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Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression

Marco Aurelio Romano · Renata Marino Romano · Luciana Dalazen Santos · Patricia Wisniewski · Daniele Antonelo Campos · Paula Bargi de Souza · Priscila Viau · Maria Martha Bernardi · Maria Tereza Nunes · Claudio Alvarenga de Oliveira

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Abstract Sexual differentiation in the brain takes place from late gestation to the early postnatal days. This is dependent on the conversion of circulating testosterone into estradiol by the enzyme aromatase. The glyphosate was shown to alter aromatase activity and decrease serum testosterone concentrations. Thus, the aim of this study was to investigate the effect of gestational maternal glyphosate exposure (50 mg/kg, NOAEL for reproductive toxicity) on the reproductive development of male offspring. Sixty-day-old male rat offspring were evaluated for sexual behavior and partner preference; serum testosterone concentrations, estradiol, FSH and LH; the mRNA and protein content of LH and FSH; sperm production and the morphology of the seminiferous epithelium; and the weight of the testes, epididymis and seminal vesicles. The growth, the weight and age at puberty of the animals were also recorded to evaluate the effect of the treatment. The most important findings were increases in sexual partner preference scores and the latency time to the first mount; testosterone and estradiol serum concentrations; the mRNA expression and protein content in the pituitary gland and the serum concentration of LH; sperm production and reserves; and the height of the germinal epithelium of seminiferous tubules. We also observed an early onset of puberty but no effect on the body growth in these animals. These results suggest that maternal exposure to glyphosate disturbed the masculinization process and promoted behavioral changes and histological and endocrine problems in reproductive parameters. These changes associated with the hypersecretion of androgens increased gonadal activity and sperm production.

Keywords Glyphosate · Endocrine disruptor · Sexual brain differentiation · Sperm production · Gonadotropins · Gonadal steroids

M. A. Romano · P. Viau · C. A. de Oliveira
Department of Animal Reproduction,
Veterinary Medicine School, University of Sao Paulo,
Sao Paulo 05508-270, Brazil

M. A. Romano (✉) · L. D. Santos · P. Wisniewski · D. A. Campos
Department of Pharmacy, State University of Centro-Oeste,
R. Simeao Camargo Varela de Sa, 03, Guarapuava,
PR 85040-080, Brazil
e-mail: maromano17@gmail.com

R. M. Romano · P. B. de Souza · M. T. Nunes
Department of Physiology and Biophysics,
Institute of Biomedical Sciences, University of Sao Paulo,
Sao Paulo 05508-900, Brazil

M. M. Bernardi
Department of Animal Pathology, Veterinary Medicine School,
University of Sao Paulo, Sao Paulo 05508-270, Brazil

Introduction

Glyphosate [N-(phosphonometyl)glycine] is a broad-spectrum herbicide (Hayes and Laws 1991; Cerdeira et al. 2007) that is effective against weeds, especially in association with transgenic glyphosate-resistant crop systems (Cerdeira et al. 2007), and represents approximately 30% of all herbicides used in agriculture (Inoue et al. 2003). Even though glyphosate is considered a low-toxic herbicide, recent studies have revealed toxic effects resulting from even low-dose commercial formulations.

A reduction in aromatase activity was observed in placental and embryonic human cells treated with low concentrations of a commercial formulation of glyphosate, which disrupted the conversion of steroids to estradiol (Benachour et al. 2007; Richard et al. 2005).

Furthermore, a recent study showed a reduction in the expression of androgen and estrogen receptors, the expression and activity of aromatase, and genotoxicity in human liver HepG2 cells exposed to low concentrations of commercial glyphosate (Gasnier et al. 2009; Benachour and Seralini 2009).

The effect of Roundup on the aromatase enzyme was also demonstrated in Leydig MA-10 tumor cell cultures, where a reduction in the expression of the steroidogenic acute regulatory protein (STAR, protein responsible for intracellular cholesterol transport) was also observed (Walsh et al. 2000).

Previous results from our laboratory demonstrated a significant reduction in serum testosterone concentrations and changes in the testicular morphology of male Wistar rats treated with Roundup during the prepubertal period, and these effects were demonstrated with even the lowest dose used in the experiment (5 mg/kg) (Romano et al. 2010). Thus, the herbicide glyphosate may be characterized as a potential endocrine chemical disruptor. Endocrine disruptors were defined by Kavlock et al. (1996) as exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes.

Based on the effects of commercial formulations of glyphosate on aromatase activity and testosterone levels, we aimed to investigate the effect of maternal glyphosate exposure during the perinatal period from gestational day (GD) 18 to postnatal day (PND) 5 on the reproductive development of male offspring. Disturbances during this phase may alter reproductive physiology and behavior (Gerardin et al. 2005; Pereira et al. 2006; Reznikov and Tarasenko 2007; Ward and Weisz 1980).

Sexual differentiation in the brain occurs during the late gestational and early postnatal days. During this period, increased aromatase expression converts circulating testosterone (produced in the fetal testes from GD18 to PND10) into estradiol in the central nervous system, which will eventually determine the gender-specific reproductive endocrinology and behavior in adults (Bakker et al. 2002; Lenz and McCarthy 2010; Phoenix et al. 1959; Sakuma 2009; Weisz and Ward 1980; Wilson and Davies 2007).

