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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.

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