FULL ARTICLE

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Perinatal exposure to an aromatase inhibitor glyphosate-base herbicide reduced male and female social behavior in juvenile age and the sexual behavior at adult female rats

Exposição perinatal ao inibidor da aromatase a base de glifosato reduz o comportamento social de machos e fêmeas e o comportamento sexual de ratas

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ABSTRACT

Objectives: The herbicide glyphosate, a pesticide used in agriculture to control weeds, both in food crops and in other agricultural areas, has been identified as an endocrine modulator through the inhibition of aromatase activity and the activation of estrogen receptors. The present study examined the effects of a glyphosate-based herbicide (Roundup® (GLY-BH) on sexual dimorphism of rats after perinatal exposure to low and high GLY-BH in males and females offspring. Methods: Two groups of pregnant rats were treated with two doses of GLY-BH (50 or 150 mg/kg) from day 15 of gestation (GD15) to postnatal day 7 (PND7). Play fighting behavior was observed at the juvenile stage and during social and sexual behaviors in adulthood. Results: Perinatal GLY-BH exposure reduced male and female body weight at 28, 75, and 90 days of age. The play fighting behavior was decreased in both sexes, but female rats were more affected. The sexual behaviors were reduced only in females. Conclusions: Perinatal exposure to both doses of GLY-BH promoted sexually dimorphic effects in both juvenile and adulthood stages. These effects were attributed to the inhibition of aromatase activity induced by exposure to GLY-BH in the perinatal period.

Keywords: Herbicide. Sexual dimorphism. Social behavior. Perinatal treatment. Aromatase inhibitor.

RESUMO

Objetivos: O glifosato é um herbicida não seletivo, usado em muitas culturas alimentares e não alimentares e em áreas não agrícolas, sendo que os produtos a base de glifosato atuam como moduladores das funções endócrinas por meio da inibição da atividade da aromatase e da ativação de receptores de estrógeno. O presente estudo avaliou os efeitos do herbicida Roundup® (GLY-BH) à base de glifosato, em comportamentos sexualmente dimórficos de ratos após exposição perinatal a doses baixas e altas de GLY-BH no período perinatal. Métodos: Ratas prenhas foram tratadas com 50 ou 150 mg/kg de GLY-BH do 15º dia de gestação (GD15) ao 7º dia de lactação (LD7). O comportamento de luta/brincar foi observado na fase juvenil e os comportamentos social e sexual na idade adulta. Resultados: a exposição perinatal a GLY-BH reduziu o peso corporal de machos e fêmeas aos 28, 75 e 90 dias de idade. O comportamento de luta/brincar diminuiu em ambos os sexos, sendo as ratas foram as mais afetadas. O comportamento sexual foi reduzido apenas nas fêmeas. Conclusões: A exposição perinatal a ambas as doses do GLY- BH promoveu tanto na idade juvenil como na idade adulta, efeitos sexualmente dimórficos. Esses efeitos foram atribuídos à inibição da atividade da aromatase induzida exposição perinatal ao GLY-BH.

Palavras-chave: Herbicida. Dimorfismo sexual. Comportamento social. Tratamento perinatal. Inibidor de aromatase.

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Introduction

Glyphosate herbicide (N-(phosphonomethyl) glycine), is a pesticide used in agriculture to control weeds, both in food crops and in other agricultural areas. This pesticide, which is also extensively used as a desiccant for non-genetically modified crops (Sørensen et al., 2021), is a systemic pesticide that is considered nontoxic to humans (Samsel & Seneff, 2013). However, several studies have shown that perinatal exposure to glyphosate or glyphosate-based herbicides (GLY-BH) induced reproductive toxicity in non-target animals such as lambs (Kafshgiri et al., 2021), fish, (Muller et al., 2021; Socha et al., 2021), cows (Kafshgiri et al., 2021) and drosophila (Muller et al., 2021). In this respect, exposure to GLY-BH in pregnant mice induced ovarian failure, altered the steroidogenesis-related gene expression, and increased oxidative stress in fetuses (Ren et al., 2018). Other studies showed decreased female fertility (Ingaramo et al., 2016; Guerrero Schimpf et al., 2017) and post-implantation resorption (Ingaramo et al., 2016). Recently, glyphosate was associated with an increased risk for thyroid diseases (Shrestha et al., 2018; Kongtip et al., 2019; Romano et al., 2021), the incidence of Non-Hodgkin lymphoma (Leon et al., 2019), and possible carcinogenic risk to humans (International Agency for Research on Cancer, 2015). The controversial endocrine properties of glyphosate and glyphosate-based herbicides were discussed recently by de Araújo-Ramos et al. (2021).

