48 h. Roundup and glyphosate were used at similar pH. All studies for each concentration were repeated three times and in three different experiments. Results of cell death are presented in Relative Luminescence Units (R.L.U.) compared to controls. SEMs are shown in all instances (Anova test $p < 0.001$ ****; $p < 0.005$ ***; $p < 0.01$ ** and $p < 0.05$ *).

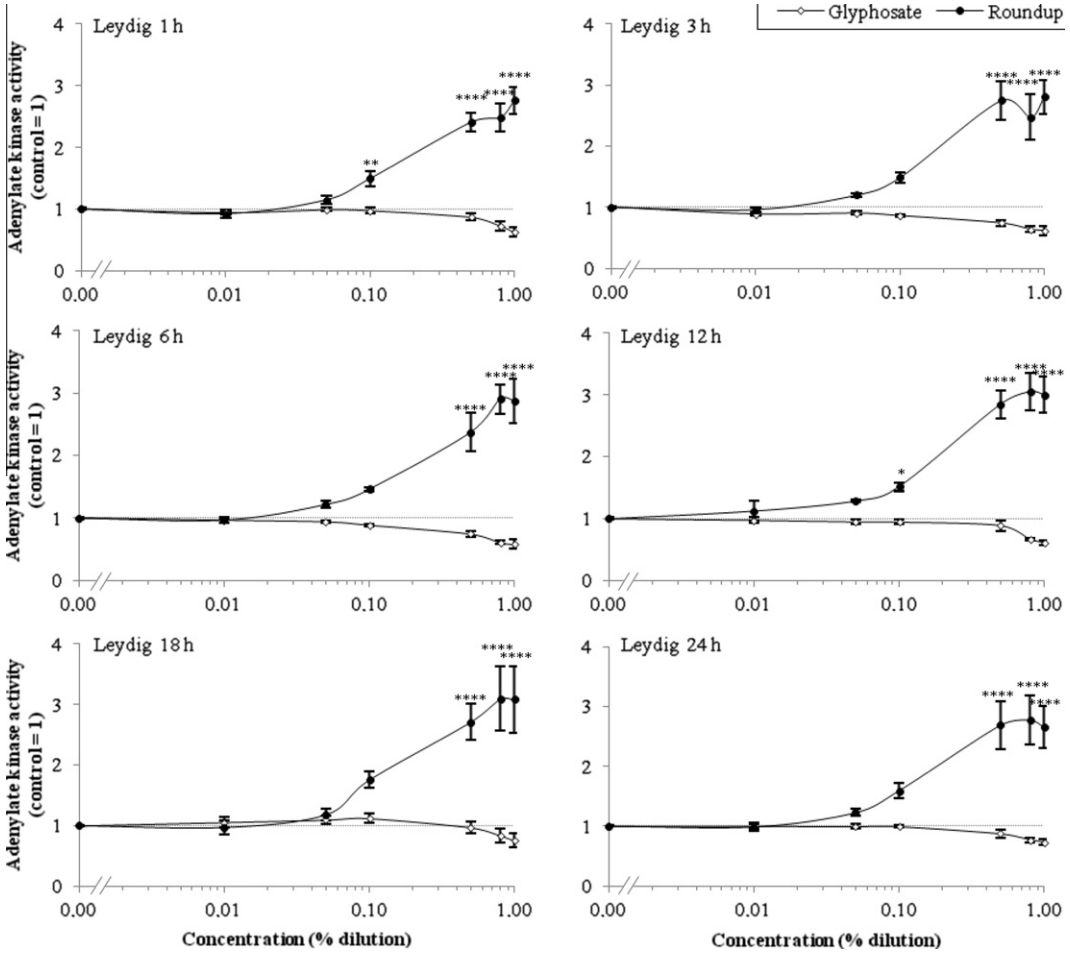


Fig. 2. Kinetics of cellular death revealed by membrane degradation in Leydig cells after 1–24 h treatments by Roundup or glyphosate alone (empty diamonds). Cytotoxicity of Roundup Bioforce® and glyphosate alone were measured through adenylate kinase activities indicating membrane degradations like previously. For more details see the caption for Fig. 1.

