PERINATAL GLYPHOSATE-BASED HERBICIDE IMPAIRED MATERNAL BEHAVIOR BY REDUCING THE STRIATAL DOPAMINERGIC ACTIVITY AND DELAYED THE OFFSPRING REFLEX DEVELOPMENT

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ABSTRACT:
Glyphosate, a non-selective herbicide, causes in mammals' cellular mutagenesis and toxic effects at the embryonic, fetal, and placental levels, even at low concentrations. This study investigated in rats the effects of perinatal exposure to glyphosate-base herbicide on maternal behavior and the hypothalamic and striatal levels of dopamine and serotonin. The pup's physical and behavioral development were observed. Glyphosate-base herbicide (50 or 150 mg/kg, per os) was administered in dams during gestation (15º gestational day to 7º lactation day. The female body weight was recorded throughout the pregnancy and lactation. The dams' reproductive performance was observed at postnatal day 2, the open field behavior at postnatal day 5 and the maternal behavior at postnatal day 6. At weaning, the dam's hypothalamic and striatal levels of dopamine and serotonin were measured. Maternal exposure to both glyphosate-base herbicide doses: i) had few effects on maternal body weight gain; ii) decreased the number and body weight of the pups; iii) impaired the maternal care; iv) both doses decreased the activity of striatal and hypothalamic

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dopaminergic systems; v) 50 mg/kg increased and 150 mg/kg decreased the serotoninergic hypothalamic activity. In offspring, no effects on physical development but a delay on reflex development. Conclusions: perinatal exposure to glyphosate-base herbicide decreased the maternal care by a reduced striatal dopaminergic activity and delayed the pup's reflex development.
Key-words: dopamine; herbicide; maternal care; pups' development; reproductive toxicology.

A ADMINISTRAÇÃO PERINATAL DO HERBICIDA À BASE DO GLIFOSATO PREJUDICOU O COMPORTAMENTO MATERNAL POR REDUZIR A ATIVIDADE DO SISTEMA DOPAMINÉRGICO ESTRIATAL E ATRASOU O DESENVOLVIMENTO DE REFLEXOS DA PROLE

RESUMO:
O glifosato é um herbicida não seletivo, que causa mutagênese celular e efeitos tóxicos embrionário, fetal e placentário, mesmo em baixas concentrações. Este estudo investigou, em ratos, os efeitos da exposição perinatal ao herbicida à base de glifosato sobre o comportamento materno e os níveis hipotalâmicos e estriatais de dopamina e serotonina. O desenvolvimento físico e comportamental dos filhotes foi observado. O herbicida (50 ou 150 mg / kg, per os) foi administrado às mães durante a gestação (15º dia gestacional ao 7º dia de lactação. O peso corporal foi registrado durante a gestação e lactação O desempenho reprodutivo das mães foi observado no dia pós-natal 2, o comportamento de campo aberto no dia pós-natal 5 e o comportamento materno no dia pós-natal 6. Ao desmame os níveis hipotalâmicos e estriatais da dopamina e da serotonina foram medidos. A exposição materna às duas doses do glifosato: i) teve poucos efeitos sobre o ganho de peso corporal materno; ii) diminuiu o número e peso corporal dos filhotes; iii) prejudicou o cuidado materno; iv) ambas as doses diminuíram a atividade dos sistemas dopaminérgicos estriatal e hipotalâmico; v) 50 mg / kg e 150 mg / kg do glifosato diminuíram a atividade hipotalâmica serotoninérgica. Na prole, não houve efeitos no desenvolvimento físico, mas observou-se atraso no desenvolvimento reflexológico. Poucos efeitos na atividade geral do filhote foram observados. Conclusões: a exposição perinatal ao herbicida à base de glifosato diminuiu o cuidado materno por uma atividade redução da atividade dopaminérgica estriatal e atrasou o desenvolvimento dos reflexos dos filhotes.
Palavras-chave: desenvolvimento dos filhotes; dopamina; cuidado materno; herbicida; toxicologia reprodutiva.

1. INTRODUÇÃO

Glyphosate (*N*-(phosphonomethyl) glycine) is a non-selective herbicide registered for use on many food and non-food crops, as well as non-crop areas where total vegetation control is desired (Cox, 1998). Studies reported that the herbicides containing glyphosate (GLY-BH) decreases spermatozoa production and pregnancy (Dallegrave *et al.*, 2007;Romano *et al.*, 2010;,Romano *et al.*, 2012). Moreover, cellular mutagenesis and toxic effects at the embryonic, fetal, and placental levels were already showed, even at low concentrations of GLY-BH (Belle *et al.*, 2012 ;Benachour *et al.*, 2007). Additionally, perinatal exposure to a GLY-BH herbicide can disrupt the hypothalamic-pituitary-thyroid axis(Souza, de *et al.*, 2017).

Previous studies from oral exposure showed that GLY-BH crosses the placenta (Poulsen *et al.*, 2009). In addition, exposure to oral GLY-BH 0.5 and 1% during pregnancy induces a variety of functional abnormalities in three cytosolic enzymes: (i) isocitrate dehydrogenase-NADP dependent (ICD), (ii) glucose-6-phosphate dehydrogenase (G6PD), and (iii) malic dehydrogenase (MD) in the liver, heart, and brain of pregnant Wistar rats and its fetuses (Daruich *et a*l., 2001). In pregnant rats, a similar behavior was observed in the activity of ICD, G6PD, and MD in both heart and liver. In these organs, both G6PD and ICD decreased sensitivity to lower concentrations of the herbicide. However, there was a higher enzymatic activity with the 1% concentration, probably as a protection mechanism by increasing the NADPH production. In the brain both GLY-BH concentrations, there was an increase in the enzymatic activity of ICD. Therefore, a poor response of the different fetus organs to both GLY-BH concentrations could be a protective mechanism of the placenta (Poulsen *et al.*, 2009).

The exposure of female rats during pregnancy and lactation periods to GLY-BH decreases glutamate uptake by glial cells in the hippocampus of exposed offspring, resulting in glutamate excitotoxicity (Cattani *et al.*, 2014). Several neurodegenerative conditions are frequently associated with glutamatergic excitotoxicity and oxidative stress (Pessoa-Pureur e Wajner, 2007). In addition, neurobehavioral alterations in the offspring were observed in Wistar rats exposed to 0.2% or 0.4% of a commercial formulation of glyphosate in the drinking water, during pregnancy and lactation (Gallegos *et al.*, 2016).