Sexual reproduction in vertebrates shows sexually differentiated behaviors to establish attraction between partners and lead to mating. Sexual dimorphic behaviors are crucial to the success of sexual reproduction to allow species survival (Semaan & Kauffman, 2010). This process is induced by sex differences in the central nervous function induced by gonadal hormones secreted early in development. In rodents, sexual differentiation occurs from the end of pregnancy until the first days of lactation Welsh et al., 2014). Thus, exposure to endocrine disruptors in these periods could interfere with male and female sexual behavior (Martini et al., 2020)

In this respect, maternal exposure to Roundup Transorb®, a commercial product of glyphosate, disrupted the masculinization process, induced behavioral changes, and histological and endocrine disturbances on the reproductive parameters of rats. These effects are associated with the hypersecretion of androgens, an increase in gonadal activity, and sperm production (Romano et al., 2012). Richard et al. (2005) showed inhibition of aromatase activity, which was not observed with the original chemical. Aromatase, an enzyme that converts androgens to estrogens, is found in gonads and the brain. Thongprakaisang et al. (2013) demonstrated Roundup Transorb® activated estrogen receptors, similarly to estradiol. These effects were attributed to an enhanced glyphosate bioavailability and/or bioaccumulation induced by the presence of Roundup adjuvants.

Concerning social behavior, evidence showed that endocrine disruptors affect the developing neuroendocrine systems leading to injuries in the juvenile, adolescent, and adult age in exposed individuals, and even in their descendants (Gore et al., 2019). Few studies examined the dual sex effects of chemicals to comparative analysis, particularly in respect to endocrine disruptors (Sandberg & Umans, 2015). This is also true for glyphosate-based herbicides. Therefore, this study took into consideration the effects of glyphosate on sexual dimorphism of rats after perinatal exposure to a GLY-BH herbicide on the behavior of male and female offspring.

Play fighting behavior at the juvenile stage and the social and sexual behavior at adult age were observed. Play behavior was chosen because it is the first expression of social behavior that is not directed towards the mothers (Vanderschuren et al., 1997). Usually, males show more play-fighting behavior than females, because it depends on the action of perinatal androgens (Meaney & Stewart, 1981; Meaney & McEwen, 1986; Meaney, 1988; Pellis et al., 1997). As a result, the examination of the possible consequences of sexual dimorphism in the adult age and on social behavior was also observed. Furthermore, male and female sexual behavior were observed because such behaviors are correlated to aromatase activity during the neonatal period. Inhibition of the enzyme's activity during the neonatal period could interfere with the rodents' brain

development of the sexual organization (Gonzalez & Leret, 1994; Gerardin et al., 2008).

Materials and Methods

Animals

Wistar rats were obtained from the Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo. For mating, the female rat was placed with one sexually experienced male (2 female/one male). The onset of pregnancy (gestation day 0) was confirmed by the presence of spermatozoa in female vaginal smears. Then, the pregnant females were housed in a temperature-controlled room between 22 ± 2°C, artificial lighting of 12 h light/12 h dark cycle, and humidity 55-65%. These females were maintained on/cage (32 x 40 x 18 cm) and received the rodent chow (Nuvilab®, Nuvital, São Paulo, Brazil) and filtered water during the experiments.

Glyphosate based-herbicide (GLY-BH)

The Roundup Transorb® from Monsanto of Brazil Ltd, São Paulo, Brazil, were used. The formulation is composed of 480 g/L of glyphosate, 648 g/L of isopropylamine salt, and 594 g/L of inert ingredients. The doses administered were 50 and 150 mg/kg of GLY-BH based on previous studies (Rocha et al., 2019), which showed that oral administration of these doses during pregnancy and lactation did not induce overt signs of maternal toxicity. These doses were below the 409 mg/kg/day, the NOAEL chronic toxic dose for rodents (Williams et al., 2000).

Exposure and experimental design

At gestation day 0 (GD0) the females were weighed, randomly divided into three groups, and housed individually. The control group received orally by gavage the vehicle (1 ml/kg of water). The experimental groups (n = 7-8 rats per group) were treated daily with 50 or 150 mg/kg of GLY-BH (1 ml/kg body weight by gavage), from GD15 to postnatal day 7th (PND7), except in the day of parturition. To avoid maternal cannibalism on the day of birth (PND1) no handling was performed. On PND2, the pups were randomly selected (6-8 pups/female) and remained until weaning (PND21) with their respective dams. This procedure was done to standardize the feeding of the offspring as the females have only 8 breasts, avoiding differences in the offspring's body weight. The remaining pups were submitted to euthanasia. On weaning day (PND21), the male and female rat pups were housed, according to sex and treatment under the same conditions as their dams. To reduce potential confounding factors associated with the litter, one rat from each litter was used for the experiments (Kirsten et al., 2015). The male and female puppies' body weight (BW) was measured at 28, 75, and 90 days of age, and the play fighting behavior was evaluated at juvenile age (PND31). At PND90-95 (adult age), both male and female offspring were evaluated on social and sexual behavior.

Behavioral procedures

The play fighting behavior

The playing behavior was evaluated in the DPN30 or DPN31, as in this period the rat exhibits the behavior with greater intensity (Pletnikov et al., 1999) and when the animal is isolated previously, there is an exacerbation of episodes of this social interaction during the subsequent pairing of animals (File & Seth, 2003). Thus, in DPN25 the rats of each sex were kept in two housing conditions: one isolated (alone in the housing box, but in the same room with other animals) and another in a group of two animals per housing box (from the same litter). These isolated or grouped animals remained under these conditions until the DPN30.