Thus, we assessed the sexual behavior of 60-day-old male rat offspring from females treated with glyphosate during the perinatal period. Among other factors, sexual behavior is influenced by hormones, so the serum concentrations of testosterone, estradiol, FSH and LH were measured. The pituitary expression of mRNA and protein content of LH and FSH was also analyzed to assess the possible glyphosate-mediated interference with their production. Changes in sex hormone serum concentrations may also affect sperm production and the morphology of the seminiferous epithelium, which were evaluated by testicular and epididymal sperm counts and the morphometric analysis of histological sections. The weight of the testes, epididymides and the seminal vesicle; the growth of the animals; and the weight and age at puberty were also recorded to evaluate the effect of the treatment on these parameters.

Materials and methods

Chemicals

The herbicide used in this study was a glyphosate-based commercial formulation of Roundup Transorb (Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, Sao Paulo, Brazil). This formulation was composed of 480 g/l of glyphosate, 648 g/l of isopropylamine salt and 594 g/l of inert ingredients.

Several additional products were also obtained for the study: Platinum SYBR green qPCR SuperMix UDG, Oligo dT and dNTPs (Life Technologies, Camarillo, CA, USA); M-MLV Reverse Transcriptase (Promega, Madison, WI, EUA); hybridization nitrocellulose membranes (Millipore, Ireland); LH, FSH and GH antibodies (Dr. A. F. Parlow, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA); GAPDH and secondary peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); BSA, protein molecular weight markers and mercaptoethanol (Life Technologies, Grand Island, NY); an Enhanced Chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK); Total Testosterone and Estradiol kits (Sigma, Solon, OH, USA); Luminex xMAP technology for LH and FSH (Milliplex MAP rat pituitary panel, Millipore, Billerica, MA, USA). All other reagents were purchased from Labsynth (Sao Paulo, SP, Brazil).

Animals, experimental design and treatment

To evaluate the effects of glyphosate male offspring sexual development, twelve 90-day-old female Wistar rats were mated in a monogamous couple, and the beginning of gestation (GD1) was confirmed by vaginal smear containing spermatozoa. Glyphosate Roundup Transorb was diluted in a watery suspension and administered to the mothers once a day, p.o. (gavage), in a volume of 0.25 ml/100 g of body weight between 7 and 8 am from GD18 to PND5. On PND4, the litters were culled to eight pups per female and kept at this proportion until weaning (PND21). At PND 60, the male rats were divided into subgroups so that the animals subjected to sexual partner preference were not subjected to other experiments. During all

experimental periods, the rats were fed with a commercially balanced mixture for rats with water available ad libitum, and they were kept in rooms with a 12:12 h dark/light cycle and a controlled temperature (23 ± 1°C). All procedures were performed according to the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation and approved by the bioethical Commission of Faculty of Veterinary Medicine and Zootechny of University of São Paulo (protocol 2197/2011). The 50 mg/kg dose was chosen based on the NOAEL (No Observed Adverse Effect Level) or NEL (No Effect Level) for glyphosate (Lu 1995) and from the results of our previous study (Romano et al. 2010). The control group was treated in the same manner with deionized water.

Sexual partner preference

The sexual partner preference was performed at PND60 according to a procedure previously described (Dahlgren et al. 1991). Briefly, a circular apparatus was used with two cages positioned on opposite sides where the stimulus animals were kept (a gonad-intact sexually mature male and a female in induced estrous). The zones nearest to the male and female stimulus animals were named male area I and female area I. There were six zones between area I and the neutral area. After a 5 min wait time for adaptation, several parameters were recorded during 20 min of observation, including the stay time in male areas I and II and in female areas I and II; however, the time spent in the neutral area was not recorded. Males from treated mothers and control mothers were submitted to the experiments alternately. The partner preference score was calculated by subtracting the total time spent in the male area from the total time spent in the female area. Thus, positive scores indicate a preference for estrous females and negative scores for sexually active males. Testing occurred 4–8 h after the beginning of the dark period.

Female stimulus animals were ovariectomized rats treated with exogenous estradiol (50 μg/kg s.c., 54 h before the tests) and progesterone (2.0 mg/kg s.c., 6 h before the tests), and they were tested for receptivity before being placed with the males.

Sexual behavior

Sexual behavior at PND60 was evaluated as previously described by Felicio et al. (1989) and Chiavegatto et al. (1989). Briefly, all animals were exposed individually to an estrus-induced female for 40 min, and several parameters were evaluated, including the number of mounts, intromissions, ejaculatory intervals, number of attempted mounts, time to first ejaculation and number of ejaculations.

Hormone measurements

The serum doses were measured by radioimmunoassay using commercial kits for total testosterone and estradiol (Coat-A-Count, DPC, Los Angeles, USA). The minimum sensitivity was 0.9932 ng/dl, and the intraassay coefficient was <4.05% for testosterone, and 1,4238 pg/ml and a CV <3.9% for estradiol. The serum FSH and LH measurements were determined by chemiluminescence immunoassay using Luminex xMAP technology (Milliplex MAP rat pituitary panel, Billerica, MA, USA). The minimum sensitivity was 2.91 pg/ml for LH and 31.0 pg/ml for FSH. The intraassay coefficient was <2.5% for LH and <4.7% for FSH.

mRNA expression of LH, FSH and GH

Messenger RNA (mRNA) levels of beta LH, beta FSH and GH were analyzed by real-time PCR. Pituitaries were homogenized in Politron, and the total RNA was extracted according to the guanidine–phenol–chloroform method (Chomczynski and Sacchi 1987). Real-time PCR was performed as recommended by Goulart-Silva et al. (2011). The quantification was performed by the ΔΔCt method, as previously described (Nolan et al. 2006; Pfaffl 2001). mRNA levels for each gene were normalized to the housekeeping gene Rpl19. All primers were generated using Primer3 Input, verified for specificity by BLAST analyses, designed to span from two different exons (http://www.ncbi.nih.nlm.gov) and verified to hairpins by OligoAnalyzer (http://www.idtdna.com/analyzer/applications/oligoanalyzer). The primer sequences are shown in Table 1.