Moreover, pups exposed to GLY-BH showed early onset of cliff aversion reflex and early auditory canal opening. At puberty and adult age, a decreased in both locomotor activity and in the anxiety, levels were also observed. Taken together, these data suggest that early exposure to GLY-BH affects the central nervous system in the offspring of rats, probably by altering mechanisms or neurotransmitter systems that regulate locomotor activity and anxiety.

For mammals, sensory, social, and hormonal experiences early in life is essential for the continuity of the infant's development. These experiences come from the mother through maternal care, and have enduring effects on the physiology and behavior of the adult organism (Melo, 2015). Disturbing the mother-offspring interaction by maternal deprivation (neglection) or exposure to adverse events, such as stress (Gerardin *et al.*, 2005), pesticides (Moniz *et al.* 1999) and heavy metals (Sant'Ana *et al.*, 2001;(Salvatori *et al.*, 2004) has negative effects on the pups mental, psychological, physiological, and behavioral health.

Considering that interferences with maternal behavior could cause developmental offspring impairment, in the present study, we investigated the perinatal effects of a GLY-BH exposure on maternal behavior and in the hypothalamic and striatal levels of dopamine and serotonin of these rats. Also, the pups physical and behavior development were observed.

2. MATERIALS AND METHODS

2.1. Animals

Wistar female rats obtained from the Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil, were mated with male rats previously tested as fertile (two females to one male were placed in each cage). The onset of pregnancy was confirmed by the observation of spermatozoa in vaginal smears (gestational day 0 – GD0). These females were individually housed in plastic cages (32 x 40 x 18 cm) with controlled room temperature (22 ± 2ºC), humidity (55-65%), artificial lighting (12 h light/12 h dark cycle, with lights on at 6 AM), free access to Nuvilab® rodent chow (Nuvital, São Paulo, Brazil), and filtered water. The animals used in this study were maintained in accordance with the guidelines of the Committee on Care and Use

of Laboratory Animal Resources of the School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (no. 2010/2010).

2.2. Glyphosate-

Experiments were performed using Roundup Transorb® (Monsanto of Brazil Ltda, São Paulo, Brazil). This formulation is composed of 480 g/L of glyphosate, 648 g/L of isopropylamine salt, and 594 g/L of inert ingredients. These doses were chosen based on previous studies (Dallegrave *et al.*, 2007) which showed that 50 and 150 mg/kg of GLY-BH administered orally during pregnancy and lactation was not capable of inducing overt signs of maternal toxicity and is considered below the NOAEL chronic toxic dose (409 mg/kg/day)(Williams *et al.* 2000).

2.3. Exposure and Experimental Design

After mating, the gestation day (GD) 0 was defined as the day when spermatozoa were detected in the vaginal smear. The dams were randomly divided in four groups, weighed and then housed individually. The experimental groups (n = 7-8 rats per group) were treated with 50 and 150 mg/kg of GLY-BH orally by gavage (1 ml/kg of body weight), once a day, from GD15 to 7º lactation day (LD) 7, except on the day of delivery. The control group was treated orally by gavage with vehicle (1 ml/kg of water). Moreover, body weight, food and water consumption were measured during gestation and lactation periods. Dams could give birth and nurture its offspring under normal conditions. The day of birth was recorded as postnatal day (PND) 1 and no handling was performed on this day. On PND 2, 6-8 offspring (3-4 males and 3-4 females) were randomly selected to homogenize the litter and remained with each dam until weaning (PND 21). On PND 21, the male and female rat pups were individually housed in polypropylene cages under the same conditions as its parents and treatments. One rat from each litter was used for offspring evaluations to minimize potential confounding factors associated with the litter (Kirsten *et al.*, 2015). The ambulatory activity dams were evaluated on LD5 in the open field and the maternal behavior and maternal aggressive behavior on LD6. At weaning on LD21, the dams were euthanatized, and the dopamine and serotonin and its metabolites levels were measured in the hypothalamus and striatum. Furthermore, the pup's physical and behavior

development were observed as previously described (Sandini *et al.*, 2014). The pups open field behavior was evaluated from LD15 until weaning (LD21).

2.4. Maternal studies

Maternal reproductive performance was observed at PND 2, including the number of born pups, pups live/dead ratio and pups body weight.

Open field test was performed at LD5, as previously described (Sandini *et al.*, 2014). Each rat was placed individually in the center of the arena and locomotion. Subsequently, rearing frequencies and immobility time were observed during 5 min. The apparatus was cleaned with a 5% alcohol/water solution between rats.

Maternal behavior: Eight pups were removed at 7 AM and placed in another cage away from their mothers, as previously described (Soto *et al.*, 2013). During this period, the body temperature of pups was preserved with a thermal blanket. Sixty minutes after the maternal separation, eight pups were returned to the cage of each dam and placed in three different corners of the box. Subsequently, the behavior was videotaped for 30 minutes. The parameters analyzed were: i) pup retrieval: latencies in seconds to retrieve and bring the first, second, and third pups to the nest; when the dam did not retrieve any of the three pups, the value of 1800 sec was attributed; ii) percentage of dams that retrieved all pups; iii) total number of pups retrieved for each dam; iv) grooming of the pups (dams were scored on how they groomed its pups, based on the levels of grooming of the control group, which were scored as: 1 = little grooming, 1-2 times; 2 = low frequency of grooming, 3-4 times; 3 = median frequency of grooming, 5-6 times; 4 = median to high frequency of grooming, 7-8 times, and 5 = high frequency of grooming, more than 10 times); v) full maternal behavior (the percentage of dams who nursed the pups with their backs arched over the pups for 3 min); and vi) nest building (scored at the end of the experiments, which were scored as 0 = no nest; 1 = poorly, defined nest; 2 = regular nest; 3 = well defined nest).

Maternal aggressive behavior: was performed 15 min after the maternal behavior test. A naïve and smaller male intruder was placed in the cage on the opposite side of the dam with their litter. Then, the session was videotaped for

10 min. In case of danger for the pups, the session was interrupted, and this analysis was excluded from the test. Danger was considered when the intruder severely attacked the pups or the dam or when the female severely attacked the intruder. Each male was used only once. After testing, the male was removed from the cage, and the dam and pups were returned to the colony´s room. Behavioral parameters were observed as previously described (Sandini *et al.*, 2014), with the following adaptations: latency to the first fight; frequencies of fights (a quick lunge by the female, which was usually followed by rolling, biting, and fur pulling directly toward the neck and back regions of the intruder); total time of fight (s), frequency of intruder sniffing of the pups and frequency of the dams sniffing of the intruder.