In the DPN30, the test consisted of introducing a grouped animal (intruder) into the housing of the isolated animal (resident), taking care that the individuals had similar weights (difference of up to 10 g). The pairing was always conducted with animals of the same treatment and same-sex. That is, controls were confronted with controls, and experimental were confronted with experimental.

Immediately before the beginning of the experiment, between 9:00 am and 11:00 am, the boxes of an isolated animal and a grouped pair were taken to the behavioral observation room, for 5 min of acclimatization. After this period, an intruder was placed in a resident's box, starting the 10-min filming.

Based on a Himmler et al. (2013) study, the following parameters were measured: 1) pinning behavior (defined as the frequency of an animal on its back with its partner standing over it and counted for both animals because both were actively involved); 2) frequency of partial rotation (defined as the frequency of incomplete partial pinning, with the rat turning its forequarters); 3) frequency of attacks (defined as the frequency of approaching another pup from the rear and then pouncing towards the nape of its neck); 4) counterattack (defined as the frequency in which the defender pup turns to face its attacker, thus not only withdrawing its nape away, but also juxtaposing its teeth between its nape and the attacker's snout); 5) dodges

frequency (the number of times the mouse evades an attack), and 6) immobility (time without movements, measured in seconds).

Between each play session, the bedding was removed, and the play apparatus was thoroughly cleaned using 5% alcohol/water solution for the elimination of odors from the previous pair. Then, new bedding was added.

Open field behavior

The open-field test was observed at PND60 (Sandini et al., 2014). Initially, the rats were individually placed in the center of the arena and immobility time in seconds, the locomotion and rearing frequencies were observed for 5 min. Between each observation, the open field was cleaned with an alcohol/water solution (5%).

Social interaction in adult age

The social interaction was observed between PND 90-95 (Novaes et al., 2012). The social apparatus was a rectangular three-chambered box. The dimension of each chamber was 41 cm (length) × 26 cm (width) × 39 cm (height). Dividing walls were made from clear acrylic doors, with small openings that allowed access between the chambers. In each of the lateral chambers, a small wire cage was placed (30x15x19cm). Before observations, the rat test was introduced in the central chamber for 10 min for habituation with the small doors closed. An unfamiliar rat was placed in one of the small, wired cages, while the other remained empty. The small doors were opened, and the behavior of the experimental rat was videotaped for 10 min. The following parameters were evaluated by two researchers who were blinded to the treatment conditions: i) the number of entries in each chamber; ii) time in seconds in each chamber; iii) frequency of nose poking towards the unfamiliar rat. After each session, the apparatus was cleaned with a 5% alcohol/water solution.

Sexual behavior

Sexual behavior was observed in a wooden box (56 x 35 x 31 cm), painted gray, with a glass front wall, an upper movable cover, and with bedding of a layer of sawdust. The behavioral studies were performed during the dark phase of the cycle, from 14:00 to 18:00 h in a room only illuminated by a fluorescent infrared lamp (Bernardi et al., 2010).

Male sexual behavior

The test was performed with inexperienced males between 90-95 days of age as described previously (Bernardi et al., 2011). The male rat was placed with adult females of our facilities in pharmacological estrus. These females were previously ovariectomized and housed in cages in a room with a partially reversed light-dark cycle. The female estrus was induced by the administration of 50.0 μg/kg 17-b estradiol and 2 mg/kg progesterone (s.c), 54 and 6 h before the experiment, respectively. For observations, the male rats were introduced in the cage of observation for habituation (5 min.). Then, the female was also introduced in the cage of observation and the session of male rat sexual behavior lasted 30 min. The parameters of sexual behavior assessed were: i) latencies to first mount, first intromission, first ejaculation, (ii) number of total mounts with or without intromissions, and (iii) number of ejaculations in 30 min.

Female sexual behavior

For the female sexual behavior observations, the female was ovariectomized, maintained in a room with a partially reversed light-dark cycle (Ferri et al., 2013) for 3 weeks, which corresponds to the time necessary for the complete elimination of ovarian hormones (Yasuhara et al., 2005). The estrous was induced by s.c. 17-b-estradiol (50.0 mg/kg) and progesterone (2.0 mg/kg), respectively, 54 and 6 h before pairing the females with an adult sexually experienced male.

The evaluated parameters were: (i) latency to the first lordosis, (ii) number of lordosis, (iii) number of mounts without lordosis. Each experimental female was evaluated once.

Statistical analysis

Homoscedasticity was verified using the F-test or Bartlett's test and normality by the Kolmogorov-Smirnov test. To analyze more than two groups and one factor, the one-way analysis of variance (ANOVA) followed by Tukey's multiple or Dunnett's multiple comparisons test was used. The two-way ANOVA followed by Tukey's multiple comparisons test was used to compare data of more than two groups and two factors. Data were presented as the means ± SEM. The levels of significance were considered if $\alpha < 0.05$. The statistical analyses were performed using GraphPad Prism® software.

Results

Body weight

Figure 1 shows the body weight of male and female rats measured at the 28, 75, and 90 days of age, exposed or not to perinatal GLY-BH.