Signs of mortality were also observed through caspases 3/7 activity reduction induced by R. This was visible in all cells in very similar profiles approximately from 0.1% (1000 ppm) with a maximal effect in 24–48 h (Fig. 3). These effects were comparable in Leydig cells for membrane degradations, and indicate clearly a major necrosis. However, G alone induced apoptosis in 48 h from 0.5% (5000 ppm) in isolated germ cells and in Sertoli and germ cells mixture (Fig. 3d, h). A minor apoptosis induction was also observed in Leydig cells from 1000 ppm. The results of kinetic study of caspase 3/7 activities on Leydig cells were fully consistent with those obtained in Fig. 2: the decrease of these activities with R was amplified between 1 and 24 h starting from 0.1% (1000 ppm) (Fig. 4), reaching rapidly its maximum (after 6 h). Moreover, after 6 h of treatment, R first then G induced a punctual apoptosis phenomenon in Leydig cells. The nuclei aspects in these cells (Fig. 5) showed clear chromatin compaction after 24 h for R (1%). This is characteristic of programmed cell death. For G alone (1%) or lower R concentrations (0.05%) on Leydig cells, always in 24 h, an increase in DNA compaction was observed. In Leydig cells, membrane degradation and caspases 3/7 measurements within 24 h were also measured in presence or absence of hCG, the human homolog of LH (Fig. 6) that physiologically stimulates steroidogenesis. The cytotoxicity of treatments on Leydig cells are thus confirmed in presence of hCG, but are slightly reduced or delayed. Concerning endocrine disruption study, R and G had no impact at non-cytotoxic doses on 3β-HSD activity in these cells (Fig. 7). However, testosterone production measured by radioimmunoassay was inhibited at 1 ppm by G and R (Fig. 8). This was not the case on the androgen and estrogen receptors (α and β) mRNA levels (Fig. 9b–d). By contrast G increases significantly but punctually (and R with a light tendency) the aromatase mRNA (Fig. 9a), at 10 ppm.

4. Discussion

We developed here a model to investigate the effects of xenobiotics on mammalian reproductive cells, and especially testicular ones, at different environmental levels. We know that the testis is a sensitive target (Toppari et al., 2010). The originality of this work lies in the study of the same chemicals on all main testicular cell types simultaneously: Leydig, and Sertoli cells exposed in association or not with germ cells.

4.1. Leydig cells

Rat Leydig cells have been already proven to be very suitable for assessing the toxicity and hormonal activities of xenobiotics (Akingbemi et al., 2004). As a matter of fact, in this experiment, Leydig cells seem to be the most differentially sensitive to R by contrast to G, by the amplitude of the effect. There was only a very light action of G on caspases 3/7 activities after 48 h. The important R impact versus G was already observed by our group for various cell lines or fresh cells, for instance in human placenta and

