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An acute exposure to glyphosate-based herbicide alters aromatase levels in testis and sperm nuclear quality

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ABSTRACT

Roundup is the major pesticide used in agriculture worldwide; it is a glyphosate-based herbicide. Its molecular effects are studied following an acute exposure (0.5%) of fifteen 60-day-old male rats during an 8-day period. Endocrine (aromatase, estrogen and androgen receptors, Gper1 in testicular and sperm mRNAs) and testicular functions (organ weights, sperm parameters and expression of the blood–testis barrier markers) were monitored at days 68, 87, and 122 after treatment, spermiogenesis and spermatogenesis. The major disruption is an increase of aromatase mRNA levels at least by 50% in treated rats at all times, as well as the aromatase protein. We have also shown a similar increase of Gper1 expression at day 122 and a light modification of BTB markers. A rise of abnormal sperm morphology and a decrease of the expression of protamine 1 and histone 1 testicular in epididymal sperm are observed despite a normal sperm concentration and motility.

1. Introduction

Exposure of human and mammalian populations to environmental and industrial contaminants represents a growing concern due to the impact of these pollutants on developmental and reproductive functions (Mathur and D'Cruz, 2011). There is an ongoing international debate as to the potential reproductive toxicity of glyphosate-based herbicides (GBH) such as Roundup. These are used worldwide in agriculture, including on edible Roundup-tolerant GMOs, and are major pollutants of rivers and surface waters (Cox, 1998; IFEN, 2007). Consequently, mammals and humans could be exposed to herbicide residues by agricultural practices, food and water (Acquavella et al., 2003). The presence of pesticide residues and/or their metabolites has been reported in the urine of families living in farms and nonfarm households (Curwin et al., 2005, 2007). Increased levels of glyphosate were also found

Abbreviations: BTB, blood–testis barrier; GBH, glyphosate-based herbicide; P450, arom aromatase; ROS, reactive oxygen species.

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in the urine of the farmer's son who lived at some distance from the sprayed fields. The contamination occurred through contact with the father (Mesnage et al., 2012). Recently, a much-debated study about the health effects of GBH exposure over a two-year period in adult rats (Séralini et al., 2013) revealed pathologies in females (increased rates of premature death and mammary tumors) and in males (increase in liver congestions and necrosis and kidney nephropathies). Paternal exposure to pesticides is increasingly recognized as causing birth defects due to pesticide-mediated alterations in germ cells (Ngo et al., 2009).

During the last two decades, the relevance of estrogens in the male gonad has been well documented (Carreau and Hess, 2010), suggesting that the androgen/estrogen balance is essential for normal sexual development and reproduction in mammals. The androgen/estrogen balance is controlled by the cytochrome P450 aromatase (aromatase, CYP19 or P450 arom), a key enzyme responsible for the irreversible bioconversion of androgen into estrogens (Simpson et al., 1994). P450 arom levels are crucial in various tissues including gonads (Sipahutar et al., 2003), and are implicated in numerous physiological functions (Simpson et al., 1997). The use of transgenic models has brought us valuable information about the involvement of P450 arom in the molecular mechanisms underlying the androgen/estrogen balance. P450 arom-deficient male mice initially fertile became infertile as the result of a disruption of spermatid production (Robertson et al., 1999). Over-expressing mice P450 arom was characterized by an elevation of estrogen production resulting in both histological and functional abnormalities in male phenotypes (Li et al., 2001). The modifications of the androgen/estrogen balance could induce pathologies such as hormone-dependent cancers for which several classes of P450 arom inhibitors have been designed (Séralini and Moslemi, 2001).

It has been hypothesized that human exposure to environmental xenoestrogens could have adverse effects on reproductive health in adult life (Stillerman et al., 2008; Meeker, 2012). Environmental contaminants could interfere with male reproductive functions consecutively to an alteration of gene expression pertinent for spermatogenesis, for the disturbance of steroidogenesis, the induction of ROS production, or the modification of the BTB integrity (Mathur and D'Cruz, 2011), or for several of these parameters. Effects of GBH as endocrine disruptor has been recently suggested in in vitro cellular models and in vivo studies consecutively to low dose exposure. Glyphosate alone could inactivate aromatase at low doses and its formulations inhibited sex steroid receptors in human placental and embryonic cells in vitro (Richard et al., 2005; Benachour et al., 2007; Gasnier et al., 2009). Until now, most studies have been carried out at low doses. Globally, the main consequences of GBH chronic exposures on reproductive function consisted of a modification of the androgen/estrogen balance and a disruption of the testicular structure (Dallegrave et al., 2007; Oliveira et al., 2007; Romano et al., 2009, 2011). A chronic treatment with a sublethal concentration of GBH in mature male New Zealand white rabbits resulted in a decrease of body weight and an alteration of sperm parameters (Yousef et al., 1995). Subchronic studies in F344 male rats treated orally with glyphosate at a very high dose of 25,000 ppm revealed a significant decrease in epididymal sperm counts (Chan and Mahler, 1992). Concerning the impact of an acute and transitory exposure of GBH, studies about reproductive functions are scarce. However, acute exposures of farmers during field treatments are frequent.

It has become imperative to reinvestigate and validate the reproductive effects of an acute exposure to GBH and to delineate the possible mechanisms implicated in the regulation of the androgen/estrogen balance (estrogen receptors ESR1 and ESR2, androgen receptor, Gper1 and P450 arom) at a molecular level in testis and epididymal sperm at different times after the end of treatment: immediately after the treatment (d68), after one cycle of spermiogenesis (19 days, d87) and after one cycle of spermatogenesis (54 days, d122). As described for Bisphenol A, we have also investigated molecular markers of the blood–testis barrier integrity (connexin-43, occludin, claudin and N-cadherin), Sertoli cell junctional proteins being important for spermatogenesis.

2. Materials and methods

2.1. Chemicals

The environmental product used for this study was Roundup Grand Travaux Plus (GT$^+$, approval 2020448, Monsanto), a commercial formulation of the GBH, composed of 450 g/L glyphosate, 607 g/L isopropylamine salt and adjuvants such as Polyoxyethylamine (POEA).

2.2. Animals and experimental design

Thirty sexually mature 60-days-old Sprague-Dawley (SD) male rats (Janvier, Le Genest Saint Isle, France) were fed and housed under standard conditions (photoperiod of a 12 h dark/light cycle and controlled room temperature) in the CURBE department (University Center of Biological Experimental Resources, Caen, France). All the procedures were performed in accordance to the French Government Regulations (Veterinary Health and Animal Protection, Ministry of Agriculture). Each group was randomized and animals had access to plain water and standard diet. Fifteen male SD rats were subjected to an acute exposure of GBH at a 0.5% dose, similar to those found in water after agricultural practices. GBH was diluted in a deionized water suspension and administered in drinking water for a short period (8 days from postnatal day (PND) 60–68) (GBH+). The water's consumption was followed every 2 days for one week before the experiment and during the protocol period. The fifteen untreated rats used as controls (GBH−) were fed and housed in the same conditions but with deionized water without added Roundup. Five GBH+ rats and five GBH− rats were systematically euthanized at three different periods after the end of treatment: immediately after the treatment (d68), after one cycle of spermiogenesis (19 days after treatment, d87 in our experiment) and after one cycle of spermatogenesis (54 days after treatment, d122 in our experiment).

