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Time- and Dose-Dependent Effects of Roundup on Human Embryonic and Placental Cells

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Abstract. Roundup® is the major herbicide used worldwide, in particular on genetically modified plants that have been designed to tolerate it. We have tested the toxicity and endocrine disruption potential of Roundup (Bioforce®) on human embryonic 293 and placental-derived JEG3 cells, but also on normal human placenta and equine testis. The cell lines have proven to be suitable to estimate hormonal activity and toxicity of pollutants. The median lethal dose (LD_{50}) of Roundup with embryonic cells is 0.3% within 1 h in serum-free medium, and it decreases to reach 0.06% (containing among other compounds 1.27 mM glyphosate) after 72 h in the presence of serum. In these conditions, the embryonic cells appear to be 2–4 times more sensitive than the placental ones. In all instances, Roundup (generally used in agriculture at 1–2%, i.e., with 21–42 mM glyphosate) is more efficient than its active ingredient, glyphosate, suggesting a synergistic effect provoked by the adjuvants present in Roundup. We demonstrated that serum-free cultures, even on a short-term basis (1 h), reveal the xenobiotic impacts that are visible 1–2 days later in serum. We also document at lower non-overtly toxic doses, from 0.01% (with 210 μM glyphosate) in 24 h, that Roundup is an aromatase disruptor. The direct inhibition is temperature-dependent and is confirmed in different tissues and species (cell lines from placenta or embryonic kidney, equine testicular, or human fresh placental extracts). Furthermore, glyphosate acts directly as a partial inactivator on microsomal aromatase, independently of its acidity, and in a dose-dependent manner. The cytotoxic, and potentially endocrine-disrupting effects of Roundup are thus amplified with time. Taken together, these data suggest that Roundup exposure may affect human reproduction and fetal development in case of contamination. Chemical mixtures in formulations appear to be underestimated regarding their toxic or hormonal impact.

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Mammals and humans may be exposed to Roundup herbicide residues by agricultural practices (Acquavella *et al*. 2004) or when the residues enter the food chain (Takahashi *et al*. 2001); glyphosate is also found as a contaminant in rivers (Cox 1998).

In our previous work, we have demonstrated that the major herbicide used worldwide, Roundup, was toxic for a human placental cell line at concentrations below that recommended for agricultural use (1–2 %, *i.e.*, with 21–42 mM glyphosate) and had endocrine-disrupting potential on estrogen synthesis at lower nontoxic doses. These cell culture experiments were performed with or without serum only on one cell model and up to 18 or 48 h, respectively (Richard *et al*. 2005). Roundup is believed to be rather specific and less toxic to the ecosystem than other pesticides; transgenic plants tolerant to this compound have even been developed following this argument (Vollenhofer *et al*. 1999, Williams *et al*. 2000). Roundup is in fact a mixture of an isopropylamine salt of glyphosate, quantitatively a minor compound called the active ingredient, and various adjuvants (Cox 1998, Cox 2004) usually considered as surfactants forming an inert part of the composition and a secret of manufacturing. All these adjuvants can be differently used depending on the formulations. Among them are ammonium sulfate, benzisothiazolone, 5-chloro-2-methyl 3(2H)-isothiazolone, FD&C Blue No. 1, glycerine, 3-iodo-2-propynyl butylcarbamate, isobutane, isopropylamine, light aromatic petroleum distillate, methyl p-hydroxybenzoate, methyl pyrrolidinone, pelargonic acid, polyethoxylated tallowamine or alkylamine (POEA), potassium hydroxide, propylene glycol, sodium sulfite, sodium benzoate, sodium salt of o-phenylphenol, and sorbic acid. These products allow for glyphosate penetration through plasmatic membranes, potentialization of its action, increased stability, and potential bioaccumulation. Glyphosate does not appear to have an herbicide action by itself.

A differential effect was noticed in our previous study in favour of Roundup, in contrast to pure glyphosate. The purpose of the present work was to study in more detail the dose-and time-dependent cytotoxicity of both compounds, up to 72 h, comparing the effects on two cell lines from human embryonic kidney and placenta. Moreover, we wanted to examine the combined effects of this chemical mixture Roundup (Bioforce® herein) on a new cellular model. We also

tested the hypothesis that Roundup and glyphosate would inhibit aromatase activity at doses lower that those producing overtly toxic effects. We determined the aromatase disruption potential in 293 cells transfected with aromatase cDNA, and examined the temperature-dependent and direct mechanism of inhibition of aromatase by glyphosate on preparations of fresh human placenta and equine testis, a tissue known to be aromatase-rich (Lemazurier *et al*. 2001).

This was of particular interest since Roundup and/or glyphosate were suggested to disturb human (Savitz *et al*. 1997) and rat pregnancies (Daruich *et al*. 2001, Beuret *et al*. 2005), mouse kidney (Peluso *et al.* 1998), rabbit spermatogenesis (Yousef *et al*. 1995), and other human tissues (Monroy *et al*. 2005).