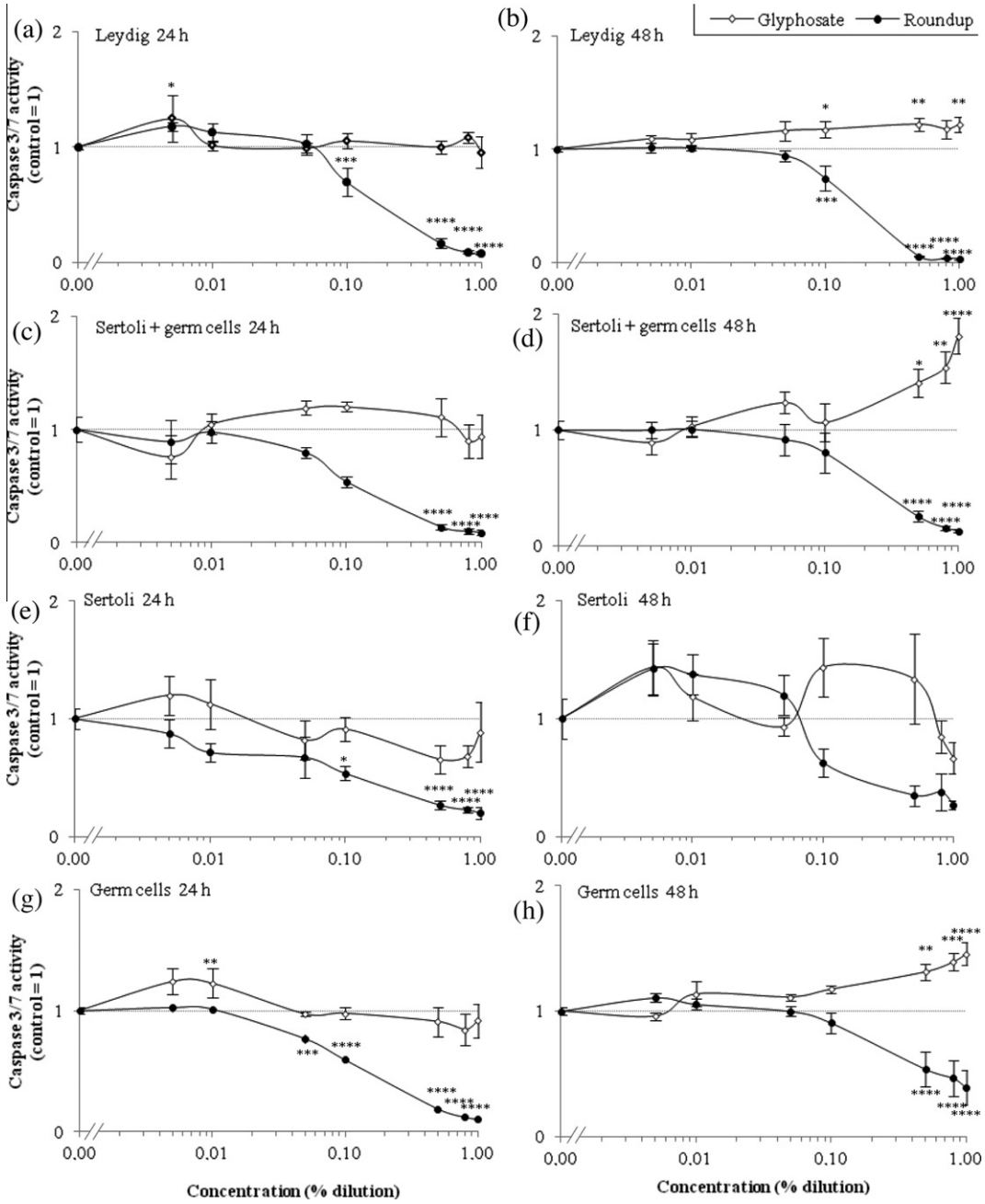


Fig. 3. Effects of Roundup or Glyphosate (empty diamonds) after 24 h (left column) or 48 h (right) on caspases 3/7 activities of Leydig (a and b), Sertoli with germ cells (c and d), purified Sertoli (e and f) and germ (g and h) cells. The effects were evaluated by the Caspases 3/7® assay. All conditions are similar as previously (see Fig. 1 legend); and studies for each concentration were repeated two or three times in three different experiments. The results are presented in relative luminescence unit and compared to non-treated cells (control = 1). SEMs are also shown in all instances (Anova test $p < 0.001$ ****; $p < 0.005$ ***; $p < 0.01$ ** and $p < 0.05$ *).

umbilical cord, embryonic kidney and liver (Benachour and Séralini, 2009; Benachour et al., 2007b; Gasnier et al., 2009, 2010, 2011; Richard et al., 2005). In other works for instance, R is also inducing toxicity on human epidermal cell lines (Gehin et al., 2005). Since in Leydig cells the membrane degradations were visible from 1 h with R, direct phenomena can occur at this level. Here we demonstrated a cytotoxic effect of R by membrane degradation at doses 1000 times lower than the commercial G-based formulation, or as underlined, 10 times under the lowest agricultural dilutions and around eight times less than the maximal level of residues authorized in GM feed. R disrupts the mammalian cell membrane and not G most probably via incorporation of adjuvants such as POEA at this level.

However after a longer exposure of Leydig cells (48 h), if the threshold of sensitivity was unchanged, the amplitude of the cell membrane degradation was divided by approximately 2. We could hypothesize a reduction of the membrane fluidity due to the interaction of adjuvants (Riechers et al., 1994), a new expression of xenobiotics excretion proteins (Melaine et al., 2002), or directly on a reduction of AK bioavailability.

Modification of membrane fluidity and/or disruption of membrane potential can induce cell death by apoptosis, because a decreased membrane potential and the pore openings can allow the release of cytochrome c and APAF-1. This can cause apoptosis via the mitochondrial pathway dependent on caspases (Sarda-Mantel et al., 2004; Vilches Troya, 2005).