2.3. Tissue collection and organ weights

Testes and epididymes were removed and weighed. Absolute weights and relative weights of testes and epididymis reported

to total body weight (mg/100 g of body weight) were recorded. After removal of the tunica albuginea, the left testes were cut into four parts before being frozen in liquid nitrogen and stored at –80 °C until RNA extraction. The entire right testes were conserved overnight in 4% paraformaldehyde, and then embedded in paraffin blocks prior to immunohistochemical examination.

The bilateral epididymal sperm was collected by cutting the cauda region of the epididymis into small pieces in IVF™ medium (In Vitro Fertilisation medium, Vitrolife) at 35 °C during 10 min and sperm parameters were analyzed. In order to obtain a pure suspension of spermatozoa necessary for RNA extraction, a selective migration of motile spermatozoa was realized for 15 min at 35 °C. Taking into account a study showing the adverse effects of pipetting and centrifugation repetitions on sperm motility (Varisli et al., 2009), sperm migration was performed using a liquid bridge between a drop containing epididymal sperm (A) and a drop of IVF™ medium (B) resulting in a lateral and selective motile sperm migration. After incubation, 200 μL of supernatant (B) was retrieved for the study sperm parameters and the extraction of sperm mRNA.

2.4. *Immunohistochemistry*

After deparaffinization in toluene, the sections (3 μm) were rehydrated in a series of ethanol grades. Antigen retrieval was carried out by heating sections in citrate buffer (pH 6.0) at 100 °C for 20 min. The slides were saturated with a Tween-PBS solution (T-PBS) containing 5% of *bovine serum albumin* (BSA) for 30 min at room temperature. Sections were incubated overnight at 4 °C with the primary antibody directed against P450 arom (1:200) (P450 arom (C-16): sc-14245, Santa Cruz Biotechnology Inc.) and washed with T-PBS. Slides were incubated with a biotinylated goat anti-mouse secondary antibody during 15 min, washed with T-PBS and incubated for 15 min with a streptavidin horseradish peroxidase complex (Dako LSAB + System HRP, ref. K0690). Immunostaining was visualized using 3,3,9-diaminobenzidene tetra-hydrochloride (Dako Liquid DAB + Substrat Chromogen system, ref. K3467). Sections were counterstained with hematoxylin and observed under a Zeiss microscope (magnification of 400×). The stained images were captured by a ScanScope® CS scanner and analyzed with ImageScope. The same procedure was carried out with the negative control characterized by the absence of the anti-aromatase primary antibody.

2.5. *mRNA expression in testicular and sperm samples*

Total RNAs from purified epididymal sperm fraction and testicular pulp were extracted using the TRI Reagent solution (Rio et al., 2010) according to a method derived from Chomczynski and Sacchi (1987). Real-time PCR was performed. Reverse transcription has been classically realized either with 250 ng of total RNA for testicular pulp or 400 ng of total RNA for spermatozoa. The polymerase chain reaction was performed on cDNA using the GoTaq® qPCR Master Mix (Promega) method. Different markers were studied (Table 1):

Table 1 – Primers used in this work for real-time PCR analysis. Primers are indicated forward (F) and reverse (R) ways. PCR efficiency, determined for each set of primers was comprised between 94.7 and 102.1%.

Rat gene	Primer sequence	GenBank accession number
Rpl19	**F** 5′ AGT ATG CTT AGG CTA CAG AA 3′ **R** 5′ TCC CTT AGA CCT GCT TGG TC 3′	NM_031103
Aromatase (P450arom)	**F** 5′ CGT CAT GTT GCT TCT CAT CG 3′ **R** 5′ TAC CGC AGG CTC TCG TTA AT 3′	M33986.1
Estrogen receptor 1 (ESRl/ERα)	**F** 5′ AAT TCT GAC AAT CGA CGC CAG 3′ **R** 5′ GTG CTT CAA CAT TCT CCC TCC TC 3′	X61098
Estrogen receptor 2 (ESR2/ERβ)	**F** 5′ CTT GCC CAC TTG GAA ACA TC 3′ **R** 5′ CCA AAG GTT GAT TTT ATG GCC 3′	U57439
Androgen receptor (Ar)	**F** 5′ TGG GGA CAT GCG TTT GGA CAG T 3′ **R** 5′ GCT GCC ACA AGT GAG AGC TCC G 3′	NM_012502.1
Gper1	**F** 5′ CCC TTG ACA GGC CAC ATA GT 3′ **R** 5′ CTC CGT GCT GTC TGG TAT GA 3′	NM_133573
Caveolin 1 (Cav1)	**F** 5′ AGT TGA TCT CTG CGC TTG GT 3′ **R** 5′ TGG TGA GGG GTT AGC GTA AG 3′	NM_031556
Occludin (Ocln)	**F** 5′ CAC GTT CGA CCA ATG C 3′ **R** 5′ CCC GTT CCA TAG GCT C 3′	NM_031329.2
Connexin 43 (Gja1)	**F** 5′ TCC TTG GTG TCT CTC GCT TT 3′ **R** 5′ GAG CAG CCA TTG AAG TAG GC 3′	NM_012567.2
Claudin-l (Cldn1)	**F** 5′ AGG TCT GGC GAC ATT AGT GC 3′ **R** 5′ TGG TGT TGG GTA AGA GGT TG 3′	NM_031699.2
N-Cadherin (Cdh2)	**F** 5′ CAG AGA GTC GCCAAATGTCA 3′ **R** 5′ TTC ACA AGT CTC GGC CTC TT 3′	NM_031333.1
Vimentin (Vim)	**F** 5′ AGA TCG ATG TGG ACG TTT CC 3′ **R** 5′ CAC CTG TCT CCG GTA TTC GT 3′	NM_031140.1
Hsd3b1	**F** 5′ AGG TCC TGA TGC CCT CTT TT 3′ **R** 5′ TGT CAT TGC TGA AGC CTT TG 3′	NM_001007719
Protamine 1 (Prm1)	**F** 5′ AGC AAA AGC AGG AGC AGA TG 3′ **R** 5′ AGA TGT GGC GAG ATG CTC TT 3′	NM_001002850.1
Histone 1 (H1t)	**F** 5′ ATC TGC TTC TGC CAA GGC TA 3′ **R** 5′ TCT TCC TGC TGC CTT CCT TA 3′	NM_012579.1

Table 2 – Organ weights after an acute exposure of GBH in control (GBH−) and treated rats (GBH+).

Organ weights (g)	d68		d122	
	GBH−	GBH+	GBH−	GBH+
Body (BW)	525 ± 2	484 ± 13	690 ± 24	670 ± 15
Testis	1.80 ± 0.05	1.86 ± 0.02	1.98 ± 0.05	1.89 ± 0.04
Epididymis	0.76 ± 0.05	0.79 ± 0.03	0.83 ± 0.01	0.81 ± 0.02
Testis weight/l00 g BW	0.34 ± 0.01	038 ± 0.01	0.28 ± 0.01	0.28 ± 0.01
Epididymis weight/100 g BW	0.14 ± 0.01	0.16 ± 0.01	0.12 ± 0.00	0.12 ± 0.00

Results are expressed as mean ± SEM and compared with controls at d68 and d122 between GBH− and GBH+. p values are not significant in any case.