The cytotoxic and/or genotoxic effects of glyphosate have been reported at several checkpoints of the ecosystem, for instance on fish (Jiraungkoorskul *et al*. 2003), tadpoles and other aquatic species (Pettersson and Ekelund 2006), but also on urchin eggs (Marc *et al*. 2002, 2004, 2005) and human cells (Richard *et al*. 2005, Monroy *et al*. 2005). The endocrine disruption provoked by this compound is less documented. However, it has a very clear target at two crucial steps of steroidogenesis in mammals: at the first rate-limiting level of mitochondrial cholesterol transport (Walsh *et al*. 2000), and at the last irreversible conversion of sexual steroids androgens into estrogens, via a direct action on the aromatase enzyme (Richard *et al*. 2005).

Aromatase is an evolutionarily well conserved cytochrome P450 enzyme. Its superfamily includes numerous proteins able to metabolize xenobiotics (Nelson 1998). Its catalytic action is ensured by the product of the *CYP19* gene (Bulun *et al*. 2003) associated with another moiety, the ubiquitous NADPH-dependent reductase as electron donor. It is considered a limiting factor involved in estrogen synthesis and, thus, in physiologic functions, including female and male gametogenesis (Carreau 2001), reproduction, sex differentiation, and even bone growth. It is also pharmacologically controlled in the treatment of estrogen-dependent cancers (Séralini and Moslemi 2001).

The cytotoxic effect of Roundup on cells, and the direct action of glyphosate on aromatase, could explain some reproduction problems at least in part. Among the two lines, the 293 cells have proven to be very suitable to estimate hormonal activity for xenobiotics after transfection (Kuiper *et al*. 1998). In contrast, JEG3 cells present natural aromatase activity and are also considered a useful model to examine placental toxicity (Letcher *et al*. 1999). These cell lines may be even less sensitive to xenobiotics than primary cultures (L'Azou *et al*. 2005); in this case, the effects measured could well be an indication of human placental toxicity *in vivo*, if sufficient contamination occurs, because the phenomena appear to be amplified with time in cells.

Materials and Methods

Chemicals

N-(Phosphonomethyl) glycine (glyphosate) was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The herbicide Roundup used in this work is the formulation available on the market called Roundup Bioforce®, which contains 360 g/L acid glyphosate, equivalent to 480 g/L of isoproplyamine salt of glyphosate, homologation 9800036, Monsanto, Anvers, Belgium. A 2% solution of Roundup (1 or 2% is recommended by the company for agricultural use, *i.e.*, 21–42 mM glyphosate) and an equivalent solution of glyphosate were prepared in Eagle's modified minimum essential medium (EMEM; Abcys, Paris, France). When their effects were compared, the pH of glyphosate solution was adjusted to the pH of the 2% Roundup solution (∼ pH 5.8). Successive dilutions were then obtained with serum-free or serum-containing EMEM. 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and all other compounds, unless specified otherwise 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 serum-free EMEM.

Cell Lines

The human embryonic kidney 293 cell line (ECACC 85120602) and the human choriocarcinoma-derived placental JEG3 cell line (ECACC 92120308) were provided by CERDIC (Sophia-Antipolis, France). Cells were grown in phenol red-free EMEM containing 2 mM glutamine, 1% non-essential amino acid, 100 U/mL of antibiotics (mix of penicillin, streptomycin, and fungizone), and 10% fetal calf serum (Biowhittaker, Gagny, France). The JEG3 cell line was suplemented with 1 mM sodium pyruvate. Fifty thousand cells per well were grown at 37°C (5% CO_2, 95% air) during 48 h to 80% confluence in 24-well plates, washed with serum-free EMEM and then exposed to various concentrations of Roundup (0.01, 0.05, 0.1, 0.5, 0.8, 1, 2%), or the equivalent concentrations of glyphosate, in EMEM serum free or not, for various times:1, 24, 48, and 72 h.

MTT Assay

This enzymatic test, based on the cleavage of MTT into a blue-coloured product (formazan) by the mitochondrial enzyme succinate-dehydrogenase (Mossmann 1983), was used to evaluate human cell viability. Cells were washed with serum-free EMEM and incubated with 250 μL MTT per well after each treatment. The plates were incubated for 3 h at 37°C and 250 μL of 0.04 N-hydrochloric acid-containing isopropanol solution were added to each well. The plates were then vigorously shaken in order to solubilize the blue formazan crystals formed. The optical density was measured using a spectrophotometer (Stratagene, Strasbourg, France) at 560 nm for test and 720 nm for reference. The differential effects between glyphosate and Roundup are measured by the surfaces between the curves by the calculation of integrals.

Measurement of Aromatase Activity in Cells

Aromatase activity was evaluated according to the tritiated water release assay (Thompson and Siiteri 1974) with a slight modification as previously described (Dintinger *et al*. 1989). This method is based on the stereo-specific release of 1β-hydrogen from the androstenedione substrate, which forms tritiated water during aromatization. The 293 cells were transfected with the human aromatase cDNA (Auvray *et al*. 1998), exposed to nontoxic concentrations of glyphosate alone or Roundup, and were washed with serum-free EMEM and incubated for 45 min with 200 nM [1β-^{3}H] androstenedione at 37°C (5% CO_2, 95% air).