Protein expression of LH, FSH and GH

Pituitaries were individually homogenized in Politron in 300 μl of homogenization buffer (0.25 M sucrose, 2 mM $MgCl_2$, 20 mM Tris–HCl) and centrifuged at 100 g at 4°C for 10 min. The supernatant was transferred, and the pellet was resuspended in 200 μl of the same buffer and recentrifuged with the same conditions. Following centrifugation at 800 g at 4°C for 10 min, the supernatant was used for total protein determination (Bradford 1976). Thirty micrograms of protein were lyophilized, resuspended in 10 μl of Laemmli buffer (62.5 mM Tris–HCl pH 6.8; 20% glycerol; 10% mercaptoethanol; 4% SDS; 0.08% bromophenol blue) and 20 μl of homogenization buffer. The samples were then submitted to SDS-PAGE on a 15% gel (electrophoresis buffer: Tris-base 25 mM, 193 mM glycine, 0.1% SDS) and transferred to a hybridization nitrocellulose membrane (Bio-Rad Laboratories Inc., USA) at 15 mV for 60 min (transfer buffer: Tris-base 25 mM, 192 mM glycine, methanol 20%). The membrane was preincubated with blocking buffer (5% nonfat dry milk,

Table 1 Primers used for real-time PCR analysis

Gene		Primer sequence	Gen_Bank accession no.
Beta FSH	Forward	5′-AAGTCGATCCAGCTTTGCAT-3′	NM_001007597
	Reverse	5′-GTCCCAGGCCTCTTACAGTG-3′	
Beta LH	Forward	5′-ATGAGTTCTGCCCAGTCTGC-3′	NM_012858
	Reverse	5′-GTGGGTGGGCATCAGAAGAG-3′	
GH	Forward	5′-TCAAGAAGGACCTGCACAAG-3′	NM_001034848.2
	Reverse	5′-GTGGCAGTTGCCAGAGTACA-3′	
Rpl19	Forward	5′-AGTATGCTTAGGCTACAGAA-3′	NM_031103.1
	Reverse	5′-TCCCTTAGACCTGCTTGGTC-3′	

PBS buffer and 0.1% Tween 20) overnight at 4°C to reduce nonspecific binding. The membranes were then incubated for 3 h at RT with either anti-LH (1:1,500), anti-FSH (1:500) or anti-GH antibodies (1:5,000) diluted in blocking buffer. GAPDH was used as constitutive control protein (1:1,000). The band detection was performed using the appropriated secondary peroxidase-conjugated antibodies (1:5,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in buffer (2.7 mM KCl, 137 mM NaCl, 8 mM $NaHPO_4$, 1.4 mM KPO_4 and 0.1% Tween 20) for 1 h at RT followed by using the Enhanced Chemiluminescence kit (ECL, Amersham Biosciences, Buckinghamshire, UK). The blots were analyzed with Image J 1.42q software (National Institutes of Health, NIH, USA). The data were normalized to GAPDH, and the results were expressed as arbitrary units (AU).

Sperm evaluation

At PND60, the sperm counts determined as previously described (Piffer et al. 2009; Robb et al. 1978). The testes and epididymis (caput, corpus and cauda) were weighed. The tunica albuginea was removed from the testes, and the parenchyma was homogenized in 5 ml saline-Triton 0.5% followed by sonication for 30 s. The samples were then diluted 10 times in saline, and the mature spermatids resistant to homogenization were counted using a hemocytometer. The four chambers were averaged, and the numbers of spermatid per testis and per gram of testis were calculated. These values were subsequently divided by 6.1 days to calculate the daily sperm production (DSP). The segments of the epididymis (caput, corpus and cauda) were cut with a scissor, homogenized, diluted and counted as described for the testes. The number of spermatozoa in each homogenate was determined, as described above, and the total numbers of spermatozoa for the parts of the epididymis were calculated. The mean time for sperm transit through the epididymis also was calculated by dividing the number of spermatozoa in each portion of the epididymis by the DSP of the associated testis.

Histology and morphometry of seminiferous epithelium

The testes were fixed in Bouin's solution for 8 h, treated with alcohol and embedded in paraffin, and cuts of 5 μm were prepared as stained laminas with hematoxylin and eosin. The laminas were observed initially with a 40× magnification, for the general organ architecture observation. Next, a magnification of 100× was used for a more detailed analysis of the seminiferous tubule architecture, as previously described (Romano et al. 2010). This included analyzing the linear morphometry of the seminiferous tubules by determining the tubular diameter (measured from the basal lamina to the basal lamina in the opposite direction), seminiferous epithelium length (from the basal lamina to the neck of the elongated spermatids) and luminal diameter. Ten fields per section per animal were selected within the histological sections in the transverse direction of the tubules. For each tubule, the averages were calculated for the measurements indicated, and the average of each field was also calculated. The measurement for each animal was obtained by measuring all the analyzed fields.