(i) estrogenic elements: P450 arom, ESR1 (ERα), ESR2 (ERß), androgen receptor (Ar), a membrane receptor Gper1 and caveolin-1; (ii) blood–testis barrier markers (occludin, connexin 43, N-cadherin and claudin-1); (iii) somatic cell markers: vimentin (Sertoli cell) and 3-beta-hydroxysteroid dehydrogenase (Hsd3b1, Leydig cell); and (iv) nuclear quality markers: protamine 1 (Prm1) (spermatid and spermatozoa) and testis-specific histone 1 (H1t) (pachytene spermatocyte). For all abbreviations, see Table 1. The PCR was performed as follows in thermocycler Stratagene Mx3005P (Agilent Technologies): an initial step at 95 °C for 3 min, then 40 cycles of 30 s at 95 °C and 60 °C for 60 s. At the end of the amplification, an increase of 0.5 °C every 10 s from 50 to 95 °C was allowed to obtain the melting curve. PCR efficiency, determined for each set of primers using a range of dilutions of reverse transcription (RT) products (1:5, 1:10, 1:20, 1:50, 1:100), was comprised between 94.7 and 102.1%. Relative mRNA levels were determined with the formula ($2^{\wedge(Ct\ gene - Ct\ ref)}$) and normalized to the housekeeping gene Rpl19 (a ribosomal protein gene) (Table 1) (Tena-Sempere et al., 2002). The average "threshold cycle" (Ct) number used for the expression of the housekeeping gene Rpl19 by real-time PCR was similar in each group (20/21 Ct). The evaluation of Ct Rpl19 in treated groups (vs. control group) was at d68 20.97 ± 0.20 (vs. 20.48 ± 0.07), at d87 21.19 ± 0.09 (vs. 20.65 ± 0.11) and at d122 21.10 ± 0.15 (vs. 20.43 ± 0.07). This phenomenon was also confirmed in vitro by Clair et al. (2012). Thus Rpl19 mRNA is a reliable marker as a constant to measure Roundup treatment effects.

2.6. Epididymal sperm parameters

For the determination of sperm concentration, an aliquot of epididymal sperm was diluted (1/50) in a fixative suspension, and then loaded in a haemocytometer chamber. Spermatozoa were then counted under a light microscope (400×) and expressed as 10^6 spermatozoa/mL. Sperm motility was expressed as a percentage of progressive motile spermatozoa. 20 μL of sperm suspension was placed on a microscope slide and covered with a coverslip. About 200 spermatozoa were counted under a light microscope (200×). For sperm viability, 10 μL of epididymal sperm was mixed with 10 μL of 0.5% eosin solution and examined after 30 s under a light microscope (400×). About 200 sperm were counted and the percentage of unstained (viable) sperm was recorded. One hundred morphologically normal and abnormal spermatozoa were also recorded according to the presence or absence of either head or tail defects found with a Zeiss microscope (1000×).

2.7. Statistical analysis

All data are presented as mean ± standard error (SEM). Considering the 3 analysis periods and the molecular parameters studied, means were compared between GBH+ and GBH− rats. Statistically significant differences from controls were determined by a Mann–Whitney test with $p < 0.005$ (***), $p < 0.01$ (**) and $p < 0.05$ (*).

3. Results

3.1. Organ weights

Testes and epididymes were regularly weighted and results were shown in Table 2. No significant changes in the absolute or relative weights of testes or epididymis were observed.

3.2. Changes in mRNA expression

In order to understand the testicular targets of GBH, we have studied molecular markers, which were either involved in the regulation of androgen/estrogen balance (P450 arom, estrogen receptors ESR1 and ESR2, androgen receptor and Gper1) or in the BTB integrity (occludin, connexin 43, claudin and N-cadherin). In Fig. 1, we have only selected the molecular markers whose expression varied between treated and untreated rats. Among molecular markers implicated in the androgen/estrogen balance, the main element disturbed was the P450 arom transcript, which increased significantly in treated rats at d68 (+53%, $p = 0.05$), d87 (+59%, $p < 0.01$) and d122 (+52%, $p < 0.05$) (Fig. 1A). The mRNAs of genes encoding for estrogen and androgen receptors were not affected, but a differential expression of Gper1 was reported at d122 (+59%, $p < 0.05$) (Fig. 1B).

For the study of BTB integrity, an elevated level of transcripts encoding for occludin was observed significantly at d87 (34%; $p < 0.05$) and no quite significantly at d122 (50%; $p = 0.06$) in treated rats (Fig. 1C). Connexin 43 mRNA levels seem to be increased but no quite significantly at d87 (58%; $p = 0.06$) and not significantly at d122 (41%; Ns) (Fig. 1D).

3.3. P450 arom mRNA and immunohistology

P450 arom is expressed in all testicular cells of the adult male rat (excepted peritubular cells), especially in Leydig cells and germ cells. The genes encoding for testicular cells markers used are: protamine 1 (Prm1) for post-meiotic cells (Klemm