The reaction was stopped by placing the plates on ice for 5 min and then centrifuging at 2700*g*, at 4°C for 10 min. After adding 0.5 mL of charcoal/dextran T-70 suspension (7%:L5%), the mixture was left at

4°C for 5 min, and then centrifuged similarly. Supernatant fractions were assessed for radioactivity by scintillation counting.

Preparation of Microsomes

Microsomal fractions (containing endoplasmic reticulum) were obtained from full-term placentas of young healthy and nonsmoking women (Centre Hospitalier Régional de Caen, France) and equine testis by differential centrifugations (Moslemi *et al*. 1997). Briefly, tissues were washed with 0.5 M KC1, homogenized in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and 1 mM Dithiothreiol DTT, and centrifuged at 20,000*g*. The supernatant was then ultracentrifuged at 100,000*g*, and the final pellet was washed twice, dissolved in the same buffer containing 20% glycerol, and stored at −70°C until use. All steps of the preparation were carried out at 4°C.

Measurement of Microsomal Aromatase Activity

Microsomal aromatase activity was evaluated by tritiated water release from radiolabelled substrate [1β-^{3}H] androstenedione as described above. Human placental microsomes (50 μg proteins) were incubated with radiolabelled androstenedione (100 pmol/tube) at 37°C for 15 min, in the presence or absence of various concentrations of Roundup or glyphosate in 1 mL total volume of 50 mM Tris-maleate buffer, pH 7.4. The reaction was started by adding 100 μL of 60 μM H^{+}-NADPH and stopped with 1.5 mL chloroform and then centrifuged at 2700*g* at 4°C for 5 min. After adding 0.5 mL of charcoal/dextran T-70 suspension (7%:1.5%) into the preparation, the centrifugation was repeated for 10 min. Aromatase activity was determined by measuring the radioactivity of 0.5 mL aqueous phase.

Purification of Aromatase Moieties and Measurement of Reductase Activity

Reductase was prepared by chromatographic separation using (ωD-aminohexyl-Sepharose 4B followed by adenosine 2′-5′-diphosphate-agarose, hydrophobic interaction, and affinity columns (Vibet *et al*. 1990), Protein concentration was determined as previously described (Bradford 1976). Reductase activity was determined by the measurement of the increasing absorbance of the preparation, corresponding to the reduction of the cytochrome C in the presence of H^{+}-NADPH (Vibet *et al*. 1990) at 550 nm for 2 min at 20°C using a Kontron-Uvikon 860 spectrophotometer. The pH of the preparation was adjusted to 7.4 by adding an appropriate volume of 10 N NaOH. After equilibration, the reaction was started by adding cytochrome C.

Inactivation Study

The inactivation was carried out as previously described (Moslemi and Séralini 1997) by pre-incubation of equine testicular microsomes (200 μg proteins) for different times (0 to 30 min) at 20°C in a 0.5 mL final volume of 50 mM Tris-maleate buffer, pH 7.4, in the presence of saturating concentration of Roundup (11.6%) or in its absence (control). Androstenedione (400 nM) or H^{+}-NADPH (60 μM) were included or not in the preincubation medium. After preincubation, the free Roundup and androstenedione were removed by adding 100 μL of charcoal/dextran T-70 suspension (2%:1%) into the medium. The mixture was then gently mixed and left at 4°C for 15 min; this was followed by a centrifugation at 350*g* at 4°C for 10 min. Residual aromatase activity was then evaluated by incubating 70 μL of the aqueous phase with 200 nM tritiated androstenedione for 15 min at 25°C, in 0.5 mL of 50 mM Tris-maleate buffer, pH 7.4, containing 60 μM H^{+}-NADPH. The efficiency of Roundup adsorption by charcoal/dextran was previously tested without preincubation.

Statistical Analysis

The experiments were repeated at least 3 times in different weeks on 3 independent cultures each time (n = 9). All data were presented as the mean ± standard error (S.E.M.). Statistical differences were determined by a Student t-test using significant levels with $p < 0.01$ (**) and $p < 0.05$ (*).

Results

Cell Viability

We tested the toxicity potential of Roundup on 293 cells derived from a human embryo, at doses (from 0.01 to 2%, *i.e.*, containing 210 μM to 42 mM glyphosate among adjuvants) below that recommended for agricultural use. We tested its effect on cell viability up to 72 h in comparison to glyphosate. We also compared the results of similar exposures on human placental JEG3 cells. The Roundup dilutions and equivalent quantities of glyphosate were adjusted to the same pH, to avoid measuring a specific action of glyphosate acidity.