Organ weights

The testes, epididymides and seminal vesicle were weighed, and the values were converted to relative weights of mg/100 g of body weight (BW) at PND60. The epididymis was previously divided into three segments: caput, corpus and cauda. The seminal vesicle was weighted with fluid (undrained) and after fluid removal (drained).

Body growth

The pups were weighted at PND21 (weaning), PND30, PND40 and PND60 to compare the body growth between the groups. Growth hormone mRNA expression and protein content in the pituitary were also evaluated at PND60.

Preputial separation (PPS)

To determinate the age at puberty, we evaluated the balanopreputial separation, which consists of the separation of the preputial membrane and the externalization of the glands of the penis (Korenbrot et al. 1977). This method was performed at PND33 and was continued once a day during the time of the balanopreputial separation, by gentle tissue manipulation. During this period, the animals were also weighed.

Statistical analysis

The variables in question were first submitted to Kolmogorov–Smirnov tests for normality and the Bartlett test for homoscedasticity. The analysis of body growth was performed using the multi-way analysis of variance for repeated measures (MANOVA) by a general linear model (GLM). The weights were compared between different groups and ages, considering the expected changes with age. The sexual behavior and day of PPS were compared among the groups using nonparametric analyses with the Mann–Whitney *U* test. The weights of seminal vesicle (drained and undrained) were compared by paired Student's *t* test. All other parameters were analyzed by Student's *t* test. All analyses were performed with Statistica 7.0 (Statsoft Inc, Tulsa, OK, USA). Statistical differences were considered significant when the value of *P* was lower than 0.05. The values were expressed as means and the standard error of the mean (±SEM) for parametric and interquartile ranges of nonparametric analysis.

Results

Changes in sexual partner preference

Sexual orientation estimates the preference for a specific gender of reproductive partner, and animals are expected to have a greater predilection for the opposite sex. Thus, to verify sexual partner preference, the males were exposed at PND60 to a sexually mature male and a female in estrous, positioned on opposite sides of the arena, so they could choose to approach the male, the female, or to remain in a neutral central area. The positive score indicates sexual preference for the female, and the negative score indicates a preference for male rats. The males from dams treated with glyphosate spent significantly more time in contact with female rats than control animals, suggesting a preference for the female gender (*t* test, $P < 0.05$, Table 2).

Table 2 Sexual partner preference in male rats perinatally exposed to a commercial formulation of glyphosate

Parameters	Time (s)	
	Control	RU 50 mg/kg
Stay time in male area I	201.8 ± 17.6[c]	88.0 ± 28.6[d]
Stay time in male area II	228.8 ± 29.0	224.0 ± 30.7
Total time in male area	430.6 ± 29.3	312.0 ± 55.9
Stay time in female area I	215.2 ± 15.9[d]	115.4 ± 25.6[c]
Stay time in female area II	287.0 ± 20.2[a]	509.2 ± 119.2[b]
Total time in female area	502.2 ± 22.7[a]	624.6 ± 110.9[b]
Partner preference score	71.6 ± 43.0[a]	312.6 ± 160.3[b]

Data are mean ± SEM.; [a, b] difference in rows—$P < 0.01$; [c, d] $P < 0.05$; Student's *t* test, $N = 5$ per group; *RU* Roundup transorb

Changes in sexual behavior

The treatment led to an increase in the latency to first mount, latency to first intromission and latency to mount after first ejaculation (Mann–Whitney *U* test, $P < 0.05$, Table 3). The other parameters did not differ significantly between the groups (Table 3).

Changes in testosterone and estradiol serum concentration

Sexual behavior may be influenced by gonadal steroids. Indeed, the levels of both hormones were different between the groups. Specifically, the group treated with glyphosate showed higher levels of testosterone and estradiol compared to control animals (*t* test, $P < 0.01$, Fig. 1).

Table 3 Sexual behavior in male rats perinatally exposed to a commercial formulation of glyphosate

Parameters	Control	RU 50 mg/kg
Latency for the first mount (min)	0.6–1.0[a]	5.2–7.0[b]
Latency for the first intromission (min)	0.6–1.0[a]	5.2–7.0[b]
Latency for the first ejaculation (min)	1.0–1.7[a]	5.5–7.0[b]
Number of incomplete mounts	11.0–12.0	15.0–17.0
Number of intromissions to first ejaculation	13.5–27.0	14.0–21.0
Total number of mounts	25.5–29.0	29.0–39.0
Latency for the first mount after first ejaculation (min)	0.5–0.9	0.2–0.6
Number of ejaculations after to first intromission in 30 min	29.0–31.0	36.0–39.0

Data are interquartile range (25–75%). [a, b] Difference in rows—$P < 0.05$; Mann–Whitney *U* test, $N = 4$ per group; *RU* Roundup transorb