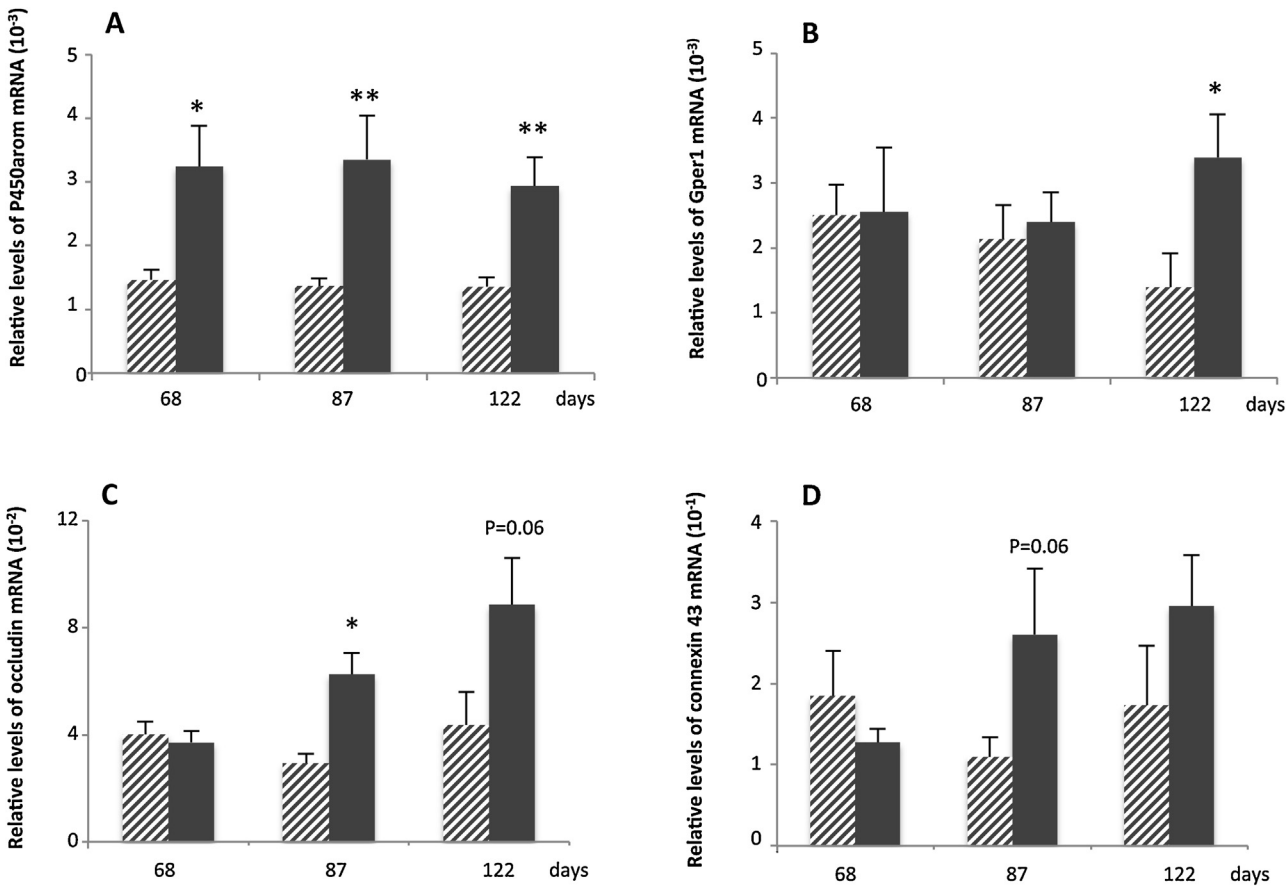

Fig. 1 – Effects of the glyphosate-based herbicide on the expression of molecular markers after an acute exposure. All molecular markers (Table 1) are studied in duplicate by qRT-PCR. The genes presenting a differential mRNA expression are: aromatase (A) (d68: 53% $p = 0.05$; d87: 59% $p < 0.01$; d122: 52% $p < 0.05$), Gper1 (B) (d68: 4% Ns; d87: 14.3% Ns; d122: 59% $p < 0.05$), occludin (C) (d68: −7.5% Ns; d87: 34% $p < 0.05$; d122: 50% Ns) and connexin 43 (D) (d68: −30.8% Ns; d87: 58% Ns; d122: 41% Ns). No significant variations of mRNAs expression for estrogen and androgen receptors were observed (ESR1 d68: −16.6%, d87: 3% and d122: −10%; ESR2 d68: 28.6%, d87: −26.2% and d122: -12.4%; Ar d68: −12.7%, d87: 11.8% and d122: −14.6%). The housekeeping gene is Rpl19. Results (GBH+, black bars) are expressed as mean ± SEM in comparison to controls (GBH−, hatched bars) at d68, d87 and d122. p values are determined by Mann–Whitney tests, $^{*}p < 0.05$ and $^{**}p < 0.01$ are considered as significant.

et al., 1989), vimentin for Sertoli cells (Bussemakers et al., 1992), Hsd3b1 for Leydig cells (Kostic et al., 2011), and testis-specific histone 1 (H1t) for pachytene spermatocytes (Sarg et al., 2009). No significant variation of the expression of specific testicular cell was found between control and treated rats at the 3 periods (data not shown). In order to understand the origin of P450 arom mRNA increase in testicular tissue, we have analyzed its relative expression related to each specific marker of testicular cells mRNA cited above. The ratios P450 arom/specific testicular markers mRNAs were significantly increased in treated rats compared to controls and were more elevated at d87 than d122 (Fig. 2). We noted an exception for the ratio P450 arom mRNA/Hsd3b1 mRNA, which remains not significantly elevated at d122. In order to localize the cell origin of the higher P450 arom expression in treated rats, we have explored the immunolocalization of the protein at d87 in treated rats compared to controls (Fig. 3). A more intensive staining of P450 arom protein was detected in all testicular cells of treated rats as well as in spermatid as Leydig cell as spermatocyte pachytene.

3.4. *Sperm parameters*

Sperm concentration, motility and viability have been studied before and after migration (Table 3). The crude sperm concentration before migration initially at $82 \pm 9\ 10^6\ mL^{-1}$ in controls at d68 presented a tendency to increase (23%; Ns) at d122, while it remained stable in GBH+ rats at d68, d87 and d122 (87 ± 5–$87 \pm 8\ 10^6\ mL^{-1}$). No modifications in crude sperm motility and viability were noted. No changes in sperm parameters (concentration, motility and viability) were noticed in the suspension of migrated sperm. By contrast, we have observed a significant decrease in normal sperm morphology in treated rats at d87 (−20%; $p = 0.001$) and d122 (−11%; $p < 0.05$).

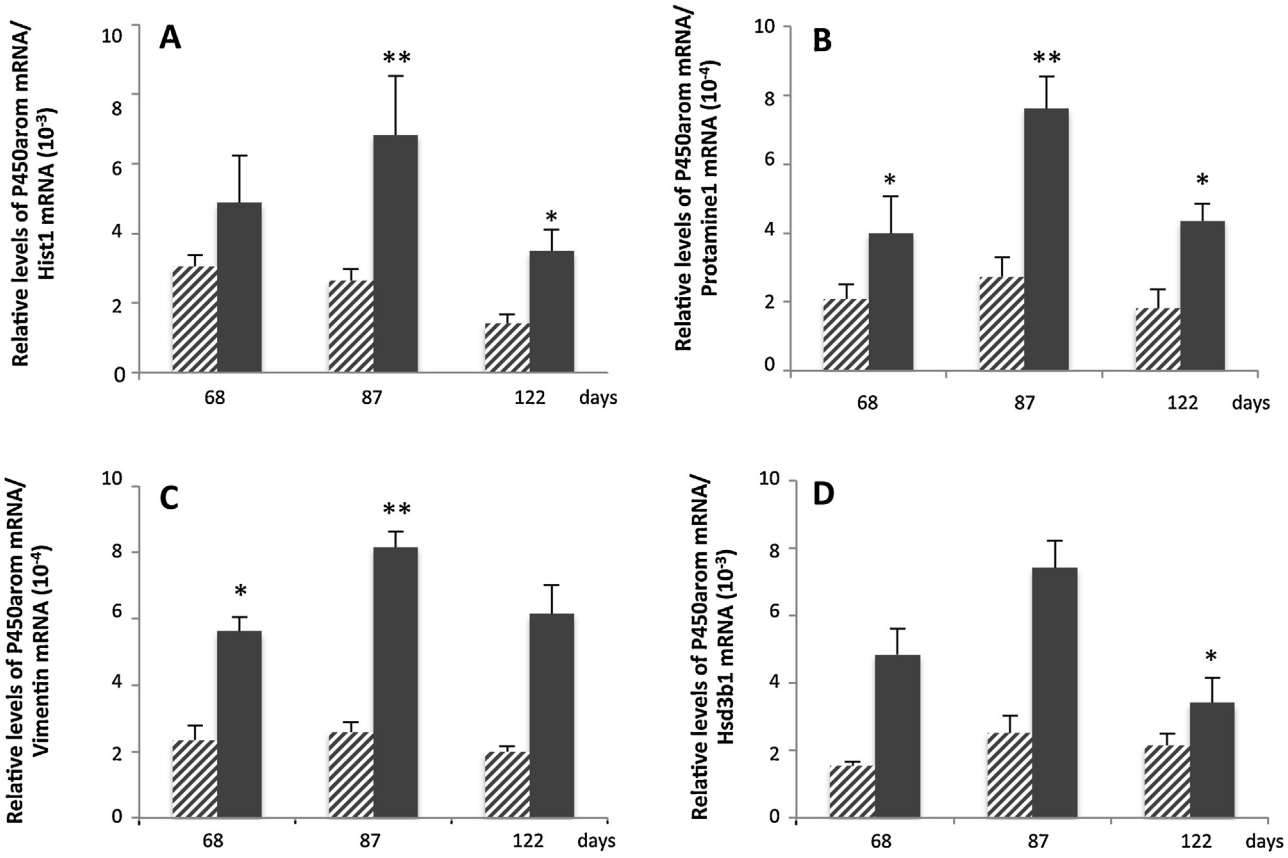

Fig. 2 – Effects of the glyphosate-based herbicide on the relative levels of aromatase mRNA related to each cell specific marker mRNA. (A) In post-meiotic cells, (B) in pachytene spermatocytes, (C) in Sertoli cells and (D) in Leydig cells. Results (GBH+, black bars) are expressed as mean ± SEM in comparison to controls (GBH, hatched bars) at d68, d87 and d122. *p* values are determined by Mann–Whitney tests, $^{*}p < 0.05$ and $^{**}p < 0.01$ are considered as significant.