Roundup always shows the highest time-and dose-dependent cytotoxicity on the 293 cell line in serum-free medium. Its toxic effect is attenuated in the presence of serum (Fig. 1A and B). Fifty percent of embryonic cells degenerate already within 1 h with 0.3% Roundup (LD_{50}) in serum-free medium. Afterwards, the LD_{50} decreases with time in the presence of serum, it reaches only 0.06% Roundup after 72 h. In all instances, Roundup is more efficient than its active ingredient, glyphosate, suggesting an additional effect provoked by the adjuvants. Moreover, the differential effect between Roundup and glyphosate, measured by the surface between the curves, permanently increases with time. This is true except when glyphosate becomes highly toxic alone, and this is only after 48 h on the more sensitive 293 cells in serum-free medium (Fig. 1B). Comparable results are obtained with the slightly less sensitive JEG3 placental cells (Fig. 2A and B). Their relative resistance is visible even with glyphosate alone. The sensitivity of the 293 cells is confirmed essentially for Roundup after 72 h (Fig. 3A and B). The cytotoxic effects of glyphosate and overall Roundup are more important after 72 h with serum in both cell lines, and differential effects between Roundup and glyphosate also become greater than after 1 h in serum-free medium. The cells were not viable in culture after 60 h without serum, but here we show that the short-term serum-free cultures optimize the xenobiotic impacts, which will in any case be visible after longer exposures in the presence of serum. In general, the serum buffers the xenobiotic impacts and the differential effect. It appears to delay the toxicity effect by 1–2 days.

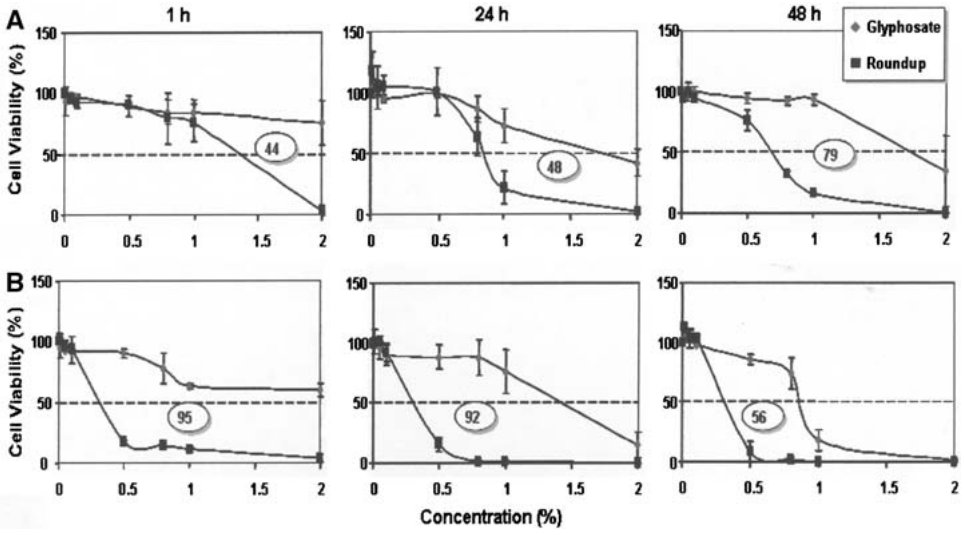

Fig. 1. Effects of Roundup and equivalent quantities of glyphosate on 293 cell viability in serum-containing medium (***A***) or in serum-free medium (***B***) for various times (1, 24, 48 h). This was evaluated by the MTT assay; the results are presented in % comparatively to nontreated cells. Cells were incubated with increasing concentrations of Roundup or equivalent concentrations of glyphosate at the same pH. The LD_{50} is indicated by a dashed line. The differential effects between glyphosate and Roundup are measured by the surfaces between the curves, and indicated within the circles in arbitrary units

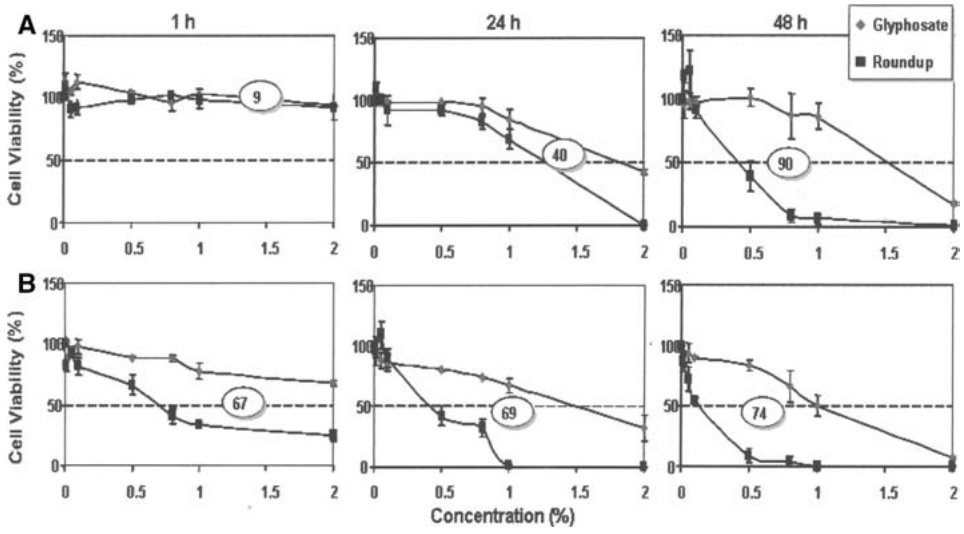

Fig. 2. Effects of Roundup and equivalent quantities of glyphosate on JEG3 cell viability in serum-containing medium (***A***) or in serum-free medium (***B***) for various times (1, 24, 48 h). The other details are indicated in the legend of Figure 1