Measurement of maternal dopamine, serotonin and its metabolites levels*:* After weaning of offspring, the dams were euthanized by rapid decapitation(BRASIL, 2018) because previous anesthesia interfere with the neurotransmitter levels (Bourgoin *et al.*, 1975). The striatal and hypothalamic areas were sampled from the dam's brain to measure the levels of dopamine and serotonin, as well as its metabolites. The brain samples were dissected on dry ice and prepared (Felicio *et al.*, 1996). Briefly, the striatum was weighed and stored at -80ºC until neurochemical analyses were carried out. Following sample collection, perchloric acid was added to the tissues, which were then homogenized by sonication for immediate determination of the monoamine levels. Dopamine (DA) and its metabolites [3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA)], serotonin (5-HT) and its metabolite [5-hydroxyindolacetic acid (5-HIAA)] were measured by HPLC (Shimadzu, model 6A) using a C-18 column (Supelco®, Sigma), electrochemical detector (Shimadzu, model 6A), sample injector (15 and 20 ml valve) and an integrator (Shimadzu, model 6A Chromatopac). Each sample was run for 18 min. The detection limit was 0.2 ng for all the analyses. Neurotransmitter turnover for DA and 5-HT was calculated by metabolite/neurotransmitter ratio.

2.5. Offspring studies

Physical and reflex development of the pups*:* The pup's body weight was measured at PND7, PND 14 and PND21. The following parameters were recorded to evaluate the physical development of the offspring: body weight (PND 2, PND

7, PND14 and PND 21), pinna detachment (PND2–PND4), hair growth (PND 4-PND8), incisor eruption (PND6–PND12), eye opening (PND11–PND17), testis descent (PND22–PND25) and vaginal opening (PND30–PND38). Animals were observed until all of them showed each parameter, subsequently the mean day of appearance was calculated.

The following reflexes were assessed in one male and one female of each litter: negative geotaxis (a minimum 90 turn after being placed face down on a 45 inclined platform for 30 s, beginning on day 2), auditory startle reflex (a sudden flinch or cessation of ongoing movement following an auditory stimulus induced by a clicker), palmar grasp reflex (pup grasps a paper clip with forepaws if stroked) and day of adult gait. All tests were carried out at the same time of the day (9:00–11:00 a.m.). The pups were separated from the mothers only in the moment of observation and were immediately returned to their home cages after observation. The mean day of appearance of each of the above parameters was calculated.

The pups motor behavior was observed as previously described (Kirsten and Bernardi, 2010). The locomotion and rearing frequencies and the time in seconds of grooming behavior were measured for 5 minutes.

2.6. Statistical analyses

The results were expressed as the means ± SEM or as percentage. Homoscedasticity was verified using an F-test or Bartlett's test. Normality was verified by a Kolmogorov- Smirnov test. One-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test were used to analyze data of more than two groups and one factor. Two-way ANOVA followed by the Tukey's multiple comparisons test were employed to compare data with and more than two groups and two factors. Kruskal-Wallis test was employed to analyze data regarding nest building, and the Chi-square test was applied to analyze data of percentages. In all cases, the results were considered significant if $p < 0.05$. The statistical analyses were performed using GraphPad Prism® 7 software.

3. RESULTS

3.1. Maternal studies

3.1.1. Body weight of the mothers during pregnancy

During pregnancy, the maternal weight increased significantly for all groups, as RMANOVA revealed significant differences during the time ($F_{8,56}$ = 453.1, p < 0.0001), without differences between treatment ($F_{2,14}$ = 0.4214, p = 0.6642) and without interaction of factors ($F_{16,112}$ = 0.8733, p = 0.6008). However, no differences were observed between groups by the Bonferroni test (Figure 1A). During lactation period, repeated measures showed significant differences during time ($F_{7,49}$ = 7.180, p < 0.0001), without difference between treatment ($F_{2,14}$ = 2.290, p = 0.1379) and without interaction ($F_{14,98}$ = 1.151 p = 0.3251). Dunnett's post hoc test showed decrease (p<0.001) in body weight in rats that received 150 mg/kg in LD1 when compared to all other groups at PND1 (Figure 1B). No differences were observed in food and water consumption between groups during gestation and lactation (data not shown).

3.1.2. Maternal reproductive performance

The body weight of dams at the postpartum was not different between groups (F2.18 =0.97; p = 0.40). The body weight of the female pups at PND2 decreased in both 50 and 150 mg/kg treatment when compared to the control group (F2.64 = 9.57; p = 0.0002), but not in the male pups of all groups (F 2,79 = 0.84, p = 0.43). GLY-BH treatment decreased the number of pups in both doses of 50 and 150 mg/kg (different (F2,17 = 18.15, p < 0.0001)), when compared to the control group (Table 1).

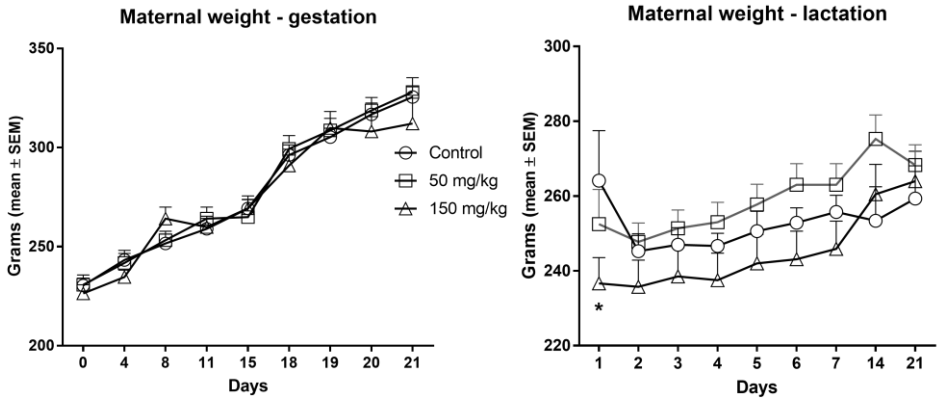


Figure. 1: Effects of perinatal exposure to 50 or 150 mg/kg of GLY-BH herbicide (by gavage, during gestational day 15 (GD15) until 7º lactation day

(LD7) on maternal weight during pregnancy (A) and lactation (B). N = 8 dams per group. Data are presented as mean ± SEM and were analyzed by repeated measure followed by Dunnett's post hoc test *p<0.05, **p<0.01 compared with control group.