3.5. *Expression of genes in epididymal spermatozoa*

All molecular markers studied in testicular tissue were tested in migrated epididymal sperm. Only a few of them have shown a significantly differential expression at d87 and/or d122, particularly H1t and Prm1, reflecting the chromatin status in mature sperm (Fig. 4). The analysis of H1t mRNA showed a decrease of 91% and 63%, respectively, at d68 and d87 and a significant difference in treated animals at d122 ($p < 0.01$). The Prm1 expression has shown a decrease of 89% at d68 and was also significantly affected at d122 ($p < 0.05$).

4. Discussion

For the first time, we have studied the modifications of gene expression encoding for testicular molecules implicated either in the regulation of the androgen/estrogen balance or in the BTB integrity after an acute exposure of GBH in adult male rats. This study was realized at three different periods following the end of GBH treatment: immediately (d68), after one cycle of spermiogenesis (d87) and spermatogenesis (d122) thus constituting the second originality of this work. We note

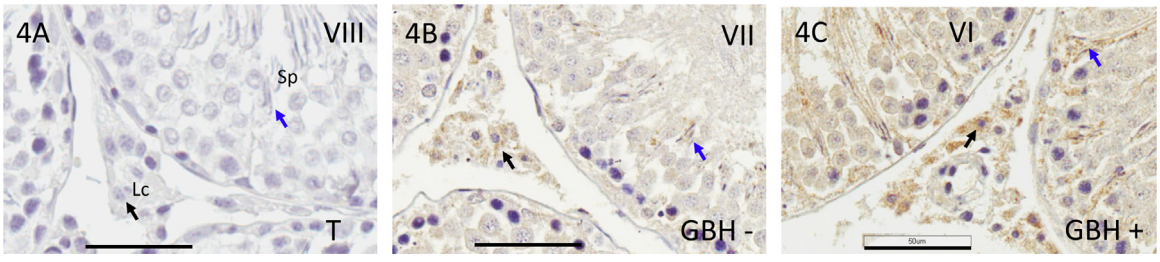

Fig. 3 – Aromatase (P450 arom) immunolocalization after glyphosate-based herbicide treatment at d87 in the seminiferous epithelium of adult rat testes. Immunostaining with an anti-aromatase antibody (1:200) (as indicated by arrows): negative control (T), no primary Ab (A); GBH−, control group (B); GBH+, treated group (C). Magnification 400×, bar in black: 50 μm. Sp, spermatids; Lc, leydig cells.

Table 3 – Epididymal sperm parameters in controls and rats exposed to glyphosate-based herbicide.

	Sperm parameters	d68			d87			d122		
		GBH−	GBH+	*p* value	GBH−	GBH+	*p* value	GBH−	GBH+	*p* value
Before selection	Concentration (10^6 mL^{-1})	82 ± 9	87 ± 5	Ns	95 ± 12	87 ± 5	Ns	101 ± 9	87 ± 8	Ns
	Motility (%)	57 ± 3	58 ± 2	Ns	59 ± 4	61 ± 2	Ns	61 ± 4	61 ± 2	Ns
	Vitality (%)	77 ± 2	79 ± 1	Ns	84 ± 2	81 ± 12	Ns	81 ± 1	83 ± 2	Ns
	Total motile sperm number (10^6)	22 ± 3	26 ± 2	Ns	28 ± 3	27 ± 2	Ns	31 ± 3	26 ± 2	Ns
After selection	Concentration (10^6 mL^{-1})	16 ± 2	22 ± 2	Ns	8 ± 1	11 ± 1	Ns	15 ± 2	12 ± 2	Ns
	Motility (%)	48 ± 1	47 ± 1	Ns	43 ± 1	37 ± 4	Ns	51 ± 4	47 ± 4	Ns
	Vitality (%)	81 ± 2	76 ± 2	Ns	80 ± 2	81 ± 2	Ns	84 ± 2	79 ± 6	Ns
	Morphology (% normal forms)	89 ± 3	90 ± 1	Ns	91 ± 1	73 ± 3	$^{**}p = 0.001$	84 ± 3	75 ± 2	$^{*}p = 0.043$
	Total motile sperm number (10^6)	1.8 ± 0.1	2.5 ± 0.3	Ns	0.7 ± 0.1	0.8 ± 0.1	Ns	1.5 ± 0.2	1.2 ± 0.2	Ns

Sperm concentration, motility, vitality and morphology are expressed as mean ± SEM in comparison to controls before and after a selective migration at d68, d87 and d122 between GBH− and GBH+. *p* values are determined by Mann–Whitney tests. Ns, not significant.

* $p < 0.05$ is considered as significant.

** $p < 0.01$ is considered as significant.

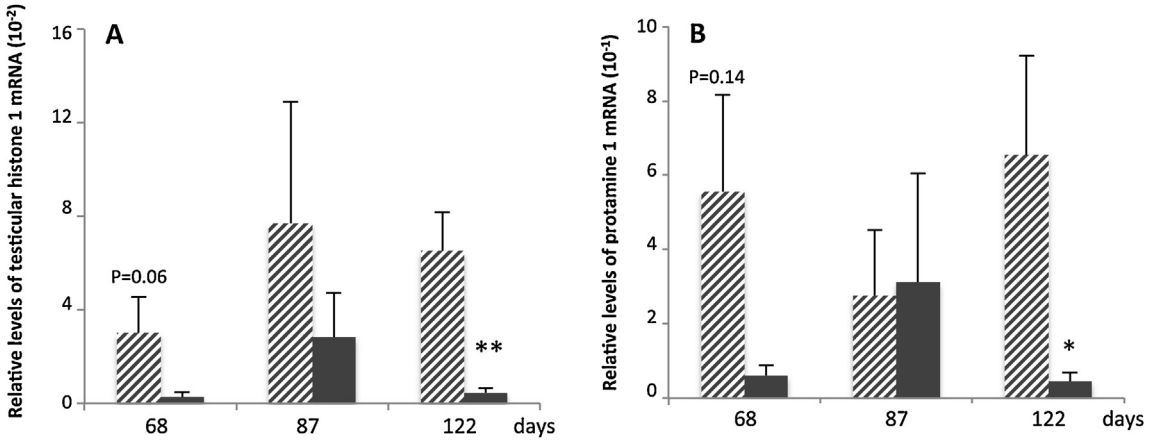

Fig. 4 – Effects of glyphosate-based herbicide Roundup on mRNA expression of nuclear markers in epididymal spermatozoa. All molecular markers studied in duplicate by qRT-PCR (Table 1) are invariant to controls (GBH−, hatched bars) except testis-specific histone 1 (H1t) and protamine 1 (Prm1). Specific mRNAs of H1t (A) and Prm1 (B) are reported to housekeeping Rpl19 and expressed as mean ± SEM at d68, 87 and 122. *p* values are determined by Mann–Whitney tests, $^{*}p < 0.05$ and $^{**}p < 0.01$ are considered as significant.