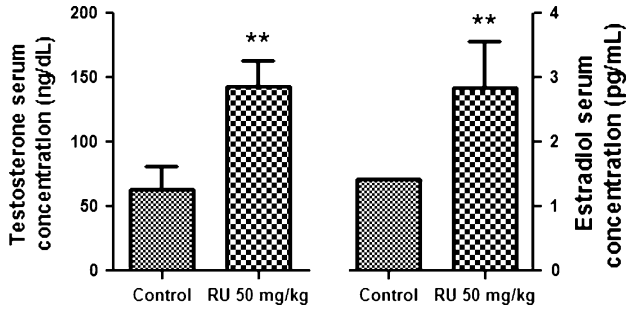

Fig. 1 Testosterone (ng/dl) and estradiol (pg/ml) serum concentrations in male rats perinatally exposed to a commercial formulation of glyphosate (RU) or control. Results are expressed as mean ± SEM. Student's *t* test; $n = 12$/group; $^{**}P < 0.01$

Changes in mRNA expression and protein content of gonadotropins (LH and FSH) in the pituitary gland and serum

The analysis of LH mRNA expression showed increased levels in treated animals (*t* test, $P < 0.05$, Fig. 2a), which was accompanied by higher amounts of LH protein in the pituitary (*t* test, $P < 0.01$, Fig. 2b) and the serum (*t* test, $P < 0.05$, Fig. 2c).

The FSH mRNA expression was increased in treated animals (*t* test, $P < 0.05$, Fig. 3A), but this was not associated with a rise in the FSH protein in the pituitary (Fig. 3b) or the serum (Fig. 3c).

Changes in sperm parameters

Because sex hormones may influence the spermatogenesis, we evaluated total sperm production, daily sperm production, sperm reserves and sperm transit at PND60 (Table 4). Glyphosate exposure during the perinatal period increased the total and daily sperm production (*t* test, $P < 0.05$). Sperm reserves were increased in the caput, corpus and cauda of the epididymis (*t* test, $P < 0.05$). Sperm transit time, however, was reduced only in the cauda of the epididymis (*t* test, $P < 0.05$).

Modifications in the morphometry of the seminiferous epithelium

Sex hormones directly influence sperm production and may alter the architecture of the seminiferous tubules. Thus, morphometry of histological sections of the testes was performed by analyzing the tubule diameter, seminiferous epithelium and the luminal diameter. Glyphosate exposure resulted in altered morphometry of the seminiferous epithelium in treated animals, causing an increase in epithelial height and a reduction in luminal diameter without changes in the tubular diameter (*t* test, $P < 0.05$, Table 5).

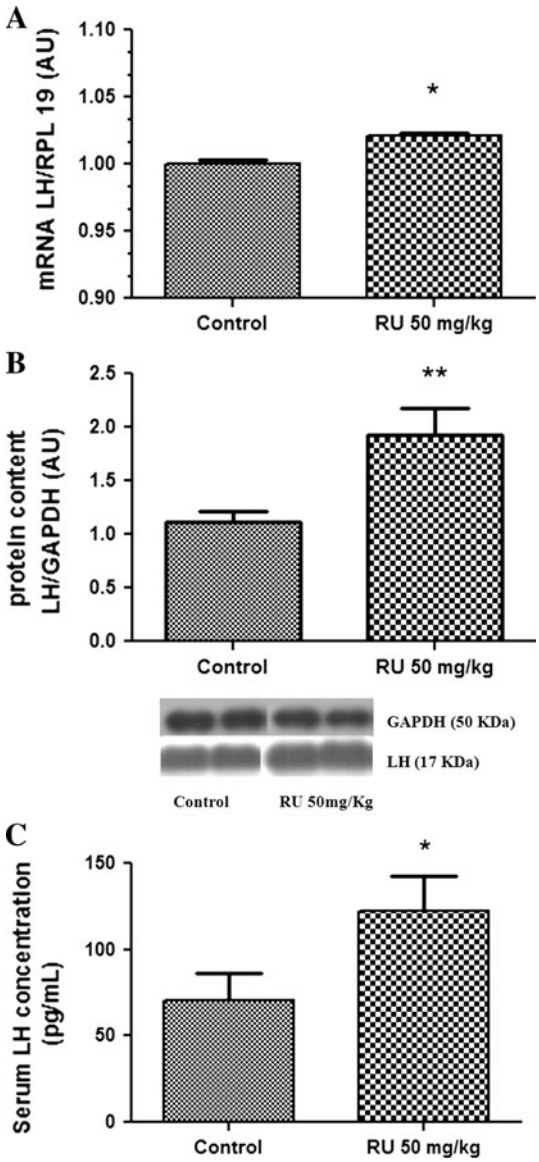

Fig. 2 LH mRNA expression (**a**), pituitary protein content (**b**) and serum concentrations (**c**) in male rats perinatally exposed to a commercial formulation of glyphosate (RU) or control. Results are expressed as mean ± SEM. Student's *t* test; $n = 8$/group; $^{*}P < 0.05$; $^{**}P < 0.01$

Glyphosate-mediated effects on the weight of the seminal vesicles and epididymis

The testes, epididymis and seminal vesicles (undrained and drained) were weighed at PND60 to determine the effect of glyphosate, because these tissues depend on sex hormones for their development and secretory activity. The weight of the testes was not different between groups. The weight of undrained seminal vesicle not altered, but the drained seminal vesicle was heavier than in the control group, suggesting that this structure contained smaller amount of fluid (paired *t* test, $P < 0.05$, Fig. 4a). The caput, corpus