3.1.3. Open field behavior

The open field test in dams performed in LD6 showed that treatment with 150 mg/kg of GLY-BH reduced dam's frequency of locomotion ($F_{2,21}$ = 4.2555, p < 0.02) and rearing ($F_{2,21}$ = 5.211, p < 0.01), and increased immobility time ($F_{2,21}$ = 4.407, p <0.021) (Table 2).

3.1.4. Maternal aggressive behavior

Treatment with 150 mg/kg of GLY-BH impaired the maternal aggressive behavior. One-way ANOVA showed differences in latency to first fight ($F_{2,21}$ = 10.10 p = 0.0008), frequency of fight ($F_{2,21}$ = 5.025, p = 0.01), and frequency that intruder sniffs pups ($F_{2,21}$ = 25.70, p = 0.0001) (Table 2). Dunnett's pos hoc test showed increased (p<0,001) in the latency to first fight in dams that received 150 mg/kg of GLY-BH, as well as decrease in the frequency of fight (p<0.01) in the same group, when compared to control group (Table 2). Furthermore, Dunnett's post hoc test showed increased intruder sniffs pups in both 50 and 150 mg/kg of GLY-BH, when compared to control group (p<0.001) (Table 3). No differences were observed in total time of fight ($F_{2,21}$ = 1.844, p = 0.1829), and neither in frequency that dams sniffs intruder ($F_{2,21}$ = 1.667 p = 0.2129) between groups.

Table 1. Effects of perinatal exposure to 50 and 150 mg/kg of glyphosate-based herbicide (by avage, during gestational day 15 (GD15) until 7º lactation day (LD7) on maternal reproductive performance. One-way ANOVA followed by the Tukey's multiple comparison test. **p< 0.01; p< 0.0001 relative to control group. N=8/group.

	Control group	**50 mg/kg**	**150 mg/kg**
Post-partum of maternal body weight	252.50 ± 9,30	244.60 ± 5.32	236.62 ± 6.90
Male pups body weight at PND2	7,60±0,20	7,50±0,30	7,0±0,30
Female pups body weight at PND2	7,30±0,20	7,20±0,10 **	6,0±0,30***
Total number of pups born	98	54	43
Number of males	32	27	25

Number of females	32	20	15
Number of dams with pups.	8/8	6/8	6/8
Mean ± SEM of pup's number	12.25 ± 0.45	6.75 ± 1.19 ***	5.37 ± 1.08***

Table 2. Maternal aggressive behavior (lactation day 6) and motor activity (lactation day 5) of dams treated with 50 and 150 mg/kg of glyphosate from gestational day 15 until lactation day 7. Data are presented as mean ± SEM. One way-ANOVA followed Dunnett's pos hoc test: *p<0.05, **p<0.01, ***p<0.001 compared with control group.

Parameters	**Control (n=08)**	**50 mg/kg (n=06)**	**150 mg/kg (n=06)**
Maternal Aggressive behavior			
Latency to first fight (sec)	104.3 ± 23.5	168.4 ± 38.7	387.5 ± 67***.
Fight (frequency)	5.8 ± 1.0	4.1 ± 1.2	1.5 ± 0.6**
Total time of fight (sec)	12.5 ± 3.7	8.6 ± 2.1	5.2 ± 1,9
Intruder sniffs pups (frequency)	14.0 ± 0.35	10.0 ± 0.5***	10.0 ± 0.5***
Dams sniffs intruder (frequency)	7,5±1,0	9,0±1,6	7,0±0,8
Open field behavior			
Locomotion frequency	23.6 ± 3.6	19,4 ± 3.2	11.2 ± 2.2*
Rearing frequency	7.2 ± 1.3	4.1 ± 1.2	2.0 ± 0.9**
Immobility time (sec)	120.6 ± 32.4	130.5 ± 21.6	210.4 ± 11.7*

3.1.5. Maternal behavior

Figure 2A showed that 150 mg/kg of GLY-BH increased latency to retrieve the second pup when compared to control group ($p<0.05$). No significant difference was found between groups in the latencies to retrieval of the first pup ($F_{2, 21} = 2.50$, $p = 0.11$, Fig. 2A), and third pup ($F_{2,21} = 1.61$, $p = 0.22$, Fig. 2A). No significant differences were observed in the number of total pups retrieved groups ($F_{2,21} = 1.21$, $p = 0.36$, Fig. 2B). Treatment with a higher dose (150 mg/kg) of GLY-BH decreased the number of pups retrieved when compared to controls (Chi-square test, $p < 0.01$). However, the lower GlyBH dose was not associated with this decrease (Chi-square test, $p > 0.05$, Fig. 2C). The frequencies that the dams groomed its pups was significantly different between groups ($F_{2,21} = 34.35$, $p < 0.0001$, Fig. 2D). In fact, Bonferroni test showed that both high and low doses of GLY-BH decreased this parameter when compared

to control group (Chi-square test, $p < 0.01$, Fig. 2E). The scores for full maternal behavior (Fig. 2E) and nest building (Fig. 2F) were reduced in both experimental groups when compared to the control group ($KW = 9.46$, $p < 0.009$).

