Effects of exposure to glyphosate in male and female mice behavior in pubertal period

Efeitos comportamentais da exposição na puberdade de camundongos machos e fêmeas ao glifosato

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Abstract

The present study aims to investigate the effects of pre-pubertal exposure of male and female mice to a commercial formulation of glyphosate on sexual dimorphism observed in animal models of emotionality, anxiety and depression. For this, mice were exposed from 23 days of age (PND) until PND 45 to glyphosate (50 mg/kg, *per os*) or saline solution, and, ten days after the end of treatments, male and female mice were observed in the open field (OF), elevated plus maze (EPM) or forced swimming test (FWT). Results showed that exposure to glyphosate: 1) reduced the locomotion frequency of male mice similarly to female mice in the OF and female mice had an increase in rearing behavior and in the immobility time; 2) reduced in male mice the motor activity both in the OF and EPM, while no effects were observed in female mice; 3) in the SWT male mice had a decreased time of float similarly female mice. We concluded that pre-pubertal exposure to glyphosate reduced in male mice the capacity of exploration in the OF and EPM tests suggesting that the herbicide interfered with the central mechanism related to brain masculinization of exploratory and anxiety behavioral models. In the FWT it was observed a decreased depressive response in male mice while in female an increased response was detected.

Keywords: Herbicide. Animal behavior. Sex differences. Exploration. Anxiety. Depression.

Resumo

O presente estudo teve como objetivo investigar em camundongos machos e fêmeas o efeito da exposição a uma formulação comercial de glifosato durante o período de pré-pubere em modelos comportamentais de emocionalidade, ansiedade e depressão. Para isto, camundongos foram expostos a partir de 23 dias de idade (dia pós-natal-PND) até o PND 45 ao glifosato (50 mg/kg, via oral) ou solução salina. Dez dias após o término do tratamento, os animais, machos e fêmeas, foram observados no campo aberto (OF), labirinto em cruz elevado (EPM) ou teste de natação forçada (FWT). Os resultados mostraram que a exposição ao glifosato: 1) reduziu de forma similar a frequência de locomoção dos camundongos em ambos os sexos; 2) reduziu a atividade motora tanto no OF como no PM em camundongos machos, sem alterações observadas em fêmeas; 3) no SWT os camundongos machos apresentaram redução no tempo de flutuação similar ao das fêmeas. Concluiu-se que a exposição pré-pubere ao glifosato reduziu em machos a capacidade de exploração no OF e EPM e no tempo de flutuação no FWT sugerindo que o herbicida interferiu com mecanismos centrais relacionados com masculinização do cérebro ligados à exploração e ansiedade. No FWT observou-se menor depressão em machos e exacerbação da resposta em fêmeas.

Palavras-chave: Herbicida. Comportamento animal. Diferenças sexuais. Exploração. Ansiedade. Depressão.

Introduction

It is known that brain differs between males and females and gender differences in rats were detected at morphological, neurochemical and functional levels (JACOBSON; GORSKI, 1981; WARD, 1984). These differences were primarily controlled by gonadal steroid hormone during the perinatal period

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– the organizational period – and in latter periods of life until adult age – the activating period (CHUNG; AUGER, 2013). Puberty is the transitional period between the juvenile and adult state in which sexual maturation begins in the hypothalamic-pituitary-gonadal (HPG) system, leading to the development of secondary sex characteristics and fertility (STOKER et al., 2000). Pubertal maturation of the HPG begins with activation of neurons that secrete gonadotropin-releasing hormone (GnRH), but its secretory activity is low and insufficient to support gonadal growth. The gradual increase in the frequency and amplitude of intermittent episodes of GnRH secretion is responsible by the onset of puberty (COX; SURGAN, 2006). GnRH are involved in the synthesis and secretion of the pituitary gonadotropins, luteinizing hormone, and follicle stimulating hormone, which stimulate the production of gonadal steroid hormones and complete the process of sperm and egg development (BECU-VILLALOBOS et al., 1997; STOKER et al., 2000). Thus, exposure to several compounds during puberty could dramatically alter male and female pubertal development, particularly in the establishment of sexual dimorphism.

Endocrine disruptors are exogenous anthropogenic chemicals (pesticides, herbicides, polychlorinated biphenyls, bisphenol A, polybrominated diphenyl ethers, phthalates and others), that are able to bind hormonal receptors of endocrine and other cells in vivo and act like hormones. These substances disrupt endocrine regulation of metabolism, reproduction and adaptive reactions of organisms and promote human and animal endocrine disorders (LAGLOVA; LAGLOV, 2012).