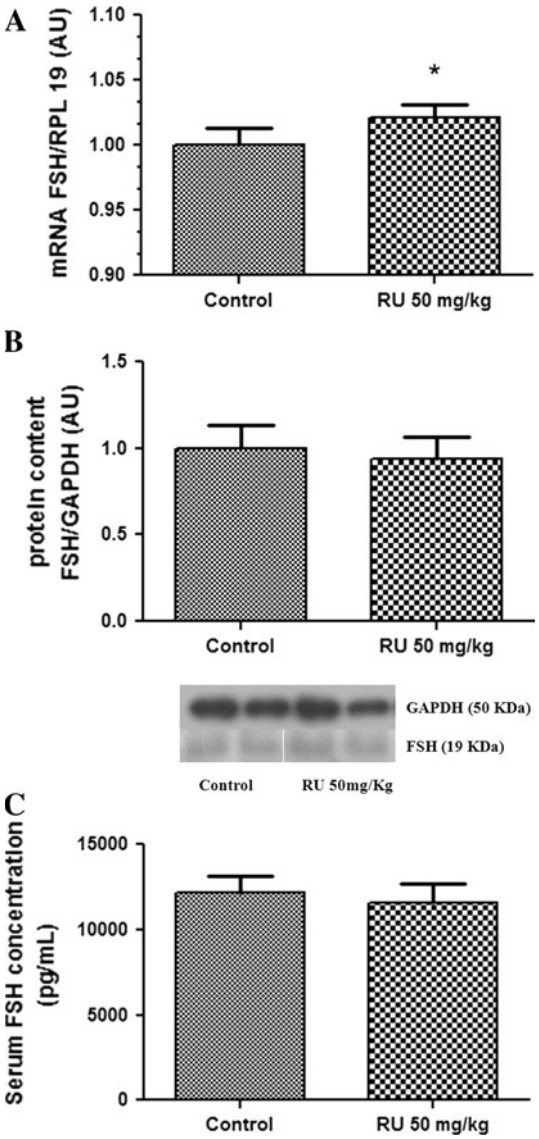

Fig. 3 FSH mRNA expression (**a**), pituitary protein content (**b**) and serum concentrations (**c**) in male rats perinatally exposed to a commercial formulation of glyphosate (RU) or control. Results are expressed as mean ± SEM. Student's *t* test; $n = 8$/group; $^*P < 0.05$

and cauda segments of the epididymis were weighted separately. The corpus and cauda, but not the caput, were heavier in the treated animals (*t* test, $P < 0.05$, Fig. 4b).

Body growth assessment

Body growth was determined by weight measurements at PND21, 30, 40 and 60 to detect any differences from weaning to PND60. The mRNA expression and pituitary protein content of GH at PND60 were not altered by glyphosate treatment. Thus, irregular growing did not jeopardize the effects of glyphosate on the reproductive axis of the offspring.

Table 4 Sperm parameters in male rats perinatally exposed to a commercial formulation of glyphosate

Parameter	Control	RU 50 mg/kg
Total sperm production ($\times 10^6$/testis)	52.34 ± 4.2[a]	99.22 ± 19.8[b]
Total sperm production ($\times 10^6$/g testis)	34.89 ± 0.2[a]	70.87 ± 1.4[b]
Daily sperm production ($\times 10^6$/testis)	8.5 ± 0.7[a]	16.3 ± 3.2[b]
Daily sperm production ($\times 10^6$/g testis)	5.7 ± 0.1[a]	11.6 ± 0.2[b]
Sperm reserves*		
Caput + corpus of epididymis ($\times 10^6$)	14.1 ± 2.6[a]	21.1 ± 2.3[b]
Cauda of epididymis ($\times 10^6$)	53.9 ± 8.2	47.7 ± 4.8
Sperm transit time trough		
Caput + corpus of epididymis (days)	1.6 ± 0.3	1.7 ± 0.4
Cauda of epididymis (days)	6.3 ± 0.8[a]	4.0 ± 0.9[b]

* Two epididymides; Data are mean ± SEM. [a, b] difference in rows—$P < 0.05$; Student's *t* test, $N = 8$ per group; *RU* Roundup transorb

Table 5 Morphometrical analysis of the seminiferous epithelium in male rats perinatally exposed to a commercial formulation of glyphosate

Groups	Tubular diameter (μm)	Epithelial height (μm)	Luminal diameter (μm)
Control	466.9 ± 14.3	91.7 ± 2.2[a]	256.9 ± 13.6[a]
RU 50 mg/kg	451.2 ± 9.6	97.7 ± 1.1[b]	238.7 ± 16.5[b]

Data are mean ± SEM. [a, b] difference in column—$P < 0.05$; Student's *t* test, $N = 8$ per group; *RU* Roundup transorb

Changes in age and body weight at puberty (PPS)

The beginning of puberty was evaluated in PPS and male rats perinatally exposed to glyphosate. The glyphosate rats showed a reduction in the age at puberty onset (Mann–Whitney *U* test, $P < 0.05$, Fig. 5a) and also in the body weight at puberty (Mann–Whitney *U* test, $P < 0.05$, Fig. 5b). However, the weights of the animals at the same age were not different, indicating that observed lower weight is merely a function of the younger age at puberty onset.