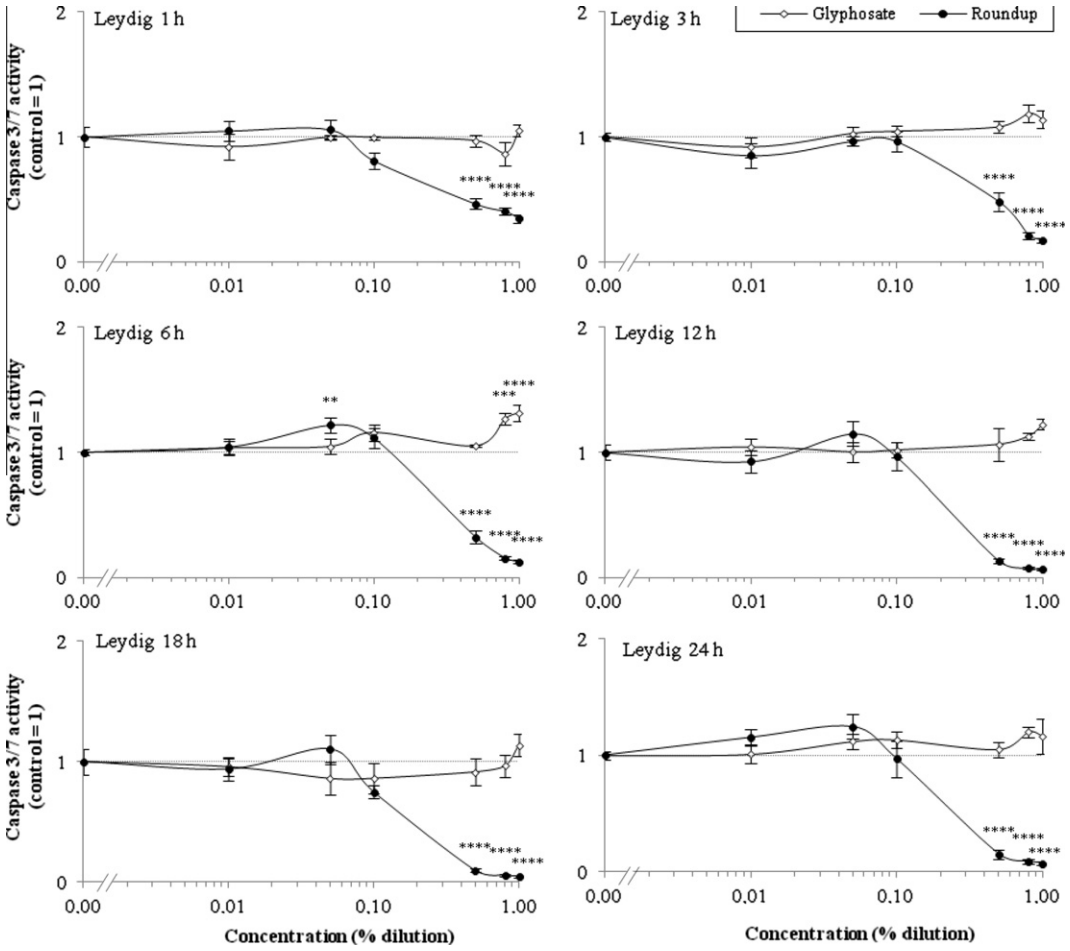


Fig. 4. Kinetics of caspases 3/7 activities in Leydig cells after 1–24 h of treatments by Roundup or glyphosate alone (empty diamonds). Cytotoxicities of Roundup Bioforce® and glyphosate alone were measured through caspases 3/7 activities like previously for all other details (see the caption for Fig. 1). All studies for each concentration were repeated two times on three manipulations.

We then tested if the cytotoxicity of R measured through membrane degradation was mostly due to necrosis, or apoptosis implying often a secondary necrosis. Necrosis is characterized by an inflammatory reaction, i.e. swelling and bursting of organelles and cells leading to a significant membrane rupture and by a release of the contents of their cytoplasm (Leist and Jaattela, 2001; Sarda-Mantel et al., 2004). Apoptosis is characterized by a nuclear condensation before the nuclear fragmentation, DNA degradation and changes in mitochondria, activation of caspases (and especially caspase 3) as well as cytoplasmic condensation, chromatin compaction and crush of the previous cell into apoptotic bodies, subsequently destroyed (Gerschenson and Rotello, 1992; Sarda-Mantel et al., 2004; Vilches Troya, 2005). Apoptosis occurs either by an extrinsic or intrinsic pathway. The extrinsic pathway is provoked through stimulation of a plasma membrane death receptor (for instance with a ligand such as a hormone, growth factor, cytokine or a toxin). The intrinsic pathway corresponds to the release of mitochondrial signals such as cell stress caused by DNA damage, heat shock, cell suicide, lack of nutrients (Brune, 2003; Csipo et al., 1998; Popov et al., 2002). These signals might accumulate during cell exposure to xenobiotics that can induce stress leading to apoptosis.