Aromatase Activity Inhibition

Roundup or glyphosate alone was observed to have another action at lower nontoxic concentrations, namely the inhibited estrogen synthesis. This was demonstrated both after 24 h on the 293 cells transfected with the aromatase cDNA (Fig. 4A), and also after 15 min on fresh human placental cellular extracts (Fig. 4B). The IC_{50} is lowered from 2.4% glyphosate after 15 min in microsomes to 0.8% after 24 h in 293 whole cells. Glyphosate acts directly and independently of the Roundup adjuvants that facilitate its action. Its activity is not solely due to its acidity, as its inhibitory activity is present after neutralization (pH adjusted to Roundup, Fig. 4B). A genomic action is thus not necessary for this endocrine disruption, but is not excluded. In fact, we noticed that the aromatase activity inhibition by glyphosate is 3 times more important at a cellular level after 24 h, than in placental microsomes after 15 min of direct contact. In addition, we also confirmed the specific action of Roundup on another mammalian aromatase. In equine testicular microsomes, the results were very comparable and we noticed and a slight pH effect (Fig. 5). In fact, we documented that Roundup is active in different tissues (cell lines from placenta or embryonic kidney, testicular or placental fresh extracts) and on two species (man and horse).

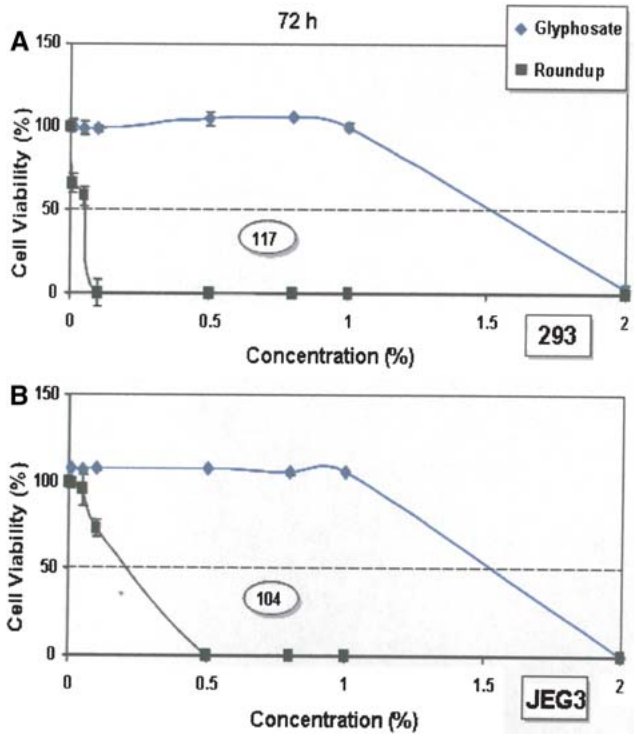

Fig. 3. Effects of Roundup and equivalent quantities of glyphosate on 293 (***A***) and JEG3 (***B***) cell viability in serum-containing medium for 72 h. Without serum, the cells do not survive 72 h. The other details are indicated in the legend of Figure 1

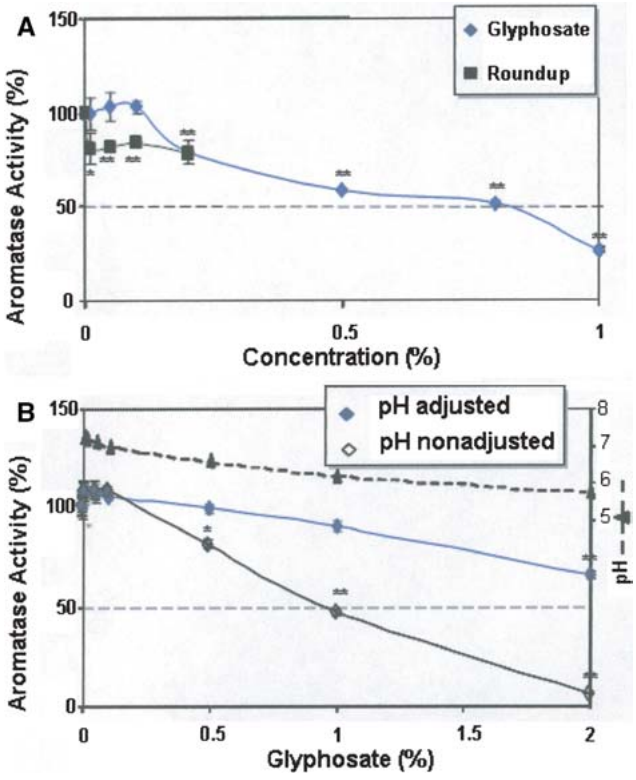

Fig. 4. Effects of glyphosate and equivalent quantities of Roundup on human aromatase activity in 293 cells in serum-free medium after 24 h (***A***) at nontoxic concentrations below 0.2 and 1% for Roundup and glyphosate, respectively. Effects of glyphosate alone on human aromatase activity in placental microsomes after 15 min and at 37°C (***B***) at pH adjusted (to the Roundup pH, - -▲- -) or nonadjusted, decreasing to pH 2.96 at 2%