first that the specific testicular mRNA markers used remain at constant levels; this is unchanged after GBH treatment. It indicates that we are not in the toxic range and thus we can study the endocrine disruption. The main disturbance observed is the significant elevation of P450 arom mRNA levels detected immediately at d68 and reaching a maximal value at d87. In our study, the interstitial tissue did not appear to be modified and whatever the cell type considered, the increased expression of P450 arom transcript and protein seemed to concern all type of testicular cells whereas no change of the expression of genes encoding for classical estrogen or androgen receptors was found. Among the other molecular markers studied those of Gper1 at d122, those of connexin 43 and occludin at d87 and d122 seem to be disturbed. Parallel studies on the expression of testicular genes and on the sperm quality have been carried out. Our study reported no differences in sperm concentration, viability and mobility but only an increase of abnormal sperm morphology at d87 and d122. The molecular impact of an acute exposure on spermatozoa has never been explored before. Our results show a significant and differential expression of H1t and Prm1 between treated and untreated rats, suggesting changes in nuclear maturity during spermiogenesis.

In our experiment, an acute dose of GBH disrupted rapidly at d68 the P450 arom expression in testes of adult male rats. Few in vitro and in vivo studies have already reported that GBH could act as a chemical endocrine disruptor by disturbing the androgen/estrogen balance. Adult drake mallards (Anas platyrhyncos) exposed to GBH presented alterations of the serum levels of testosterone associated with changes in the expression of androgen receptors restricted to the testis (Oliveira et al., 2007). According to the period of GBH exposure in rats, serum testosterone levels have been found to be increased i.e. during perinatal period (Romano et al., 2011) or decreased: pregnancy and lactation (Dallegrave et al., 2007) and prepubertal exposure (Romano et al., 2009). Serum estradiol levels have been sparsely studied and seem to be less affected: reduced concentration only at low doses in drakes (Oliveira et al., 2007) and higher level in perinatal exposure in rats (Romano et al., 2011). The variation of steroid hormone level could be explained by a modification of the Star protein

(Walsh et al., 2000) or P450 arom expressions. In in vitro cellular models (JEG 3 and 293 cells), the P450 arom activity seems to be increased or decreased according to the time of GBH exposure (1 h or 24 h) (Richard et al., 2005). Our study is the first one in vivo showing a rapid increase of mRNA and protein P450 arom expression immediately after an acute exposure of GBH probably inducing a transient modification in the androgen/estrogen balance. Recently in adult rats, it has been reported that 0.1 ppb of GBH inverts the androgen/estrogen ratio after one year exposure (Séralini et al., 2013). This data seems to be in agreement with the work of Romano et al. (2011): the effects of gestational maternal exposure induces an increase of serum LH, testosterone and estradiol concentrations in 60-day-old male offspring and consequently an increase of P450 arom activity (Romano et al., 2011). We could postulate that a repetition of acute exposure could at long-term disrupt the androgen/estrogen balance in adult and thus severely delaying the process of spermatogenesis.

The P450 arom enzyme, which is mainly localized in Leydig cells and post-meiotic cells, represents the main source of estrogen in adult rats (Carreau and Hess, 2010). Estrogens interact with classical nuclear estrogen receptors (ESR1 and/or ESR2) and induce the activation of signaling pathways controlling spermatogenesis (Edwards, 2005). But estrogens can bind other receptors such as a G protein-coupled seven transmembrane spanning receptor called GPR30/Gper1, rapidly and directly activating signaling pathways that modulate the spermatogenic process. In rats, expression of Gper1 is detected in pachytene spermatocytes (Chimento et al., 2010) and also in round spermatids (Chimento et al., 2011). Gper1 can influence genes involved in the balance between cellular proliferation/differentiation and apoptosis (Lucas et al., 2010; Chimento et al., 2012). In GC-2 cells, an immortalized mouse pachytene spermatocyte-derived cell line, the ESR1 and Gper1 activation results in the activation of the EGFR/ERK/c-jun pathway and a bax up-regulation associated with apoptosis mechanism (Chimento et al., 2012). In immature rats Sertoli cells, activation of Gper1 by 17β-estradiol (E2) induces rapid signaling pathway via phosphorylation of mitogen-activated protein kinase 3/1 and modulation gene expression involved with apoptosis (Lucas et al., 2010, 2011; Royer et al., 2012). Based on similar chemical structure, xenoestrogens may mimic such nongenomic actions of estrogens. Recently, a study has shown that the monobutyl phtalate (MBP), active monoester metabolite of Di(*n*-butyl) phtalate, potentially interfere with the estrogen action through Gper1 in Sertoli cells by stimulating its gene expression (Hu et al., 2013). These results illustrate that Gper1 may be regarded as a sensitive biochemical marker for observing the rapid response of testicular cells to environmental estrogens and particularly GBH.

An acute exposure of GBH could also alter the BTB integrity as shown in our study. Sertoli cells junctional proteins of tight (occludin and zonula occludens-1), anchoring (N-cadherin) and gap (connexin 43) junctions play a pivotal role in the maintenance of spermatogenesis and spermiogenesis. It was reported that the Sertoli cell junction at the BTB acts as an early target of environmental testicular toxicants, including BPA using a SerW3 Sertoli cell line (Fiorini et al., 2004). A neonatal exposure of BPA impairs the expression of Sertoli cell junctional proteins in the testes of adult rats (Salian et al., 2009). BPA has also been described as increasing the testicular ROS production by down-regulation of the anti-oxidative enzyme, it can also modify the BTB integrity by disruption of cellular junctions via the metabolic pathway PI3K/c-Src/FAK (Wong and Cheng, 2011). We can imagine a negative impact of high dose of GBH on the BTB integrity but further study must be realized in order to confirm this hypothesis.

The mechanism of action of GBH is now confirmed in vivo. If we knew before that the Roundup was inhibitor of the estrogen dependent pathway, showing an inhibition on P450 arom and estrogen and androgen receptors (Gasnier et al., 2009), the in vivo action appears to be a membrane signaling pathway. Only Gper1 mRNA level has been changed and not the nuclear steroid receptors, and the P450 arom mRNA has been increased to some level, possibly as a retroaction. Only the P450 arom mRNA has been disturbed rapidly and significantly after Roundup treatment and for all periods. This confirms the endocrine disrupting effect of Roundup in vivo, and indicate that the membrane is reached to some extent like it was already demonstrated in vitro on adenylate kinase (Clair et al., 2012) and the mitochondrial succinate dehydrogenase effects (for mitochondria) at higher doses (Gasnier et al., 2009).