The caspases 3/7 activities as apoptosis indicator collapsed after 1 h in Leydig cells, with no increase at all (except a very light one after 6 h) but a decrease; this indicates no significant apoptosis in comparison to the basal activity in controls. We deduced that the cellular death was mostly due to necrosis, overall after adenylate kinase release, without excluding a light apoptosis visible after 24–48 h of G treatment. At this time, most necrotic degraded cells were not visible anymore (Fig. 5).

Leydig cells under hormonal treatment (hCG; Fig. 6), like in an *in vivo* context, were still reactive to R. This stimulation of steroid synthesis by the LH substitute had even a slightly protecting effect from necrosis on these cells; it induces vitality as observed in other cases (Nagai, 1992). Hormones are important for survival and physiological function of testicular cells. Endocrine disruption was then studied on mRNA levels for aromatase, androgen and estrogen (α and β) receptors, 3β-HSD activity and testosterone production at non cytotoxic doses, i.e. environmental ones, also found in urine agricultural workers and their families (1 ppm). In this work, the only endocrine effects in our conditions were a testosterone decrease by R and G and an aromatase mRNA increase by G. The latter may be secondary to an inhibition of this enzymatic activity previously shown, due to a direct interaction of G in the aromatase active site (Richard et al., 2005). The testosterone production inhibition and the consecutive recovery may be due to an inhibition in the early steroidogenic pathway for instance on StAR but not on 3β-HSD activity (Walsh et al., 2000).

An endocrine disruption in testicular cells could result in adverse effects on the reproductive system including epigenetics ones in offsprings (Anway et al., 2005), and more importantly it could provoke a decrease in sperm count and sperm production during adulthood, a decrease in the serum testosterone level at puberty and an increase of abnormal sperms in rats (Dallegrave