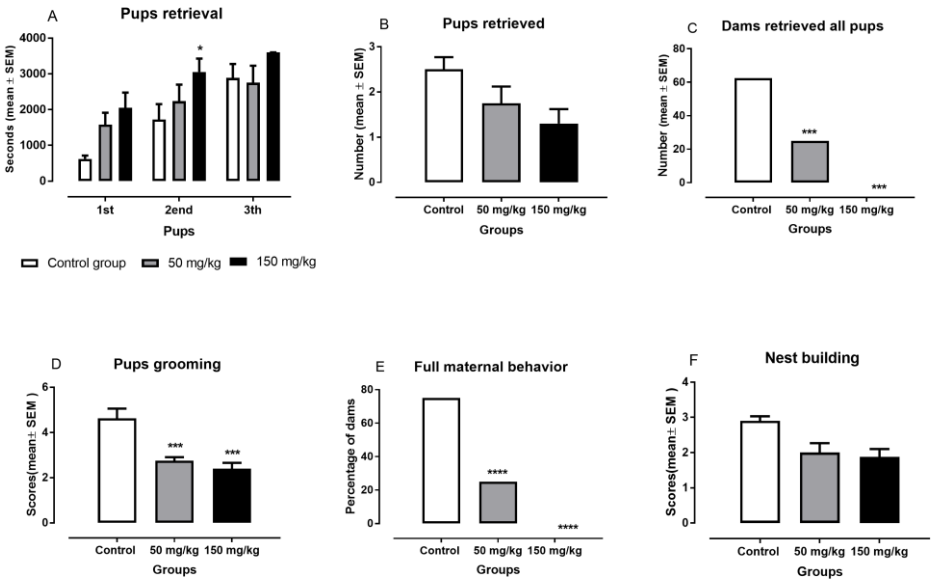


Figure 2: Maternal behavior of dams exposed to 50 or 150 mg/kg of GLY-BH herbicide (by gavage, during gestational day 15 (GD15) until 7º lactation day (LD7). A = retrieval latencies; B = pups retrieved, n = 3; C = retrieval of all pups; D = grooming of pups; E = full maternal behavior; and F = nest building. Data are presented as mean ± SEM or percentage. N = 8 dams/group. Two-way ANOVA was followed by the Bonferroni test for retrieval of pups, * $p < 0.05$ ** $p < 0.01$; ***$p < 0.001$ in relation to the control group. Kruskal-Wallis test of nest building, *** $p < 0.001$. Chi –square test to data in percentage, **$p < 0.01$.

3.1.6. Levels of neurotransmitter and metabolites in dams

In the hypothalamus, one-way ANOVA showed significant differences in DA levels ($F_{2,15} = 15.87$, $p < 0.0002$), in DOPAC/DA turnover ($F_{2,15} = 111.6$, $p < 0.0001$), 5HIIA/5-HT turnover ($F_{2,15} = 24.01$, $p < 0.0001$) and NA levels ($F_{2,15} = 11.71$, $p < 0.0009$) between groups. Dunnett's post test showed decreased DA levels in dams that were exposed to 50 ($p<0.01$) and 150 ($p<0.001$) mg/kg of GLY-BH; increased in DOPAC/DA turnover in dams that received GLY-BH 50 ($p<0.01$) and 150 ($p<0.0001$) mg/kg; decreased ($p<0.0001$) in turnover 5-HT in both groups; and increased ($p<0.001$) NA levels in the dams that received GLY-BH 150 mg/kg during GD15 to LD7 (Table 3).

In the striatum, one way ANOVA showed significant differences in DA levels ($F_{2,15} = 7.341$, $p < 0.006$); DOPAC levels ($F_{2,15} = 26.13$, $p < 0.0001$), DOPAC/DA turnover ($F_{2,15} = 97.37$, $p < 0.0001$); 5-HT levels ($F_{2,15} = 31.58$, $p < 0.0001$) and in turnover 5HIIA/5-HT ($F_{2,15} = 64.50$, $p < 0.0001$) between groups. Dunnett's post test showed decreased ($p<0.01$) in DA levels in dams that were exposed to GLY-BH 150, decreased ($p<0.0001$) DOPAC levels in both groups; decreased ($p<0.01$) DOPAC/DA in dams that were exposed GLY-BH 50 mg/kg; increased ($p<0.0001$) in 5-HT levels and decreased ($p<0.0001$) turnover 5HIIA/5-HT in damns of GLY-BH 150 mg/kg; and increased turnover 5HIIA/5-HT in dams that received GLY-BH 50 ($p<0.01$) and 150 ($p<0.0001$) mg/kg during GD15 to LD7 (Table 3).

Table 3. Brain monoamine and metabolite levels (ng/g of tissue) of dams on 21º lactation day (LD21) exposed to 50 and 150 mg/kg of glyphosate or control group, during gestational day 15 (GD15) until LD7. Data are presented as mean ± SEM. One way-ANOVA followed Dunnett's pos hoc test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared with control group. Dopamine (DA); 3,4-dihydroxyphenylacetic acid (DOPAC); homovanilic acid (HVA); serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA). One-way ANOVA followed by the Bonferroni test. **p< 0.01; ***p¸0.001 relative to control group.

	Control (n=06)	**50 mg/kg (n=06)**		**150 mg/kg (n=06)**	
	Hypothalamus				
DA	454.9 ± 33.5	335.6 ± 15.8**	↓	274.7 ± 14.7***	↓
DOPAC	105.4 ± 21.5	121,5 ± 17,0		175.7 ± 36.8	
DOPAC/DA	0.25 ± 0.001	0.34 ± 0.01**	↑	0.62 ± 0.03****	↑
HVA	40.8 ± 3.5	47.6 ± 2.6		32.7 ± 1.6	
HVA/DA	0.09 ± 0.002	0.20 ± 0.04*	↑	0.10 ± 0.03	
5-HT	3348.1 ± 578	4411.2 ± 687		5568.4 ± 598	
5HIAA	3654.9 ± 345	2540.2 ± 321		3455.9 ± 433	
5HIAA/5-HT	1.10 ± 0.09	0.63 ± 0.03***	↓	0.62 ± 0.02****	↓
	Striatum				
DA	5985.7 ± 791	3399.9 ± 1001		1790.9 ± 450**	↓
DOPAC	2781.6 ± 325	1008.4 ± 115****	↓	841.0 ± 118****	↓
DOPAC/DA	0.40 ± 0.008	0.30 ± 0.005**		0.41 ± 0.005	
HVA	1142.0 ± 300	790.1 ± 89	↓	961.8 ± 59	
HVA/DA	0.19 ± 0.08	0.21 ± 0.09		0.51 ± 0.09	
5-HT	2688.2 ± 274	3055.9 ± 163		4948.1 ± 195****	↑
5HIAA	1351.1 ± 148	1870.0 ± 253		1239.6 ± 311	
5HIAA/5-HT	0.50 ± 0.01	0.61 ± 0.02**	↑	0.27 ± 0.03****	↓
NA	349.0 ± 62	272.7 ± 59		300.4 ± 65	

3.2. Offspring studies

3.2.1. Pups physical, reflex and motor development

No differences were observed in the body weight of male and female pup rats during development. Also, no differences were observed regarding physical development among all groups (Table 4), except for the vaginal opening were the 50 mg/kg GLY-BH showed a delay relative to the control group. Concerning the reflex development, a delay in the surface righting reflex in GLY-BH 150 mg/kg group, in the day of adult gait in the GLY-BH 50 mg/kg group and at both GLY-BH doses in the auditory startle (Table 4).