We further purified the reductase enzyme moiety from the aromatase-rich equine testis, to confirm the level of action of the herbicide on the aromatase complex. A direct temperature-dependent effect was observed on both enzymatic moieties, because not only was the cytochrome P450 aromatase (Fig. 6A) affected, but also the purified reductase (Fig. 6B), to a lesser extent. The inhibition was maximal at body temperature or higher, and it was 3 times higher on the aromatase complex than on reductase alone. We confirmed and clarified this functioning between 25°C and 37°C with human placenta. Aromatase in fresh cellular extracts present a greater sensitivity to Roundup (increasing with temperature) than to glyphosate (Fig. 7A and B). When glyphosate is mixed with the adjuvants, its effect is 2–3 times more important. Moreover, we demonstrate for the first time that the Roundup inhibition is partially irreversible on the aromatase activity (Fig. 8) because in the presence of Roundup during the preincubation, the enzyme is partially inactivated. In contrast, the substrate protects the active site to some extent.

Discussion

In this work, we demonstrated a cytotoxic effect of Roundup for the first time on human embryonic cells, as well as endocrine disruption in this new model at lower nontoxic levels. This major herbicide is used worldwide and composed of glyphosate and a mixture of various adjuvants. The 293 cells were shown to be suitable for the estimation of hormonal activity of xenobiotics after aromatase transfection (Kuiper *et al*. 1998), in particular since they are themselves deprived of steroidogenesis. Our results also confirmed and extended our previous study on the human placental JEG3 cell line (Richard *et al*. 2005). This cell line is considered to be a useful model for examining placental toxicity (Letcher *et al*. 1999). Our studies also revealed that the embryonic cells are more sensitive than the placental ones.

The use of transformed or cancer-derived cell lines allows longer experiments than in primary cultures; moreover, the established cell lines may be less sensitive to xenobiotics than their normal counterparts (L'Azou *et al*. 2005), but still we measure here important impacts of Roundup. In this case, the timing and effects measured may be more important *in vivo* if living tissues are exposed to comparable contamination. Of course, the metabolism in the body will moderate these actions. However, we demonstrate irreversible inhibition and the exposures are also often longer *in vivo.* Thus, our models offer at least a good indication of the potential toxicity of Roundup during agricultural use. We have also worked here with fresh human placenta to determine whether the endocrine disruption by Roundup observed in the cell lines could also be evoked in the microsomal fraction obtained from fresh, normal tissue.

When used in agricultural practice, the formulated concentrated commercial Roundup is diluted on the farm. The farmers

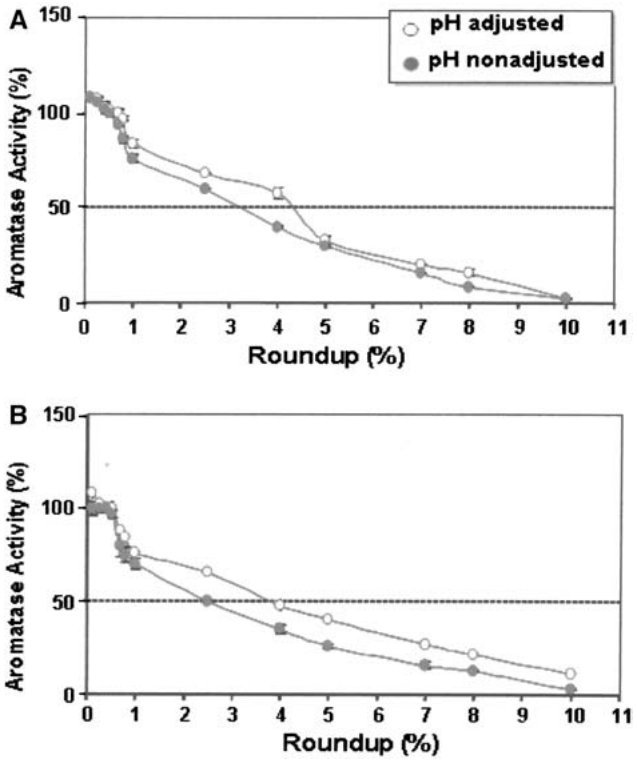

Fig. 5. Effects of Roundup on aromatase activity in human placental microsomes (**A**) and in equine testicular microsomes (**B**). The enzymatic activity was measured with pH nonadjusted decreasing to 4.88 at 10% (●) or pH adjusted to 7.4 (○) at 25°C after 15 min. The IC_{50} is indicated by a dashed line