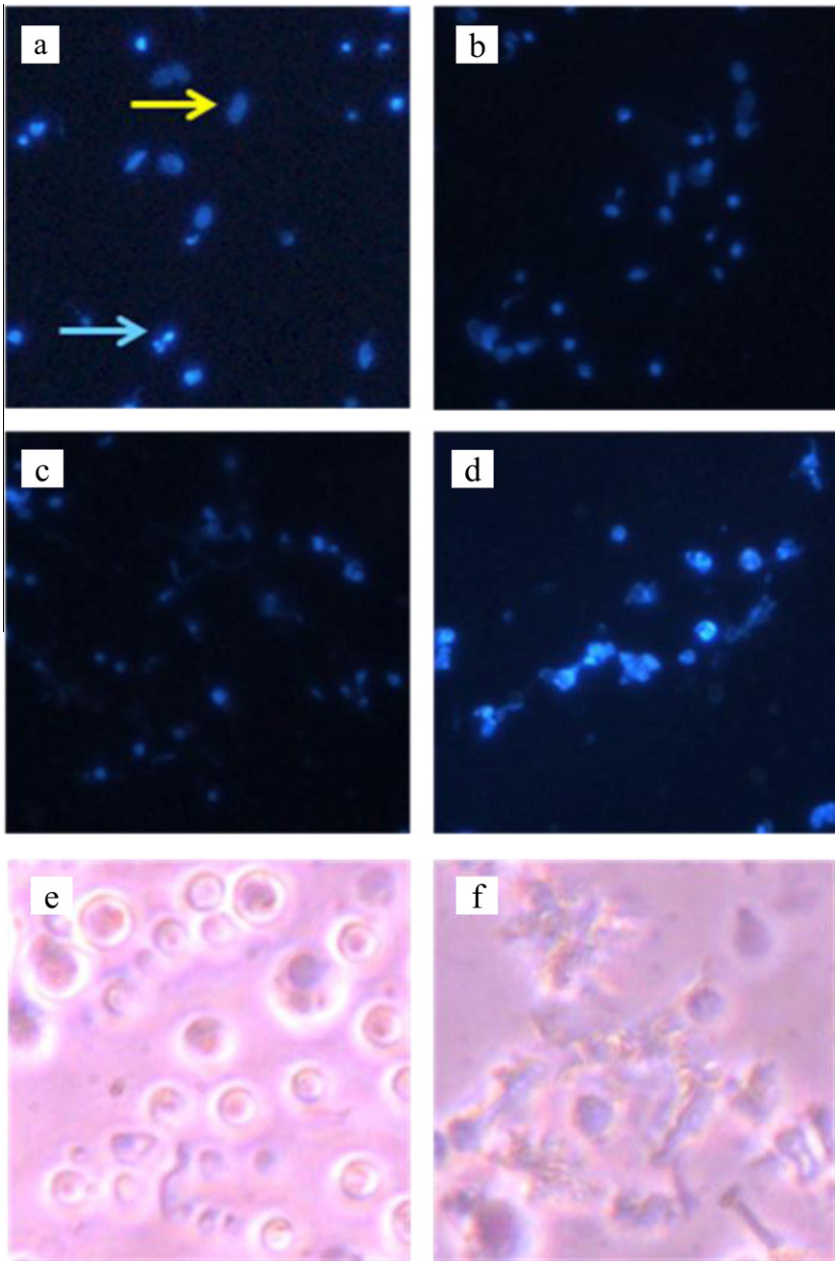


Fig. 5. Study of chromatin condensation by DAPI-labeling (a–d) and cellular aspect (e and f) on primary Leydig cells cultures in DMEM/Ham F12 medium after 24 h of treatment by Roundup or glyphosate alone. Bright condensation of chromatin revealed apoptotic cells (magnification 400×). Yellow arrow shows normal chromatin condensation and the blue arrow evidences compacted chromatin. DAPI labeling in (a) control cells, (b) cells treated with glyphosate 1%, (c) Roundup 0.05%, (d) Roundup 1%; (e) control unstained cells and (f) with Roundup 1%.

et al., 2007). After exposure to G, various effects were also observed in male rabbit reproductive health like a reduced body weight, libido, ejaculate volume, sperm concentration, osmolarity of semen, and an increase of abnormal or dead sperm (Yousef et al., 1995).

4.2. Sertoli cells, germ cells alone and Sertoli/germ cells co-cultures

R also induces a caspases 3/7 collapse for all cells, indicating a necrosis with a smaller membrane degradation than with Leydig cells, and mostly in 24 h. This is again in contrast to G impact alone, which corresponds to a clear caspases induction in germ cells and Sertoli/germ cells associations. This appears to be due to germ cells sensitivity, since Sertoli cells are not significantly reactive. Moreover G could penetrate more easily in germ cells due to membrane specificities. We know that G alone may penetrate into cells (Gasnier et al., 2011). Whether it corresponds to an *in vitro* artifact on isolated cells which have to be checked in *in vivo* experiments. However, it is well known that germ cells do undergo significant apoptosis during their differentiation (Petre-Lazar et al., 2007), which could well be amplified by environmental stressors (Anway et al., 2006). It has been observed that rats fed with R-treated transgenic plants undergo ultrastructural disorders in their Sertoli cells, such as transcription disruptions, nuclear and reticulum changes, possibly due to herbicide residues (Vecchio et al., 2004).

Surprisingly, while germ cells have less transporters to mediate cellular efflux of xenobiotics than Sertoli or Leydig cells (Melaine et al., 2002; Tribull et al., 2003), they were at a membrane degradation level insensitive to G. It was the same in Sertoli associated

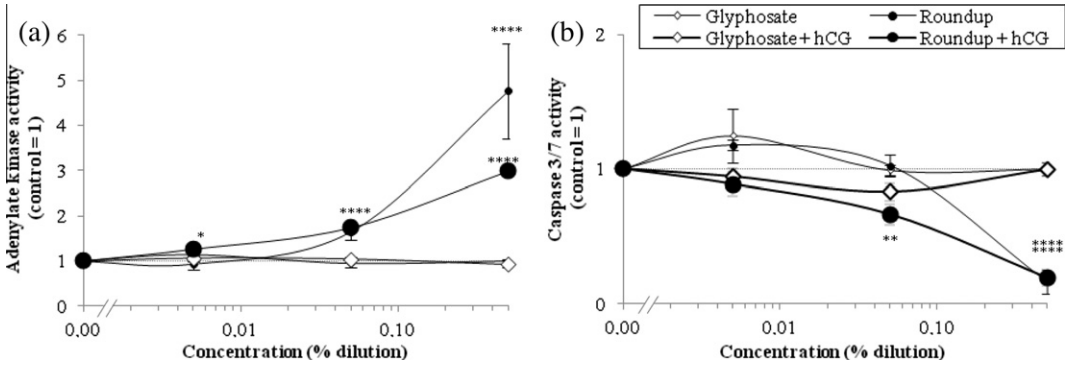


Fig. 6. Effects of Roundup or Glyphosate alone (empty diamonds) in medium complemented or not with hCG (larger symbols) after 24 h of treatment on Leydig cells. Cytotoxicites of Roundup Bioforce® and glyphosate alone were evaluated on primary cultures of Leydig cells through adenylate kinase (a) and caspases 3/7 (b) activities as previously described (see caption for Figs. 1 and 3). All studies for different concentrations were repeated three times and in three different experiments. SEMs are also indicated.