The male pups open field behavior showed increased locomotion frequencies mainly at weaning after exposure to GLY-BH dose relative to the control group (Figure.3). On LD 16, female pups showed increased locomotion frequency and in grooming behavior, respectively after exposure to the low and high GLY-BH doses.

Table 4. Physical, reflex and motor behavioral development of pup rats from dams exposed to 50 or 150 mg/kg by oral route to glyphosate-base herbicide (Roundup®). from gestation day 15 to lactation day 7. Data are presented as means ± SEM of the day of parameter occurrence N = number of litters (one pup / litter). One-way ANOVA followed by the Bonferroni test. *p< 0.05; **p< 0.01; **p< 0.001 relative to the control group.

Parameters	Control group	GLY-BH 50 mg/kg	GLY-BH 150 mg/kg	F, *df*, P
Physical development	N=16	N=12	N=12	
Incisor eruption	10.0±0.6	10.4±0.5	10.1±1.4	F (2, 37) = 1.3, P=0.2847
Eyes opening	13.5±0.3	13.0±0.3	13.2±0.2	F (2, 37) = 0.85, P=0.4366
Ear unfolding	2.5±0.1	2.3±0.2	2.7±0.2	F (2, 37) = 1.37, P=0.2666
Hair growth	6.5±0.2	6.3±0.2	6.2±0.3	F (2, 31) = 0.33, P=0.7238
Testis descent	19.8±0.2	20.0±0.3	19.7±0.2	F (2, 31) = 0.32, P=0.7304
Vaginal openings	34.3±0.5	32.3±0.9*	34.5±0.5	F (2, 31) = 3.53, P=0.0415
Reflex development	N=16	N=12	N=12	
Palmar grasp reflex	5.8 ± 0.2	5.7 ± 0.1	5.8 ±0.4	F (2, 31) = 0.03, P=0.9756
Surface righting reflex	5.1 ± 0.1	5.1 ± 0.1	6.0 ± 0.1***	F (2, 31) = 24.25, P<0.0001
Negative geotaxis reflex	6.1 ± 0.2	6.2 ± 0.2	6.0 ± 0.1	F (2, 31) = 0.22, P=0.8064
Day of adult gait	10.7 ± 0.2	12.5 ± 0.5**	11.6 ± 0.3*	F (2, 31) = 8,26, P=0,0013
Auditory startle reflex	12.2 ± 0.1	13.1 ± 0.2**	12.3 ± 0.3	F (2, 37) = 5,90, P=0,006

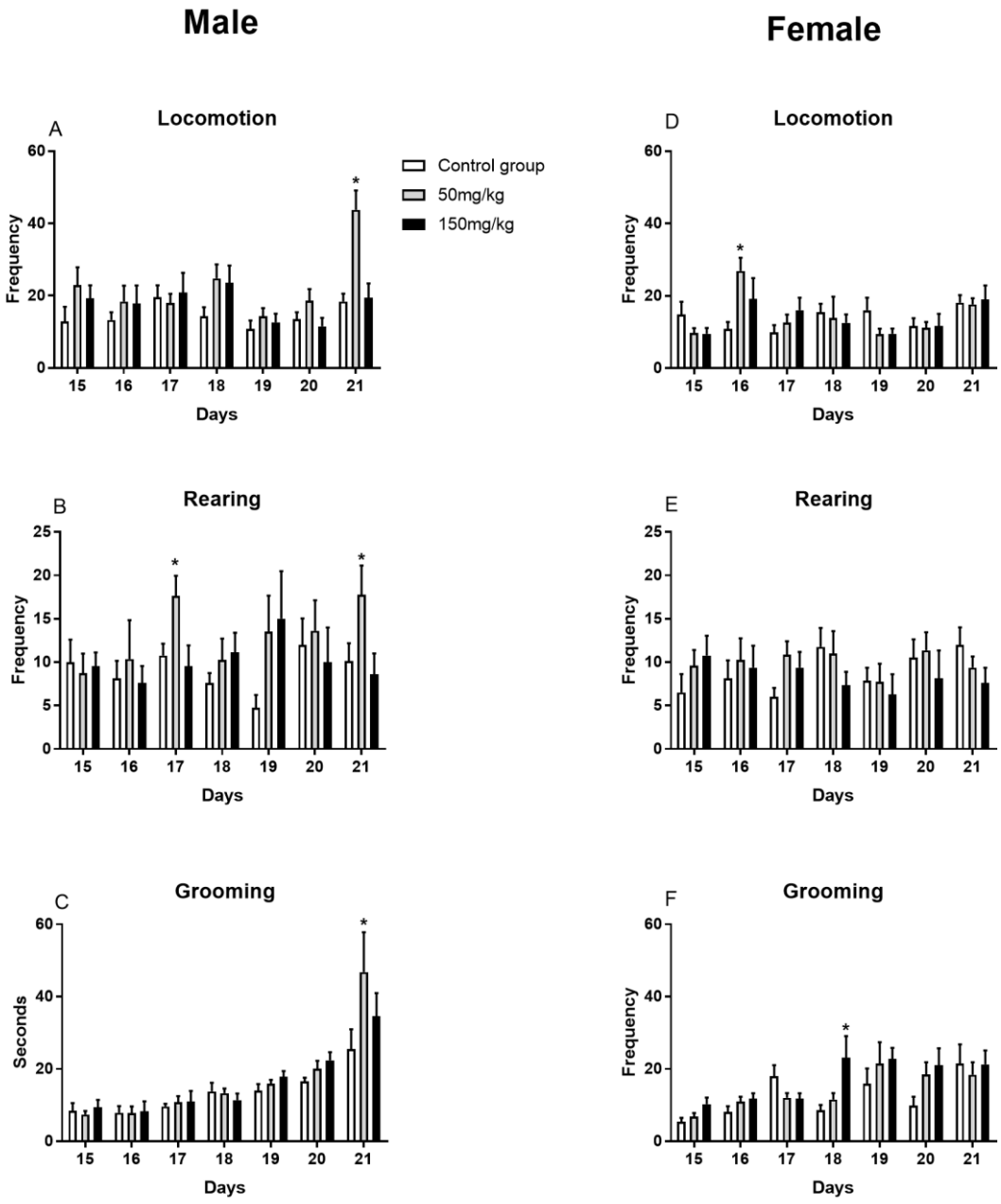


Figure 3. General activity of male and female pup rats observed from the lactation day 15 to weaning from dams exposed to 50 or 150 mg/kg of GLY-BH herbicide (by gavage, during gestational day 15 (GD15) until 7º lactation day (LD7). Data are presented as mean ± SEM. Two-way ANOVA followed by the Tukey's multiple comparisons test. * p< 0.05 relative to control group.