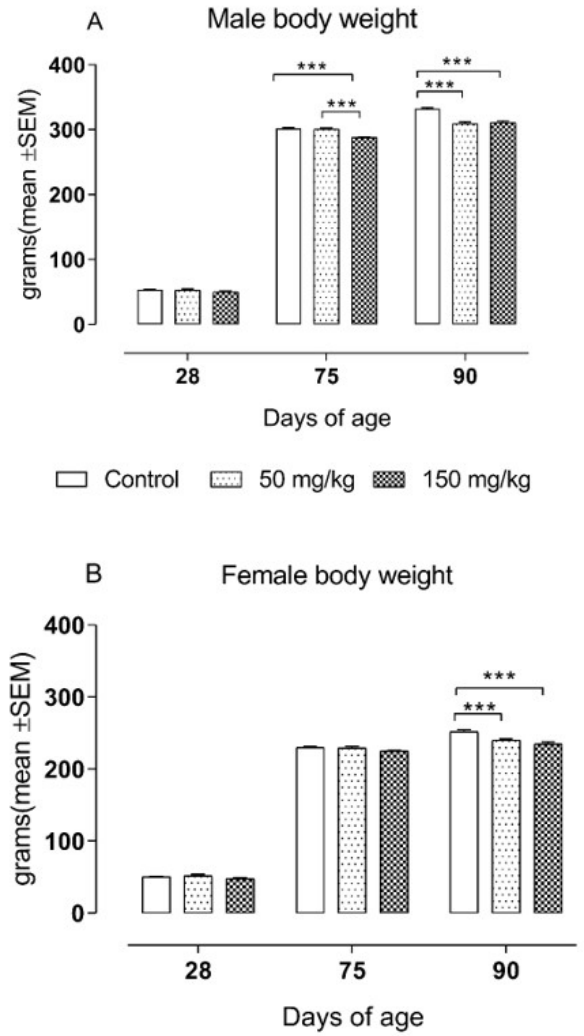

Figure 1 – Male and female body weight of rats from dams perinatally exposed to 50 or 150 mg/kg of GLY-BH, *per oz,* from gestational day 15 until postnatal day 7. The results are presented as mean ± SEM. N = 8/group). *** p< 0.001. Two-way analysis of variance followed by Tukey's multiple comparisons test.

In male rats (Figure 1A), significant differences were observed between treatments (F (2, 63) = 0.20886, p<0.0001) and days of age (F (2, 63) = 41.13, p<0.0001) with interaction (F (4, 63) = 16.48, p<0.0001). Our results demonstrated a decreased body weight at PND75 and PND90 in male offspring treated with 150 mg/kg GLY-BH and at PND90 in male offspring treated with 50 mg/kg GLY-BH when either group was compared to respective controls. Furthermore, at PND75, the body weight of the 150 mg/kg treatment group remained smaller compared to the 50 mg/kg male GLY-BH treatment group.

The body weight of female offspring (Figure 1B) was influenced by treatment (F (2, 63) = 14.44, p <0,0001) and days of age (F (2, 63) = 10155, p<0,0001) with interaction (F (4, 63) = 5,653, p=0,0006). Our results demonstrated a decrease in body weight at PND90 in female offspring treated with GLY-BH at both doses when compared to respective controls.

The play fighting behavior

Data of play fighting behavior of the pups are shown in Figure 2. Our results demonstrated that both doses of glyphosate impaired the pinning behavior in female GLY-BH treated offspring (Figure 2A), but not in the male-treated offspring. An effect of sex was observed in the frequency of attacks (Figure 2B) and counterattacks (Figure 2D), where female rats showed low levels when compared to males. While female 50 mg/kg GLY-BH treated offspring presented higher immobility time when compared to male control, treatment with 150 mg/kg GLY-BH suppressed this difference (Figure 2F). Statistical results are described in Supplementary Table I.

Male and female open-field behavior

No significant differences were observed in either male or female offspring in all behavioral parameters observed in the open field (data not shown).

Social behavior in adult age

The 50 mg/kg GLY-BH treated female offspring showed an increased frequency of social behavior in the unfamiliar rat chamber (Figure 3C); however, with decreased nose poking frequencies relative to the control group (Figure 3E); no differences were reported concerning sexual behavior. No differences were observed in either male or female GLY-BH treated offspring in other relative parameters that were measured. Statistical analysis is shown in Supplementary Table 2.

Male sexual behavior

Table 1 shows that no significant differences in male sexual behavior were observed between groups.

Female sexual behavior

The parameters of the sexual behavior of female rats are shown in Figure 4. Our results showed increased latencies to the first lordosis (F (2, 21) = 10.6, p=0.0007, Figure 4A) in GLY-BH treated offspring perinatally with 50 and 150 mg/kg with the control group. In addition, the number of lordosis was reduced in these rats relative to control (F (2, 21) = 11.69, p=0.0004, Figure 4B). The number of mounts was not different between groups (F (2, 21) = 0.0119, p=0.9882, Figure 4C).