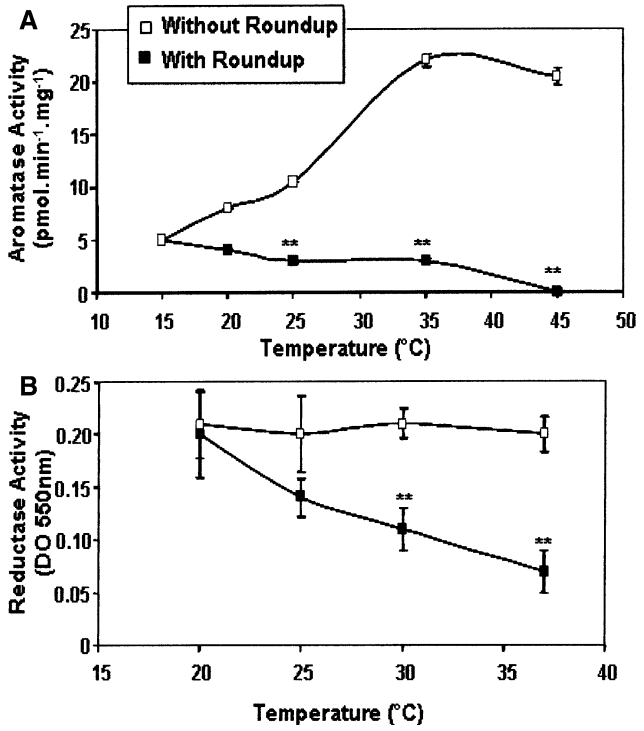

Fig. 6. Temperature influence on aromatase activity (***A***) and on reductase activity (***B***) in equine testicular microsomes or with the purified enzyme moiety, respectively. The enzymatic activity was measured in the absence (□) or in the presence of Roundup at IC_{50} (■)

are then often exposed to concentrated solutions (100%, *i.e.*, 2.13 M glyphosate), and then during spraying to more diluted solutions, up to 1–2%, the latter corresponding to the maximal concentrations used on the cells in this work. Pregnant women with embryonic and placental cells could be exposed during repeated herbicide preparations and generally only a few precautions are applied, since Roundup is believed to be one of the most environmentally friendly pesticides (US EPA 1998, Williams *et al*. 2000).

Our data demonstrated that as little as 0.01% Roundup, within only 24 h, provoked a significant reduction of 19% of estrogen production in transfected 293 cells. Estrogens are known to be necessary for normal fetal development. This Roundup dose became toxic after 72 h of exposure. Serum-binding proteins, including albumin, can buffer the xenobiotic bioavailability (Seibert *et al*. 2002), as we have observed, and our serum-free cultures allowed a shortening of the experiments to mimic longer-term effects, since within 1 h we obtained results comparable to those after 1–2 days in serum.

The endocrine effect was linked to glyphosate, which was directly able to inhibit aromatase in cells, and in the microsomes formed not only by the endoplasmic reticulum out of placental fresh cells but also from equine testis. Glyphosate also inhibited aromatase activity independently of its acidity, and on both enzyme moieties (reductase and cytochrome P450 aromatase). However, the acidity presented very little partial impact in contrast to the formulation Roundup. This interaction was not only demonstrated to be direct with the aromatase active site (Richard *et al*. 2005), it was also found to be temperature-dependent in our work on enzymatic catalytic activity, and all these impacts were promoted by the adjuvants in all instances. It is also suggested that the adjuvants allow a better solubilization of glyphosate and the latter are more active with the increase of the temperature. An indirect pathway on the aromatase gene expression was also observed in JEG3 cells (Richard *et al.* 2005). The action of Roundup disturbing the transcriptional activity of another crucial enzyme has been demonstrated (Marc *et al*. 2005) for the hatching of the sea urchin eggs. In addition, when the cytotoxic effect was noticed in this work, it was due to disruption of the mitochondrial enzyme succinate-dehydrogenase, implicated in a cellular viability process.

Our models are then pertinent to the study of Roundup toxicity. If the agents that it contains bioaccumulate, in case of contamination of a pregnant woman, it is likely that the placenta and embryo will be reached by significant levels of those. Pesticide adjuvants and surfactants, which are present in Roundup, are used in herbicide formulations to favor stability and penetration of the active ingredient into cell membranes (Cox 1998). These adjuvants amplify the cellular effects of herbicides not only in plants but also in animal models (Marc *et al*. 2002, Walsh *et al*. 2000, Nosanchuk *et al*. 2001). Some of them may eventually stick to DNA and bioaccumulate in new and not usually detected forms (Peluso *et al*. 1998). Thus, partial Roundup elimination does not exclude the action of some metabolites at cellular levels, since at least some of the Roundup residues have been