4. DISCUSSION

Maternal GLY-BH exposure reduced significantly the maternal behavior and maternal aggressive maternal behavior, showing a reduced maternal and defensive care of pups.

The regulation of maternal behavior in rats occurs in two phases (Numan, 1994). Firstly, the natural onset of maternal behavior occurs at parturition and is controlled by several pregnancy-related hormones (estrogen, progesterone, prolactin and oxytocin). Secondly, the maintenance of maternal behavior occurs during the postpartum period and is controlled primarily by nonhormonal factors (e.g., the multisensory stimuli provided by pups).

In female rats, high levels of nuclear estrogen receptors are control by the medial preoptic area (MPOA), the main region for mediating estrogen stimulation of maternal behavior, as well as the medial and cortical amygdale, the nucleus accumbens, and the paraventricular nucleus. In addition, other limbic and hypothalamic structures are normally established during the second half of pregnancy and are necessary for estrogen stimulation to maternal behavior(Numan *et al.* 2006).Taken together, the present results suggest that GLY-BH administered at the second half of pregnancy induced hormonal deregulations that impairs the maternal behavior.

Retrieval and nesting behavior are indicative of maternal motivation, representing an active voluntary response that reflects interest and attraction toward pup-related stimuli ,(Olazábal *et al.*, 2013). In fact, transport and protection of young, nursing, and nestbuilding behavior can be easily understood as important for infant survival. Nursing behavior may be more indicative of a reflexive maternal response ,(Teodorov *et al.* 2010). Nursing may also be transiently activated as a reflex when the female wanders near the pups and they crawl under her (Stern e Lonstein, 2001). Overall, in the present study, results suggest that these maternal reflexes and motivation decreased by GLY-BH exposure, since the time of pup retrieval was increased, and the number of pups retrieved decreased with a high dose of GLY-BH.

Furthermore, decreased maternal grooming in both GLY-BH doses was also observed. In the rat, particularly the maternal licking and grooming, are important regulators of pups' development of endocrine and emotional responses to stress (Champagne e Curley, 2009). Thus, this lack of pup

grooming by the mother can alter the pup's endocrine development and its response to stress later in life (Suchecki, 2018).

Maternal defense is a behavior showed by all dams to defend its litter against a potential threat, and could be observed by the a dam's aggressive behavior toward an intruder (Bosch, 2011). The function of maternal aggression has been suggested to protect pups from infanticide of non-parental conspecifics or usurpation of the mother's territory by intruders (Lonstein, 2005). Overall, the present results showed that maternal aggressive behavior decreased in females treated with GLY-BH, mainly in the high dose. Therefore, this pesticide induces dams to not guarantee the protection of its pups from environmental threats. In fact, during maternal aggressive behavior, the dams showed increased time to first fight and decreased frequency to fight the intruder. However, the frequency of the intruder sniffing pups was decreased in both GLY-BH doses. Thus, perinatal GLY-BH exposure decreased maternal aggressive behavior(Zhang *et al.*, 2004).

For Rosenblatt *et al*. (1994), nest defense or maternal aggression is an integral part of the pattern of maternal behavior in the rat, distinguished by being directed at an intruding animal that approaches the nest and litter. It differs in many ways from maternal care, which is directed at the young, and from nest building while maternal aggression is dependent upon the young. Together, maternal care and maternal aggression comprise the pattern of maternal behavior.

In the open-field test, we observed a decreased locomotion and rearing behaviors and an increased immobility time after both GLY-BH doses. Thus, a motor impairment cannot be ruled out on the decreased maternal care despite only the high GLY-BH dose reduced the pup's retrieval.

As DA systems are involved with motivation (Ikemoto e Bonci, 2014), and with the motor control (Grillner e Robertson, 2016), the levels of striatal and hypothalamic levels of DA and its metabolites were studied. In addition, the serotonin and its metabolite were measured because there are evidences that GLY-BH repeated administration depletes serotonin (Anadón *et al.*, 2008).

The present data showed a decreased striatal DA and DA metabolite levels, which might be consistent with observed motivational and motor impairments in

female rats perinatally exposed to GLY-BH. Concerning the striatal and mesolimbic dopaminergic systems and maternal behavior (Teodorov *et al.* 2010), extensive lesions of the dopaminergic system may impair maternal behavior. In fact, dopamine receptor antagonists infused locally into the nucleus accumbens inhibited the retrieval and licking components of maternal behavior, suggesting that these behaviors require DA. Increased levels of DA and its metabolites DOPAC and HVA in the extracellular space of the ventral striatum after separated mother rats and pups suggest a role in the motivational components of the behavioral response (Silva *et al.,* 2001).

Regarding dopaminergic and serotoninergic central systems effects of exposure during 5 days to 75, 150 and 800 mg/kg of GLY-BH to rats. Anodon *et al*.(2008) showed that this herbicide caused depletion of serotonin, dopamine and its metabolites in a dose-dependent way, in the frontal cortex, midbrain and striatum. More recently, Hernández-Plata et al. (2015) showed that repeated injections in male Sprague-Dawley rats of 50, 100, or 150 mg GLY-BH/kg over 2 weeks (three injections per week) caused locomotor hypoactivity immediately after each injection, which was also observed 2 days after the last injection in the rats exposed to the highest dose.

GLY-BH decreased specific binding to D1-DA receptors in the nucleus accumbens, when measured 2 days after the last GLY-BH injection. Moreover, GLY-BH decreased basal extracellular DA levels and high-potassium-induced DA release in the striatum (Hernández-Plata *et al.*, 2015). In fact, repeated GLY-BH exposure promoted hypoactivity by decreased in specific binding to D1-DA receptors in the nucleus accumbens, and acute exposure to GLY-BH affects the striatal DA levels. Therefore, we suggest that the impaired general activity, maternal behavior and maternal aggressive behavior induced by the herbicide are, in part, consequence of blockage of the striatal and mesolimbic systems. Furthermore, because excessive grooming decreased by D1 receptor antagonist SCH 23390 (Wimersma Greidanus, Van *et al.*, 1989), the decreased maternal grooming behavior could be resulted from the perinatal exposure to the herbicide.