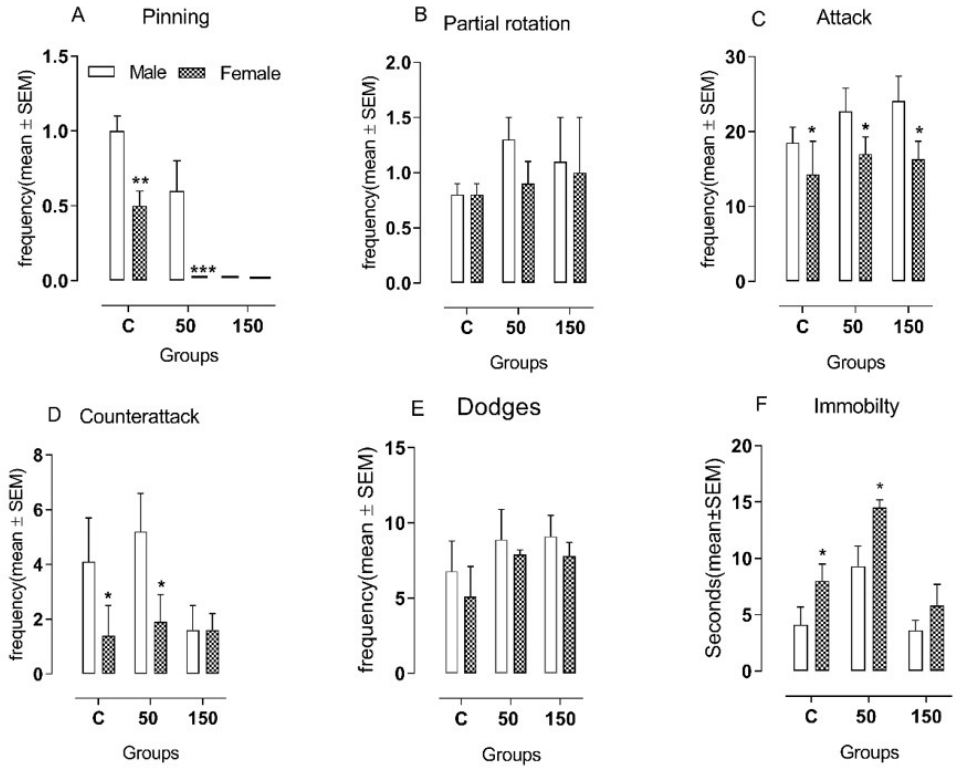

Figure 2 – Male and female play-fighting behavior from dams perinatally exposed to 50 or 150 mg/kg of GLY-BH, *per oz,* from gestational day 15 until postnatal day 7 observed in the juvenile age. - pinning frequency; -frequency of partial rotation; - attack frequency; - counterattack frequency; - dodges frequency; (F)- seconds of immobility. The results are presented as mean ± SEM. N = 6/group. * p< 0.05, ** p< 0.01 relative to male rat of the same treatment. Two-way ANOVA followed by Tukey's multiple comparisons test.

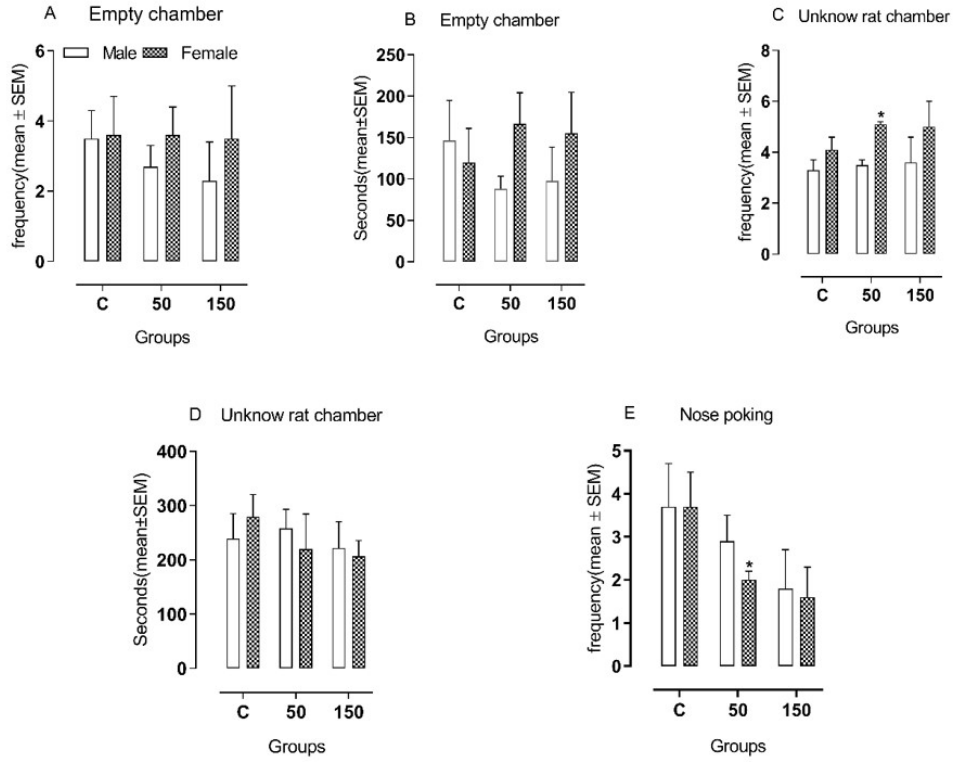

Figure 3 – Male and female social behavior from dams perinatally exposed to 50 or 150 mg/kg of GLY-BH, *per oz,* from gestational day 15 until postnatal day 7 observed in adult age. - empty chamber frequency; –seconds in the empty chamber; - unknown rat chamber frequency; - seconds in the unknown rat chamber; – frequency of nose poking. The results are presented as mean ± SEM. N=8/group. * P< 0.05 relative to male rats of the same treatment. Two-way ANOVA by Tukey's multiple comparisons test.