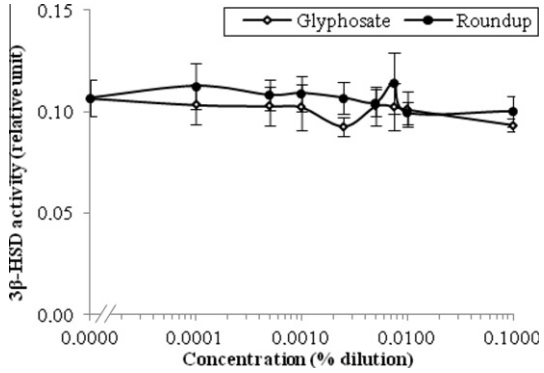


Fig. 7. Effects of Roundup Bioforce® or Glyphosate in DMEM/Ham F12 medium after 24 h of treatment on 3β-HSD Leydig primary cells activity in culture cells. The cells were exposed to different doses of Roundup Bioforce® or equivalent non-cytotoxic doses of glyphosate in DMEM/HamF12 medium in 96-wells plate at 32 °C (5% CO_2, 95% air) during 24 h. Roundup and glyphosate were used at similar pH. All studies for each concentration were repeated three times and for three different manipulations. SEMs are shown in all instances (Anova test $p < 0.001$ ***; $p < 0.01$ ** and $p < 0.05$ *).

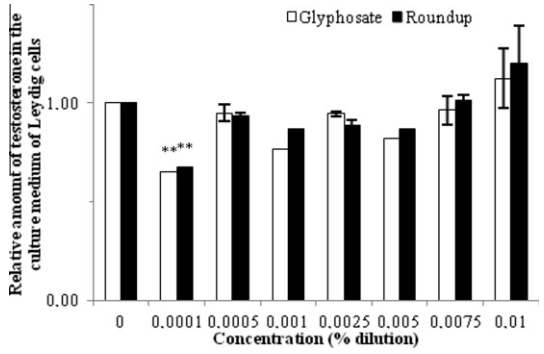


Fig. 8. Effects of Roundup Bioforce® and glyphosate on the production of testosterone by Leydig cells in primary cultures after 24 h of exposure to various non-cytotoxic concentrations. Changes in production of testosterone secreted into the culture medium of primary Leydig cells previously prepared and incubated at 32 °C in the presence or absence of xenobiotics was measured using the radioimmunoassay method. All operations for each concentration were repeated three times on three different manipulations. The SEMs are indicated in the figure (Anova test $p < 0.001$ ****; $p < 0.005$ ***; $p < 0.01$ and ** $p < 0.05$ *).

to germ cells; their association was protective, as shown in other studies (Benbrahim-Tallaa et al., 2002).

In conclusion, the general sensitivity of all cells to R either through direct membrane degradation or caspases 3/7 disruptions can be explained either by a combined sensitivity to G and adjuvants forming R, or to a major sensitivity to some adjuvant(s) alone. Previous studies on human umbilical cord fresh cells, and cell lines from embryo and choriocarcinoma indicate in fact a great sensitivity to POEA alone first, the main adjuvant; but this also did not exclude a combined effect with G. *In vivo* studies on rats (Adam et al., 1997) reached similar conclusions.

From 24 h R always induced necrosis in all testicular cells, and only germ cells and to a lesser extent Leydig cells were affected by apoptosis after 48 h of G alone. The differences in structures of cell membranes of the three testicular cell types used for this work may explain the differential effects of G and R on cells by differential membrane composition, which impacts fluidity and membrane resistance. Moreover it has been shown previously that a chemical can induce apoptosis or necrosis depending on the applied dose because of bioaccumulation, genomic effect or oxidative stress (Kanduc et al., 2002; Uezono et al., 2001). It is also known that R and G cause a dose dependent increase in sister chromatid exchange in human cells, with a greater effect of R (Bolognesi et al., 1997). In addition, we should note that a recent study reports that AMPA, the main metabolite of G (in R) is clastogenic on human cells *in vitro* and genotoxic both *in vitro* and *in vivo* (Manas et al., 2009). As previously described, these DNA disruptions like the ones observed by nuclear shrinkage in our work could lead to necrosis. People working with herbicides are typically exposed for much longer and repeatedly to the tested doses, however this has to be confirmed *in vivo*.