Discussion

Glyphosate is an herbicide associated with transgenic resistant crop systems (Cerdeira et al. 2007) and represents approximately 30% of all herbicides used in agriculture (Inoue et al. 2003). Glyphosate-mediated toxic effects shown in recent studies have led to the characterization of this herbicide as a potential endocrine chemical disruptor.

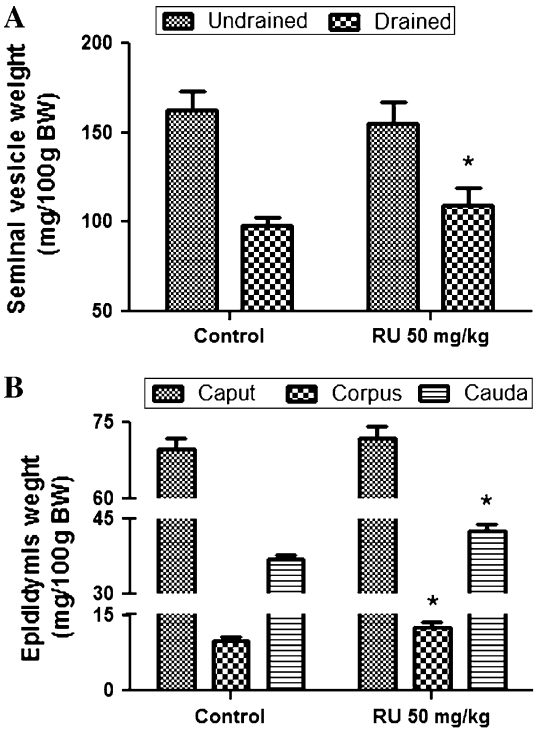

Fig. 4 Seminal vesicle (**a**) and epididymis (**b**) weights in male rats perinatally exposed to a commercial formulation of glyphosate (RU) or control. Results are expressed as mean ± SEM. Paired Student's *t* test (**a**) and Student's *t* test (**b**); $n = 16$/group; $^*P < 0.05$

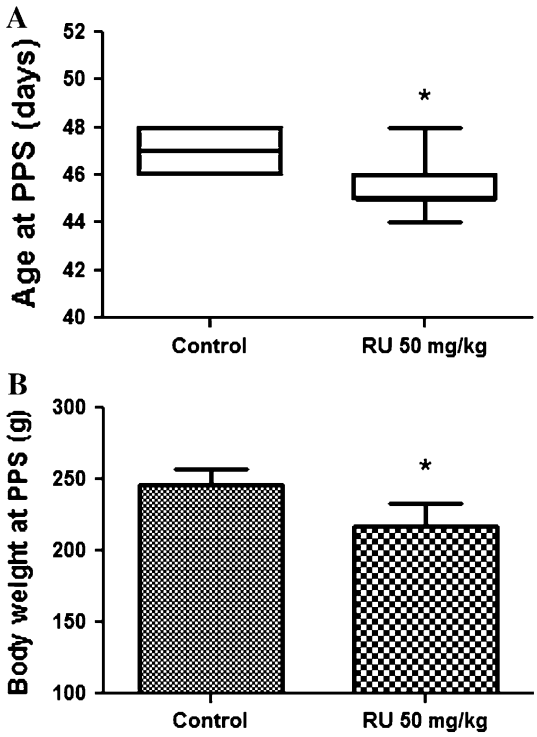

Fig. 5 Age (**a**) and body weight at PPS (**b**) in male rats perinatally exposed to a commercial formulation of glyphosate (RU) or control. Results are expressed as mean ± SEM. Mann–Whitney *U* test (**a**) and Student's *t* test (**b**); $n = 16$/group; $^*P < 0.05$

The focus of this study was to verify the toxicological effects of a commercial formulation of glyphosate, which is known to alter aromatase activity (Benachour et al. 2007; Gasnier et al. 2009; Richard et al. 2005) and decrease serum testosterone concentrations (Romano et al. 2010). To accomplish this, we investigated the reproductive development of male offspring exposed to glyphosate during the critical period of sexual hypothalamic differentiation.

We observed changes in most of the parameters evaluated in this study, suggesting that maternal exposure to glyphosate disturbed the masculinization process. Gonadal hormones modify the organization of the central nervous system during the perinatal period (Bardin and Catterall 1981; MacLusky and Naftolin 1981; Phoenix et al. 1959; Ward and Weisz 1980). Starting from GD18, fetal testes begin to produce testosterone, which plateaus and subsequently declines to basal levels around PND10 (Lenz and McCarthy 2010). Importantly, central nervous system aromatase, which converts testosterone into estradiol, is concomitantly expressed with the rise in serum testosterone concentration (MacLusky and Naftolin 1981). This stage is critical for the establishment of the characteristic reproductive behavior in adulthood, as shown in aromatase knockout mice (ArKO) that are characterized by degenerated sexual performance compared to wild-type animals (Bakker et al. 2002). The increase in serum testosterone during the perinatal period also changes the threshold for androgen sensitivity in target tissues, which is important for the development and activity of these tissues at puberty (Hoepfner and Ward 1988).

Sexual orientation is the preference for a specific gender for a reproductive partner, and it has an expected predilection for the opposite sex. The sexual partner preference score was increased approximately four-fold in the glyphosate-treated animals, which may be related to higher serum testosterone concentrations, as observed by Harding and Velotta (2011) in castrated rats treated with high amounts of testosterone.