Table 1 – Male sexual behavior of rats from dams perinatally exposed to GLY-BH (50 or 150 mg/kg, by gavage*)* from gestational day 15 until postnatal day 7. The immobility time was presented as mean ± SEM of seconds. The locomotion and rearing were presented as mean ± SEM of frequencies. N = number of rats/groups

Parameters	Control group	Glyphosate (mg/kg)	
	(N=8)	50 (N=8)	150 (N=8)
Latency to first mount (s)	25.3±5.7	21.4±3.7	23.4±4.1
Latency to first intromission(s)	45.50±13.8	61.3±10.9	57.7±11.0
Latency to first ejaculation (s)	1846±347	1321±187	1449±199
Number of total mounts	33.7±10.0	30.1±5.4	28.7±6.3
Total ejaculations	3.73±1.0	5.65±0.8	3.69±0.6

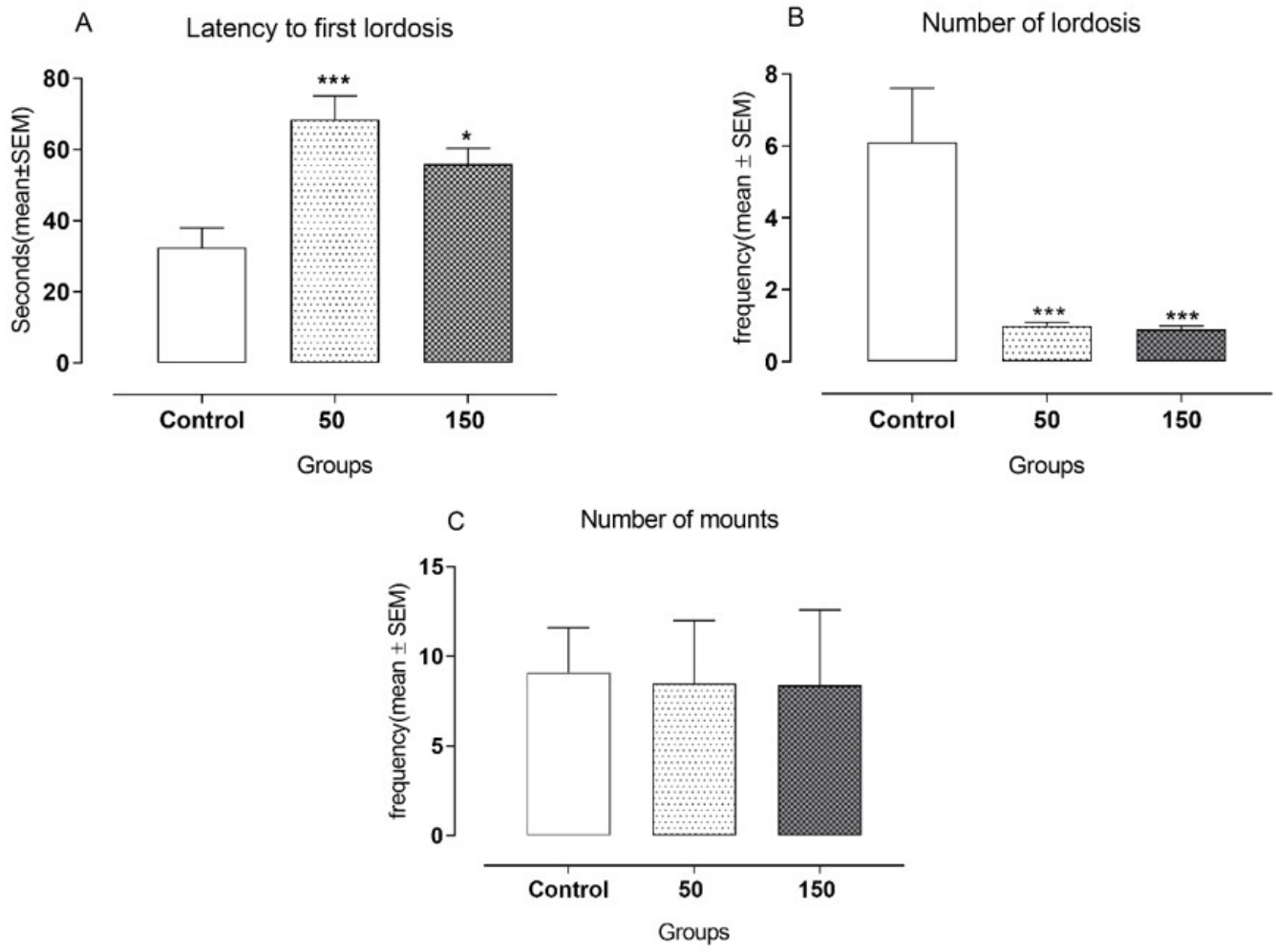

Figure 4 – Female sexual behavior of rats from dams perinatally exposed to 50 or 150 mg/kg of GLY-BH, *per oz,* from gestational day 15 until postnatal day 7. - latency to the first lordosis; -number of lordosis; -number of mounts. The results are presented as mean ± SEM. N = 8/group. * $p < 0.05$, *** $p < 0.001$. One-way ANOVA followed by Tukey's multiple or Dunnett's multiple comparisons test.