Our study reported no difference in sperm concentration, viability and mobility but only an increase of abnormal sperm morphology at d87 and 122. With very high dose of GBH or G alone, the major effects observed are an alteration of sperm parameters (Yousef et al., 1995; Chan and Mahler, 1992). Adult Wistar rats exposed in utero to a chronic dose of 50 ppm/day presented an impairment of spermatogenesis (Dallegrave et al., 2007). The molecular impact on spermatozoa has never been explored before. Our results show a significant and differential expression of H1t and Prm1 between treated and untreated rats, suggesting changes in nuclear maturity during spermiogenesis. Protamines confer a high order of DNA packaging in sperm necessary for normal sperm function (Carrell et al., 2007). An immature chromatin (unprocessed nuclear proteins and/or poorly condensed chromatin) has been shown to reduce capacity to fertilize oocytes or, in the event of fertilization, may be responsible for defective early implantation (Oliva, 2006).

Considering the acute exposure of farm workers each year and the increasing use of GBH to cultivate GMOs, the investigation of the reproductive effects in terms of molecular targets in testis and sperm is crucial. We conclude that an acute exposure of GBH causes molecular changes in the reproductive function. In fact, changes in P450 arom (mRNA and protein) during the entire experimental period could interfere with the androgen/estrogen balance. This increase of P450 arom suggests a rise in estradiol, which acts via the receptors used the nongenomic pathway such as Gper1. Moreover, the increase of occludin and connexin 43 expression involves probably a modification of BTB integrity leading to alteration of both sperm morphology and nuclear quality. These modifications appear maximum at d87 and decrease afterwards. These transient effects must be confirmed on a larger sample and a longer recovery period covering two cycles of spermatogenesis following a GBH exposure. Consequences are that sperm production is unaffected but the modifications observed in the expression of nuclear quality markers raise questions about

sperm efficiency. We can hypothesize that the repetition of successive exposures of GBH at sub-agricultural doses could alter the mammalian reproductive system over the long term.

Authors' contributions

ECM carried out sperm parameters, immunohistological and molecular studies. SG carried out the animal protocol and molecular studies. GES directed the protocol, the fund raising and the final version of manuscript. IGD co-directed the research. All authors read and approved the final manuscript. All authors contributed equally to the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Transparency document

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

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REFERENCES

Acquavella, J.F., Alexander, B.H., Mandel, J.S., Gustin, C., Baker, B., Chapman, P., Bleeke, M., 2003. Glyphosate biomonitoring for farmers and their families: results from the farm family exposure study. Environ. Health Perspect. 112, 321–326, http://dx.doi.org/10.1289/ehp.6667.

Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Séralini, G.E., 2007. Time- and dose-dependent effects of roundup on human embryonic and placental cells. Arch. Environ. Contam. Toxicol. 53, 126–133, http://dx.doi.org/10.1007/s00244-006-0154-8.

Bussemakers, M.J., Verhaegh, G.W., van Bokhoven, A., Debruyne, F.M., Schalken, J.A., 1992. Differential expression of vimentin in rat prostatic tumors. Biochem. Biophys. Res. Commun. 182, 1254–1259.

Carreau, S., Hess, R.A., 2010. Oestrogens and spermatogenesis. Philos. Trans. R. Soc. B: Biol. Sci. 365, 1517–1535, http://dx.doi.org/10.1098/rstb.2009.0235.

Carrell, D.T., Emery, B.R., Hammoud, S., 2007. Altered protamine expression and diminished spermatogenesis: what is the link? Hum. Reprod. Update 13, 313–327, http://dx.doi.org/10.1093/humupd/dml057.

Chan, P., Mahler, J., 1992. NTP technical report on the toxicity studies of glyphosate (CAS No 1071-83-6) administered in dosed feed to F344/N rats and B6C3F1 mice. Toxic. Rep. Ser. 16, 1–D3.

Chimento, A., Sirianni, R., Casaburi, I., Ruggiero, C., Maggiolini, M., Andò, S., Pezzi, V., 2012. 17β-Estradiol activates GPER- and ESR1-dependent pathways inducing apoptosis in GC-2 cells, a mouse spermatocyte-derived cell line. Mol. Cell. Endocrinol. 355, 49–59, http://dx.doi.org/10.1016/j.mce.2012.01.017.

Chimento, A., Sirianni, R., Delalande, C., Silandre, D., Bois, C., Andò, S., Maggiolini, M., Carreau, S., Pezzi, V., 2010. 17β-Estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. Mol. Cell. Endocrinol. 320, 136–144.

Chimento, A., Sirianni, R., Zolea, F., Bois, C., Delalande, C., Andò, S., Maggiolini, M., Aquila, S., Carreau, S., Pezzi, V., 2011. Gper and ESRs are expressed in rat round spermatids and mediate oestrogen-dependent rapid pathways modulating expression of cyclin B1 and Bax. Int. J. Androl. 34, 420–429, http://dx.doi.org/10.1111/j.1365-2605.2010.01100.x.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159, http://dx.doi.org/10.1006/abio.1987.9999.

Clair, E., Mesnage, R., Travert, C., Séralini, G.-É., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 26, 269–279, http://dx.doi.org/10.1016/j.tiv.2011.12.009.

Cox, C., 1998. Herbicide factsheet – glyphosate (Roundup). J. Pesticide Reform. 18, 3–15.

Curwin, B.D., Hein, M.J., Sanderson, W.T., Nishioka, M.G., Reynolds, S.J., Waerm, E.M., Alavanja, M.C., 2005. Pesticide contamination inside farm and nonfarm homes. J. Occup. Environ. Hyg. 2, 357–367.

Curwin, B.D., Hein, M.J., Sanderson, W.T., Striley, C., Heederik, D., Kromhout, H., Reynolds, S.J., Alavanja, M.C., 2007. Urinary pesticide concentrations among children, mothers and fathers living in farm and non-farm households in Iowa. Ann. Occup. Hyg. 51, 53–65.

Dallegrave, E., Mantese, F.D., Oliveira, R.T., Andrade, A.J.M., Dalsenter, P.R., Langeloh, A., 2007. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch. Toxicol. 81, 665–673, http://dx.doi.org/10.1007/s00204-006-0170-5.

Edwards, D.P., 2005. Regulation of signal transduction pathways by estrogen and progesterone. Annu. Rev. Physiol. 67, 335–376, http://dx.doi.org/10.1146/annurev.physiol.67.040403.120151.

Fiorini, C., Tilloy-Ellul, A., Chevalier, S., Charuel, C., Pointis, G., 2004. Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. Reprod. Toxicol. 18, 413–421, http://dx.doi.org/10.1016/j.reprotox.2004.01.002.

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.-C., Séralini, G.-E., 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 262, 184–191, http://dx.doi.org/10.1016/j.tox.2009.06.006.

Hu, Y., Li, D., Lu, Y., Han, X., 2013. Monobutyl phthalate induces the expression change of G-Protein-Coupled Receptor 30 in rat testicular Sertoli cells. Folia Histochem. Cytobiol. 51, 18–24, 10.5603/FHC.2013.003.

IFEN, 2007. Report on pesticides in waters. Data 2005.

Klemm, U., Lee, C.H., Burfeind, P., Hake, S., Engel, W., 1989. Nucleotide sequence of a cDNA encoding rat protamine and the haploid expression of the gene during rat spermatogenesis. Biol. Chem. Hoppe. Seyler 370, 293–301.