The mating test is usually employed to determine the sexual performance in male rats that are placed for a specified time in contact with sexually receptive females. During this specific time, the parameters of latency to first mount, number of intromission, number of mounts and number of ejaculations are evaluated (Meisel and Sachs 1994; Harding and McGinnis 2003, 2004, 2005; Pfaus et al. 2001). In relation to copulatory behavior, we observed an increase in the latency to first mount, latency to first intromission, latency to mount after first ejaculation, suggesting an herbicide-mediated effect on the libido of the animals. The copulatory latency is used to evaluate arousal and sexual motivation (Saito and Moltz 1986; Mendelson

and Pfaus 1989). However, some authors have questioned the efficiency of this method to assess the motivational component because it measures an effect of the peripheral nervous system (Sachs 2007) rather than the central nervous system (Bancoft 2005). Thus, our study was based on classical assessments of sexual behavior, without evaluating the motivational component.

We observed an increase in estradiol serum concentrations, likely due to the increase in testosterone, which leads to greater conversion of estradiol (Bardin and Catterall 1981; Knudsen and Max 1980). Estradiol is known to stimulate the sexual arousal in both females and males (Harding and Velotta 2011; Max and Knudsen 1980; Heindel and Kimberley 1989); however, it appears that this did not occur in these animals, suggesting that hormone signals may have been misinterpreted by the central nervous system.

The age at puberty is an important component of reproductive development and is influenced by sex hormones, which can determine its anticipation or delay. In rats, the age at puberty onset can be evaluated by the separation of the preputial skin from the glands of the penis (Korenbrot et al. 1977; Stoker et al. 2000). Balanopreputial separation (PPS) is triggered by the rise of serum testosterone concentrations in the prepubertal period (Korenbrot et al. 1977). Thus, the early onset of puberty observed in this study suggests that the production of this hormone was initiated precociously in these animals. In addition, testosterone levels were also elevated, which may have contributed to change in the age at puberty onset. Because the rats reached puberty at an earlier age, the animals had lower body weights at puberty.

However, the animal body weights throughout the experimental period and the analysis of GH mRNA expression and pituitary content at PND60 revealed no differences between groups, suggesting that the effects of prepubertal administration of glyphosate are restricted to reproductive development and function.

The epididymis and vesicular glands, which are androgen-dependent tissues, were used as parameters for evaluating the effects of testosterone level variations. We observed an increase in the weight of the cauda and corpus of the epididymis, likely due to the increased sperm produced by these animals. The seminal vesicles showed a lower content.

Besides the changes observed in the gonadal hormones, the evaluation at PND60 showed altered gonadotropins, with elevated LH mRNA expression, protein content in the pituitary gland and serum. The FSH mRNA expression was increased, while the protein and serum concentrations remained unaltered. The gonadotropins are produced and released by the stimulation of hypothalamic GnRH, and testosterone exerts negative feedback on LH production. Thus, we may speculate that the glyphosate treatment may have caused (1) a permanent stimulus of LH production and (2) failure in the interpretation of the negative feedback mechanism.

Because sex hormones may influence the spermatogenesis, we evaluated total sperm production, daily sperm production, sperm reserves and sperm transit at PND60. Perinatal glyphosate exposure increased the total and daily sperm production in the testes, which is associated with the increase in the height of the germinal epithelium of seminiferous tubules in histological sections. In addition, we assessed the sperm content in epididymis segments, noting that sperm reserves were increased in the caput, corpus and cauda segments. Sperm transit time, however, was reduced only in the cauda of the epididymis. These observations may be related to the increased testosterone, estradiol and LH serum concentrations.

One study showed that glyphosate Roundup administration from the perinatal period to lactation did not induce maternal toxicity but caused reproductive problems in male offspring, including a decrease in the number of sperm in the cauda epididymis affecting the daily production of sperm in adult life. In this study, an increase in the sperm morphological pathology and lower levels of testosterone at puberty were observed (Dallegrave et al. 2007). However, the exposure to glyphosate in the study conducted by Dallegrave et al. (2007) was not limited to the perinatal period (the phase of hypothalamic sexual differentiation) but was also extended to the prepubertal period. As previously demonstrated by our group, exposure to glyphosate during this period causes an anti-androgenic effect, causing a reduction of serum testosterone and in the height of the seminiferous epithelium (Romano et al. 2010). The number of Sertoli cells formed in the prepubertal period under the influence of FSH determines the final size of the testes (Almiron and Chemes 1988) and the future sperm production in adult animals (França et al. 2000; Orth et al. 1988; Silva et al. 2006; Mruk and Yan Cheng 2004). Our findings suggest that elevated hormone levels increased the proliferation of the epithelium of the seminiferous tubules causing increased sperm production in animals treated by glyphosate. In addition, we observed increased epididymal transit, which is influenced by levels of sex hormones (Sharpe 2001).

This study shows, for the first time, the effects on the reproductive development of male offspring from dams treated with glyphosate only in the perinatal period. We conclude that the exposure promotes behavioral changes and histological and endocrine problems in reproductive parameters and these changes are reflected by a hypersecretion of androgens and increased gonadal activity, sperm production and libido. It is not known whether the dose used in this study is in fact the levels of exposure of

population to the glyphosate herbicide. However, it was shown that women occupationally exposed to this herbicide have reproductive disorders (Richard et al. 2005). Future studies should be conducted to establish possible dose-dependent effects.

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