Discussion

Previously, it was observed that maternal exposure to both doses, 50 and 150 mg/kg of GLY-BH from the GD15 to PND7, had few effects on maternal body gain weight, although such treatment impaired maternal care of offspring (Rocha et al., 2019). Presently, male rats perinatally treated with 150 mg/kg GLY-BH showed decreased BW at 75 and 90 days of age, while in females, BW decrease was observed at 90 days of age. Also, both offspring perinatally with 50 mg/kg of GLY-BH demonstrated a reduction in BW at 90 days of age. Thus, we suggest that the decreased body weight of the rats at 75 and 90 days of age resulted from reduced maternal care. In addition, these findings indicate that male offspring were more susceptible to perinatal effects of GLY-BH when compared to female offspring.

The pinning behavior is considered the most important expression of play behavior (Panksepp & Beatty, 1980), in which the objective of the rat is to wrestle the opponent

onto its back and stand over him/her (Panksepp et al., 1984). The experience of pinning the opponent is considered critical to the development of sexual competence by males at adult age (Gollin, 1985). Juvenile males exhibit a greater frequency of pinning than females during play fighting (Pellis & Pellis, 1990), this behavior being sexually dimorphic. Our studies showed that this behavior was abolished in both male and female offspring perinatally exposed to the highest GLY-BH dose. Female rats from dams that received 50 mg/kg did not demonstrate impairment in pinning behavior, while male offspring showed significant impairment. Thus, these data corroborated the decreased pinning behavior observed in female control offspring; leading to an early effect on females from dams that were treated with 50 mg/kg. Additionally, it is known that pinning behavior can be influenced by the actions of steroid sex hormones during the perinatal period (Meaney et al., 1985).

The same explanation could be attributed to the counterattacks and the increased time of immobility in female control rats relative to male rats. We observed a reduced frequency of attacks and counterattacks and an increased immobility time in the female control group relative to male controls. As a result, both GLY-BH doses reduced the play fighting in male and female offspring, but this effect is independent of the GLY-BH perinatal exposure.

On social behavior, the female offspring from dams treated with 50 mg/kg showed an increased frequency of entering the unfamiliar rat chamber but, at the same time, showed a decrease in the frequency of nose poking. Thus, these females only explored the new rat but did not show a specific social interaction. Nose poking is a known important social behavior because it is related to collaborative social behavior (Łopuch & Popik, 2011). Due to observations of no effects on sex, we suggest that the decreased nose poking behavior was a consequence of perinatal exposure to the low GLY-BH dose only in female rat offspring.

Both GLY-BH doses reduced female sexual behavior while male sexual behavior was unaffected. Previous studies showed that Roundup formulation, as well as other glyphosate-based herbicides, could be responsible for the aromatase disruption *in vitro* and *in vivo* (Richard et al., 2005; Cassault-Meyer et al., 2014). The androgen/estrogen balance is controlled by aromatase, a key enzyme responsible for the irreversible conversion of androgens to estrogen (Simpson et al., 1994). As a result, we believe that the perinatal and lactational exposure to GLY-BH disrupted aromatase activity, leading to the impairment of sexual behavior in female offspring. In addition, a precocious vaginal opening was previously shown in female pups from dams perinatally treated with 50 mg/kg GLY-BH, but not with the perinatal treatment of 150 g/kg of the herbicide (Rocha et al., 2019). In this respect, the early sign of puberty in a female rat is the canalization or opening of the vagina, and this event is estrogen-dependent (Critchlow & Bar-Sela, 1967).

Before the first ovulation, the serum levels of aromatized androgens, testosterone, and androstenedione are increased (Uilenbroek et al., 1975; Parker & Mahesh, 1976; Andrews et al., 1980; Ojeda & Urbanki, 2004). In this respect, administration of aromatized androgens to prepubertal rats induces vaginal opening (Ojeda & Urbanki, 2004; Leibowitz et al., 2009), and its action on the vaginal epithelium is exerted after local aromatization and conversion of androgen to estrogens (Stocco, 2012). Therefore, inhibition of aromatase increases the time of androgen exposure/action, which is per the observed vaginal opening and reduced female sexual behavior. These results implicate GLY-BH action as an aromatase inhibitor. This finding agrees with previous studies showing that pre- and perinatal exposure to another aromatase inhibitor, letrozole, affected male and female rats' sexual orientation. Prenatal exposure to letrozole reduces the volume and hypothalamic cells number, which are proposed as the neural bases of sexual preference/orientation in male rats (Olvera-Hernández et al., 2017), and induces feminization of male rats (Olvera-Hernández et al., 2015). Moreover, neonatal exposure to letrozole impaired male sexual competence (Gerardin et al., 2008) and induced male feminization (Olvera-Hernández & Fernández-Guasti, 2015). Furthermore, perinatal exposure to another aromatase inhibitor, clomiphene citrate, reduced male and female sexual behavior and induced female masculinization (Oliani et al., 2015) as well as male feminization (Pereira et al., 2003). These data suggest that females were more sensitive to perinatal exposure to treatment with GHY-BH. Finally, we attribute GLY-BH impairment of female sexual behavior to an indirect hormonal effect through inhibition of aromatase enzyme during perinatal exposure.