Kostic, T.S., Stojkov, N.J., Bjelic, M.M., Mihajlovic, A.I., Janjic, M.M., Andric, S.A., 2011. Pharmacological doses of testosterone upregulated androgen receptor and 3-Beta-hydroxysteroid

dehydrogenase/delta-5-delta-4 isomerase and impaired leydig cells steroidogenesis in adult rats. Toxicol. Sci. Off. J. Soc. Toxicol. 121, 397–407, http://dx.doi.org/10.1093/toxsci/kfr063.

Li, X., Nokkala, E., Yan, W., Streng, T., Saarinen, N., Wärri, A., Huhtaniemi, I., Santti, R., Mäkelä, S., Poutanen, M., 2001. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. Endocrinology 142, 2435–2442.

Lucas, T.F.G., Pimenta, M.T., Pisolato, R., Lazari, M.F.M., Porto, C.S., 2011. 17β-Estradiol signaling and regulation of Sertoli cell function. Spermatogenesis 1, 318–324, http://dx.doi.org/10.4161/spmg.1.4.18903.

Lucas, T.F.G., Royer, C., Siu, E.R., Lazari, M.F.M., Porto, C.S., 2010. Expression and signaling of G protein-coupled estrogen receptor 1 (GPER) in rat sertoli cells. Biol. Reprod. 83, 307–317, http://dx.doi.org/10.1095/biolreprod.110.084160.

Mathur, P.P., D'Cruz, S.C., 2011. The effect of environmental contaminants on testicular function. Asian J. Androl. 13, 585–591.

Meeker, J.D., 2012. Exposure to environmental endocrine disruptors and child development. Arch. Pediatr. Adolesc. Med. 166, 952–958.

Mesnage, R., Moesch, C., Le Grand, R., Lauthier, G., Spiroux de Vendômois, J., Gress, S., Séralini, G.-E., 2012. Glyphosate exposure in a farmer's family. J. Environ. Prot. 03, 1001–1003, http://dx.doi.org/10.4236/jep.2012.39115.

Ngo, A.D., Taylor, R., Roberts, C.L., 2009. Paternal exposure to Agent Orange and spina bifida: a meta-analysis. Eur. J. Epidemiol. 25, 37–44, http://dx.doi.org/10.1007/s10654-009-9401-4.

Oliva, R., 2006. Protamines and male infertility. Hum. Reprod. Update 12, 417–435, http://dx.doi.org/10.1093/humupd/dml009.

Oliveira, A.G., Telles, L.F., Hess, R.A., Mahecha, G.A.B., Oliveira, C.A., 2007. Effects of the herbicide Roundup on the epididymal region of drakes Anas platyrhynchos. Reprod. Toxicol. 23, 182–191, http://dx.doi.org/10.1016/j.reprotox.2006.11.004.

Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G.-E., 2005. Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716–720.

Rio, D.C., Ares Jr., M., Hannon, G.J., Nilsen, T.W., 2010. Purification of RNA using TRIzol (TRI reagent). Cold Spring Harb. Protoc 2010, http://dx.doi.org/10.1101/pdb.prot5439, pdb.prot5439.

Robertson, K.M., O'Donnell, L., Jones, M.E.E., Meachem, S.J., Boon, W.C., Fisher, C.R., Graves, K.H., McLachlan, R.I., Simpson, E.R., 1999. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. Proc. Natl. Acad. Sci. 96, 7986–7991.

Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., Souza, P.B., Viau, P., Bernardi, M.M., Nunes, M.T., Oliveira, C.A., 2011. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Arch. Toxicol. 86, 663–673, http://dx.doi.org/10.1007/s00204-011-0788-9.

Romano, R.M., Romano, M.A., Bernardi, M.M., Furtado, P.V., Oliveira, C.A., 2009. Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Arch. Toxicol. 84, 309–317, http://dx.doi.org/10.1007/s00204-009-0494-z.

Royer, C., Lucas, T.F.G., Lazari, M.F.M., Porto, C.S., 2012. 17β-Estradiol signaling and regulation of proliferation and apoptosis of rat sertoli cells. Biol. Reprod. 86, 108, http://dx.doi.org/10.1095/biolreprod.111.096891.

Salian, S., Doshi, T., Vanage, G., 2009. Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis. Toxicology 265, 56–67, http://dx.doi.org/10.1016/j.tox.2009.09.012.

Sarg, B., Chwatal, S., Talasz, H., Lindner, H.H., 2009. Testis-specific linker histone H1t is multiply phosphorylated during spermatogenesis. Identification of phosphorylation sites. J. Biol. Chem. 284, 3610–3618, http://dx.doi.org/10.1074/jbc.M805925200.

Séralini, G.E., Mesnage, R., Defarge, N., Gress, S., Hennequin, D., Clair, E., Malatesta, M., Spiroux de Vendomois, J., 2013. Answers to critics: why there is a long term toxicity due to a Roundup-tolerant genetically modified maize and to a Roundup herbicide. Food Chem. Toxicol. 53, 476–483.

Séralini, G.-E., Moslemi, S., 2001. Aromatase inhibitors: past, present and future. Mol. Cell. Endocrinol. 178, 117–131.

Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., 1994. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15, 342–355.

Simpson, E.R., Zhao, Y., Agarwal, V.R., Michael, M.D., Bulun, S.E., Hinshelwood, M.M., Graham-Lorence, S., Sun, T., Fisher, C.R., Qin, K., Mendelson, C.R., 1997. Aromatase expression in health and disease. Recent Prog. Horm. Res. 52, 185–213, discussion 213-214.

Sipahutar, H., Sourdaine, P., Moslemi, S., Plainfosse, B., Seralini, G.-E., 2003. Immunolocalization of aromatase in stallion leydig cells and seminiferous tubules. J. Histochem. Cytochem. 51, 311–318, http://dx.doi.org/10.1177/002215540305100306.

Stillerman, K.P., Mattison, D.R., Giudice, L.C., Woodruff, T.J., 2008. Environmental exposures and adverse pregnancy outcomes: a review of the science. Reprod. Sci. 15, 631–650.

Tena-Sempere, M., Barreiro, M.L., Gonzalez, L.C., Gaytan, F., Zhang, F.-P., Caminos, J.E., Pinilla, L., Casanueva, F.F., Dieguez, C., Aguilar, E., 2002. Novel expression and functional role of ghrelin in rat testis. Endocrinology 143, 717–725.

Varisli, O., Uguz, C., Agca, C., Agca, Y., 2009. Various physical stress factors on rat sperm motility, integrity of acrosome, and plasma membrane. J. Androl. 30, 75–86, http://dx.doi.org/10.2164/jandrol.107.004333.

Walsh, L.P., McCormick, C., Martin, C., Stocco, D.M., 2000. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environ. Health Perspect. 108, 769.

Wong, E.W.P., Cheng, C.Y., 2011. Impacts of environmental toxicants on male reproductive dysfunction. Trends Pharmacol. Sci. 32, 290–299, http://dx.doi.org/10.1016/j.tips.2011.01.001.

Yousef, M.I., Salem, M.H., Ibrahim, H.Z., Helmi, S., Seehy, M.A., Bertheussen, K., 1995. Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. J. Environ. Sci. Health B 30, 513–534, http://dx.doi.org/10.1080/03601239509372951.