These dopaminergic improvements were not restricted to one brain area, but to the striatum and hypothalamus. Hypothalamus plays a critical role for grooming control. There is a specific area known as hypothalamic "grooming area", which

consists of parts of the hypothalamic paraventricular nucleus and of the dorsal hypothalamic area (Roeling *et al.* 1993).

Oxytocinergic neurons are present in the hypothalamic "grooming area", thus, oxytocin is possibly involved in grooming behavior (Roeling, *et al.*, 1993). Grooming behavior induced by oxytocin is mediated by dopaminergic and opioid neurotransmission and administration of haloperidol completely blocked oxytocin-induced grooming(Drago *et al.*, 1986). Therefore, it is possible that the decreased hypothalamic dopaminergic activity is involved with the decreased of maternal grooming behavior by oxytocinergic pathways.

Maternal responses in rodents include a wide variety of behaviors, ranging from nursing, pup retrieval and nest building to maternal aggression. Dopamine has been strongly associated with the onset and maintenance of maternal behavior as well as pup-induced maternal behavior (Silva *et al*. 2001), while serotonin is more strongly associated with aggression that could affect maternal behavior ((Johns *et al.*, 2005). Maternal defense is a behavior showed by all dams to defend their litter against a potential threat and it manifests as a dam's aggressive behavior toward an intruder (Olivier e Mos, 1992). The function of maternal aggression has been suggested to protect pups from infanticide of non-parental conspecifics or usurpation of the mother's territory by intruders (Lonstein, 2005). Several studies showed decreased aggression as a result of increased serotonin levels. However, maternal aggressive behavior depends on the serotonin receptor subtype that was stimulated by an agonist or antagonist (Olivier e Mos, 1992).Therefore, GLY-BH exposure during prenatal and postnatal periods can alter the serotoninergic system and, consequently, maternal care. Each component of the maternal repertoire seems to be associated with a unique neuronal circuit, of which serotonin is one of the main components. Previous studies showed that the most important areas related to maternal behavior and maternal aggressive behavior are: (i) the hypothalamic medial preoptic area, (ii) the limbic bed nucleus of the *stria terminalis,* (iii) amygdalar complex, (iv) nucleus accumbens and (v) ventral tegmental area (Teodorov *et al.,* 2010). Taken together, the present results showed changes in serotonin activity on striatal areas caused by GLY-BH exposure, suggesting a deregulation of this system and non-monotonic dose response, once we observed increased turnover in the low GLY-BH dose and decreased GLY-BH in the highest dose.

Serotonin acts either indirectly or directly, and this neurotransmitter has a very important influence in the regulation of maternal behavior (Angoa-Perez e Kuhn, 2015). By indirect action, serotonin receptors (5HTIA, 5HT2A and 5HT2C receptors) have been described as mediators of the activation of hypothalamic-pituitary-adrenocortical function, and direct stimulation of at least these types of receptors mediate the release of oxytocin (Jørgensen *et al.*, 2003), prolactin and adrenocorticotropic hormone (Bagdy, 1995). Besides, the regulation of these molecules is fundamental for maternal behaviors, and serotonin can also influence other important parameters, such as the expression of estrogen receptors (Ito *et al.*, 2014) and the secretion of vasopressin (Jørgensen *et al.*, 2003). The direct action of serotonin on maternal behavior was based on pharmacological manipulations, as well as transgenic models designed to target more specific elements within the serotonin system (Angoa-Pérez e Kuhn, 2015). Thus, we suggest that exposure to GLY-BH alters the basal 5-HT levels and its metabolites and this may have contributed directly or indirectly to impaired maternal care. However, further studies are necessary to understand the link between GLY-BH, 5-HT and maternal care.

The impairment in maternal care and maternal aggressive behavior can be attributed to dopaminergic hypoactivity in the striatum (Hernández-Plata *et al.*, 2015), caused by repeated exposure to GLY-BH during gestation and lactation. In addition, the reduced activity in the open field test was also a likely consequence of the dopaminergic hypoactivity. Regarding the striatum serotonin, GLY-BH exposure during prenatal and postnatal periods in dams caused a deregulation of this system and non-monotonic dose-response.

Despite GLY-BH-induced maternal impairment, rat pups' BW gain was not affected by the maternal treatment suggesting that mothers nurture properly their pups. Moreover, physical development was not modified by perinatal exposure to the pesticide, except for the vaginal opening. The external sign of female puberty is vaginal canalization, when the vaginal channel opens, which usually occurs a day after the first course of gonadotropin release (Terasawa, 2001). Perinatal exposure to 50 mg/kg of GLY-BH delayed the day of vaginal opening, which denoted possible interference with gonadotropin release.

The righting reflex and surface righting reflex responses of rats reflect both motor development and activity that are guided by the vestibular system and by

the cerebellum (Altman e Sudarshan, 1975), which imply in a possible interference of GLY-BH maternal exposure with these brain areas.

In rats, the adult gait develops from rostral–caudal as well as ventral–dorsal gradient during nervous system development (Shriner *et al.*, 2009). In fact, delay in the day of adult gait suggest that maternal perinatal exposure to GLY-BH also affected the temporal pattern of neurogenesis in spinal cord of rats (Nornes e Das, 1974).

The regulation of postural adjustments include vestibular, exteroceptive (e.g.,tactile) and proprioceptive systems (Altman e Sudarshan, 1975). A delay in the maturation of the surface righting reflex, which occurred in pups of dams that were exposed to 150 mg/kg, suggests an influence on the maturation of CNS structures and a possible damage primarily in neuronal myelination (WU, 2008).

Startle responses in rats can be induced by the stimulation of the cochlear nuclear complex and by the activation of trigeminal and/or vestibular systems (Yeomans, 2002).The present results showed a delay for the startle reflex appearance in pups from dams that were exposed to 50 mg/kg, suggesting an influence on the maturation of the vestibular system.

However, it remains to be investigated whether these impairments on reflexological and motor development were a consequence from the release of GLY-BH via milk or indirectly by the reduced maternal care. Finally, the possible late consequences of this maternal exposure to the herbicide are being studied.

5. CONCLUSIONS

In conclusion, the perinatal exposure to GLY-BH reduced the maternal care by a decreased striatal dopaminergic activity and delays the pups reflexological development.

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7. AUTHORS CONTRIBUTIONS

All authors contributed equally in the work.

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