Taken together, the *in vitro* disturbances by R on testosterone and aromatase in rat testicular cells around 1–10 ppm at non toxic levels, underline the environmental endocrine disruptions possible by G-based herbicides. Moreover Leydig cells are exposed to this kind of environmental doses (Acquavella et al., 2004) because 1 ppm was found in human urine and thus was present in blood. When 10 ppm of G are given to rats, half was still found in plasma 15 h later (Anadon et al., 2009). Moreover, in testis, Leydig cells and blood vessels are in fact very close.

We know in addition that pesticide residues may bioaccumulate because of adjuvants. In our works, the presence of adjuvants in commercial formulations is decisive in inducing most herbicide side effects; this is why the present regulatory *in vivo* tests with G alone to study chronic toxicity are hardly relevant. It is thus necessary to revise the safety of formulations (Mesnage et al., 2010; Monosson, 2005; Tichy et al., 2002). Our studies also represent another method of investigation for understanding the possible effects of xenobiotics on the decline of male reproductive functions.

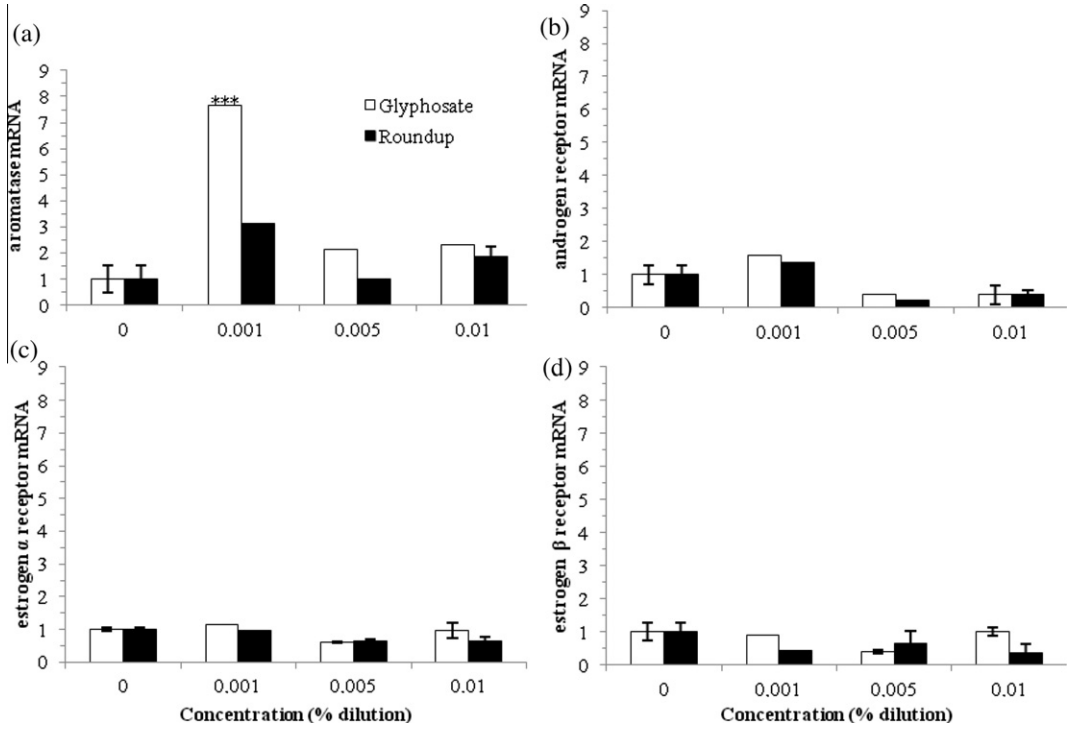


Fig. 9. Impacts of glyphosate and Roundup on the mRNA levels of aromatase, estrogen α and β and androgens receptors. After 24 h exposure of Leydig cells to various non-cytotoxic concentrations of Roundup or glyphosate in 6-wells plates, the cell pellets were recovered and total RNA extracted by the method of TriReagent® (Sigma–Aldrich). All operations for each concentration were repeated three times on three different manipulations. The SEMs are indicated in the figure (Anova test $p < 0.001$ ****; $p < 0.005$ ***; $p < 0.01$ and ** $p < 0.05$ *).

Conflict of interest statement

The authors declare that they have no conflict of interest.

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