Conclusion

Perinatal exposure to both GLY-BH assessed doses decreases male and female play fighting behavior at the juvenile age. In adult age, the sexual behavior was reduced only in female offspring. These sexual dimorphic effects were attributed to the inhibition of aromatase activity induced by the perinatal exposure to GLY-BH herbicide.

Conflict of Interest

All authors declare no conflict of interest.

Ethics Statement

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the School of Veterinary Medicine, University of São Paulo, Brazil (permit no 2010/2010). All efforts were made to minimize suffering. The experiments were performed in accordance with good laboratory practice protocols and with quality assurance methods.

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Supplementary Material

Supplementary Table 1 - Play fighting was observed at 30 days of the age of rats from dams perinatally exposed to 50 or 150 mg/kg of GLY-BH by gavage from gestational day 15 until postnatal day 7. The immobility time was presented as mean ± SEM of seconds. The remained parameters were presented as mean ± SEM of frequencies. N = number of pairs.

Parameters	Control group	GLY-BH (mg/kg)		
		50	150	F (2,15); p
		male		
	(N=6)	(N=6)	(N=6)	
Pinning	1.0±0.1	0.6±0.2	0*	14.88; 0.0003
Partial rotation	0.8±0.1	1.3±0.2	1.1±0.4	2.48; 0.12
Attack	18.5±2.1	22.7±3.1	24.1±3.3	1.01;0.38
Counterattack	4.1±1.6	5.2±1.3	1.6±0.9	2.01; 0.17
Dodges	6.8±2.0	8.9±2.0	9.1±1.4	0.49; 0.62
Immobility (s)	4.1±1.6	9.3±1.8	3.6±0.9#	4.52; 0.03
		female		
	(N=6)	(N=6)	(N=6)	
Pinning	0.5±0.1	0*	0*	24.01; <0.0001
Partial rotation	0.8±0.1	0.9±0.2	1.0±0.5	0.10; 0.91
Attack	14.3±4.4	17.0±2.3	16.3±2.4	0.19; 0.82
Counterattack	1.4±1.1	1.9±1.0	1.6±0.6	0.07; 0.93
Dodges	5.1±2.0	7.9±0.3	7.8±0.9	1.55; 0.25
Immobility	8.0±1.5	14.5±0.7*	5.8±1.9	9.67; 0.002

One-way ANOVA was followed by the Bonferroni test. * Significant different relative to the control group and # significantly different from 50 mg/kg.

Supplementary Table 2 - Social interaction was observed at the adult age of rats from dams perinatally exposed to 50 or 150 mg/kg of GLY-BH by gavage from gestational day 15 until postnatal day 7. The times were presented as mean ± SEM of seconds. The remained parameters were presented as mean ± SEM of frequencies. N = number of pairs.

Parameters	Control group	GLY-BH (mg/kg)		
		50	150	F (2,21); p
Male	**(N=8)**	**(N=8)**	**(N=8)**	
Frequency in the empty chamber	3.5 ± 0.8	2.7 ± 0.6	2.3 ± 1.1	0.50; 0.61
Frequency in the unknow rat chamber	3.3 ± 0.4	3.5 ± 0.2	3.6 ± 1.0	0.06; 0.94
Time in the empty chamber (s)	146.5 ± 48.4	88.0 ± 15.3	97.6 ± 40.7	0.98; 0.39
Time in the unknow rat chamber (s)	239.3 ± 46.0	258.5 ± 34.6	221.9 ± 48.6	0.04; 0.99
Nose poking	3.7 ± 1.0	2.9 ± 0.6	1.8 ± 0.9	1,26; 0.30
Female	**(N=8)**	**(N=8)**	**(N=8)**	**F (2,21); p**
Frequency in the empty chamber	3.6 ± 1.1	3.6 ± 0.8	3.5 ± 1.5	0.02; 0.99
Frequency in the unknow rat chamber	4.1 ± 0.5	5.1 ± 0.1	5.0 ± 1.0	0.72; 0.50
Time in the empty chamber (s)	119.8 ± 41.1	166.3 ± 37.8	155.2 ± 49.6	0.32; 0.73
Time in the unknow rat chamber (s)	279.3 ± 41.1	220.0 ± 64.5	207.0 ± 28.7	0.67; 0.52
Nose poking	3.7 ± 0.8	2.0 ± 0.2	1.6 ± 0.7	3.1; 0.06