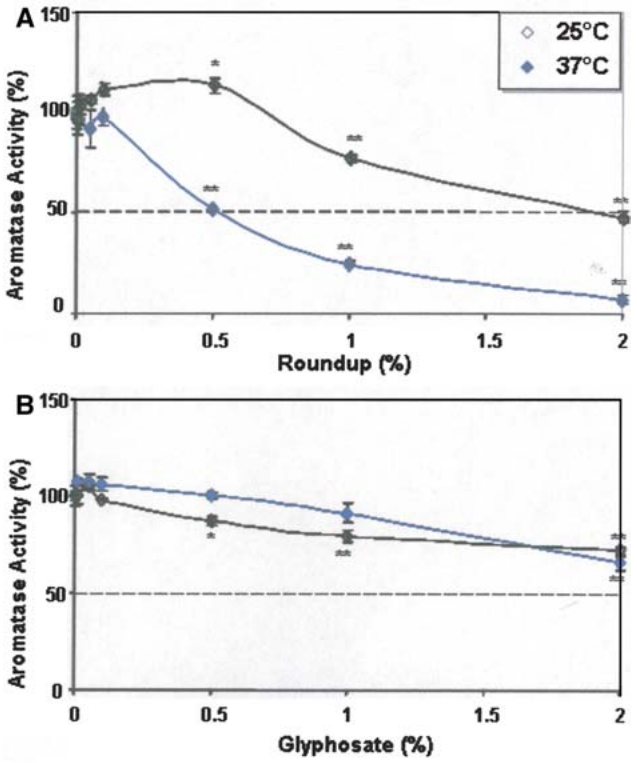

Fig. 7. Comparative effects of Roundup (***A***) and equivalent concentrations of glyphosate (***B***) on human placental aromatase activity in microsomes at 25 and 37°C after 15 mm. The pH was adjusted for glyphosate to the Roundup pH (Fig. 4B). Asteriks (*): $p < 0.05$; (**): $p < 0.01$. Significant effects in comparison to the 100% control

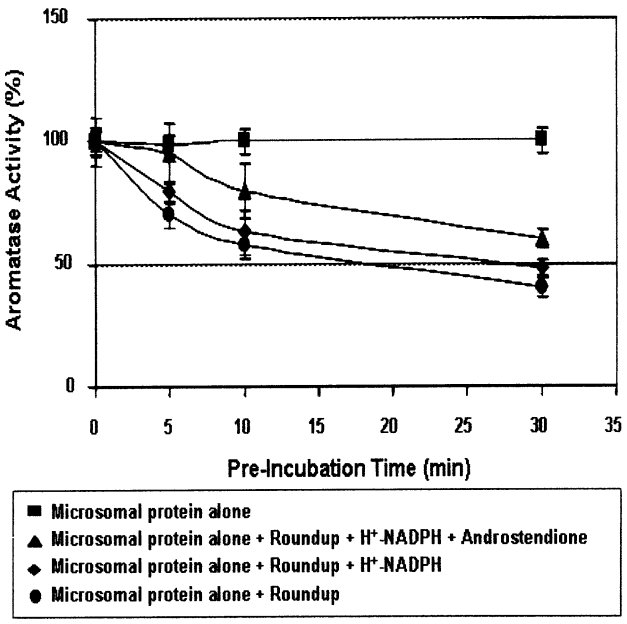

Fig. 8. Partial inactivation of equine aromatase by Roundup. Equine testicular microsomes were pre-incubated in different conditions (see below), when indicated with a saturating concentration of Roundup (11.6%) and 60 μM H^+-NADPH, plus 400 nM androstenedione, in 50 mM Tris-Maleate buffer, pH 7.4 at 20°C for different times. After Roundup removal, aromatase activity was evaluated

demonstrated to be strongly and more permanently bound to mammalian tissues.

This potential bioaccumulation could also induce or explain amplified effects with time. Thus, our results are in favour of the recruitment of other synergistic signalling pathways of action. This is why we have also analyzed the cell viability from 1 to 72 h, which has confirmed a drastic amplification of the cytotoxic effect of Roundup with time. Unfortunately, farmers are exposed often at least for many weeks to this regularly used product, which is also a common contaminant of rivers (Cox 1998). Considering all the data taken together, we cannot conclude like Williams *et al*. (2000) that the effects of the surfactants are antagonistic rather than synergistic.

Finally, we characterized in this work the differential sensitivity for Roundup and glyphosate of human embryonic cells, placental-derived cell lines, and fresh tissue extracts from human placenta and mammalian testis. Moreover, we confirmed the potential endocrine disruption of Roundup in all models on estrogen synthesis. As Roundup was more active than its claimed active ingredient in all instances, the formulation adjuvants probably allow a better cell penetration and stabilization of the product. Chemical mixtures in formulations may thus be underestimated regarding their toxic or hormonal impact (Tichy *et al*. 2002, Lydy *et al*. 2004, Monosson 2005). Most of the tests undertaken in a regulatory context are in fact performed with the active ingredient alone *in vivo* for one or two years (Williams *et al*. 2000). For instance, toxicity was not measured for Roundup treatments during more than 22 days with rats and rabbits. The potency for endocrine modulation was not assessed with the Roundup mixture at all, but only with glyphosate or POEA alone (Williams *et al*. 2000). Consequently, our experiments with Roundup should be also conducted on entire organisms *in vivo.* As emphasized by Brian *et al*. (2005), we can conclude that the failure to account for the combined effects, in particular with adjuvants, will undoubtedly lead to the underestimation of potential hazards, especially at the endocrine disruption level, and hence to erroneous conclusions at a regulatory level regarding the risk that they provoke. Thus, the toxic or hormonal impact of chemical mixtures in formulations appears to be underestimated.

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