Glyphosate is a highly effective herbicide used to control weeds in several crops. According to the US Environmental Protection Agency, both glyphosate and its metabolite, aminomethylphosphonic acid (AMPA) are classified in the lower category of toxicity. However, commercial products contain surfactants that can contain acid glyphosate and adjuvants such as polyethoxylated tallowamine (COX; SURGAN, 2006). Its surfactants are generally considered dilutants for regulatory purposes. Several hormonal and reproductive effects were reported to this herbicide (GASNIER et al., 2009; ROMANO, 2012). Walsh et al. (2000) demonstrated that a glyphosate commercial formulation, inhibited steroidogenesis by disrupting expression of the steroidogenic acute regulatory (StAR) protein, showing that this commercial formulation of the herbicide has the potential to disrupt reproductive function in animals. It has also been shown to disrupt the animal cell cycle in urchin egg (MARC et al., 2004; MARC; MULNER-LORILLON; BELLE, 2004).

The glyphosate dose, employed (50 mg/kg), was chosen based on previous studies of our group (ROMANO et al., 2010) and in the NOAEL dose proposed by Lu (1995). The period of glyphosate administration (PND 23 to PND45) corresponds in male mice to the juvenile period, i.e., the pre-pubertal and pubertal period, at which time the first mature spermatozoa appear in the vas deferens (OJEDA; ADVIS; ANDREWS, 1980).

In this respect, one of the most important contributions of experimental studies about sex differences in the organization of the brain is the incidence of neurological and psychiatric diseases is also highly dependent on sex.

The present study aims to investigate the effects of pre-pubertal exposure of male and female mice to a commercial formulation of glyphosate on sexual dimorphism observed in animal models of emotionality, anxiety and depression.

Material and method

Animals

Male and female adult BALB/c mice, 14-16 weighing on average (15 ± 2g, n = 85), approximately 21 days old, provided by the School of Medicine Veterinary, São Paulo University were used. At arrival, the animals were housed in individual microisolator

cages with controlled temperature (22-26°C) and humidity (50-65%) in an artificially lighted rooms on a 12-h light/12-h dark cycle (lights on at 7:00 am) with free access to food and water. Animals were used in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources of the Universidade Paulista (Protocol CEP/ICS/UNIP 025/08). The experiments were performed in accordance with safe laboratory practice protocols and quality assurance methods.

Glyphosate

Experiments were completed using Roundup Transorb® (oral administration, 480 g/L of glyphosate, 648 g/L of isopropylamine salt and 594 g/L of inert ingredients, *per os* Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil).

Behavioral models

Open field test

The open field (OF) test was used to assess the effects of glyphosate exposure on emotionality and exploration. A white, circular, wooden arena was built as previously published by Broadhurst (1960) with modifications based on the size of the mice. The background of the arena is divided into three concentric circles, which in turn are divided into 19 straight segments of equal areas. The circular wooden arena remains inside a wooden case 48 cm from the floor. The apparatus was placed in a sound-proof room with dim light (55 lux at the OF arena). For the OF test, each animal was placed in the center of the arena and was observed for 5 min. Animals in the control and experimental groups were observed alternately, in the light phase of the cycle, between 2:00 and 5:00 p.m. The OF was cleaned with a 5% alcohol solution between the sessions to remove any odors. The parameters assessed were the frequency of locomotion, rearing and defecation as well as immobility duration. A unit of locomotion was defined as the frequency of an animal entering with its four paws in one area of the arena floor. One unit of rearing corresponds to a standing position on the hindlimbs, with the trunk perpendicular to the floor, head tilted up and the forelimbs touching the walls of the arena. Immobility was defined as the period in seconds, during which the animal was not engaged in any motor activity (head, trunk and limbs were still). Rearing and locomotion frequencies were recorded with a manual counter, and immobility time was measured with a stopwatch.

Elevated plus-maze (EPM) test

The EPM is a commonly used test to evaluate anxiety (PELLOW et al., 1985). It was used a EPM made of black-painted wood with two open arms and two closed arms (25 x 5 x 15 cm). The apparatus was elevated 55 cm above the ground and placed in a sound-proof room with dim light (55 lux at the EPM arena). Each mouse was placed individually in the central square of the EPM apparatus and allowed 5 min of free exploration. The parameters recorded consisted of the number of entries into the open and closed arms, the time spent exploring the open and closed arms, time in the center and the total number of crossings the EPM center. Exploratory behavior was determined by the number of entries into the closed arms and the number of crosses in the center of the EPM. Data for frequency events were recorded with a manual counter, and the duration of time was measured with a stopwatch. The mice employed in this experiment were the same animals observed in the OF and they were observed immediately after the OF test.

Forced swim test (FWT)

The FWT proposed by Porsolt, Le Pichon and Jalfre (1977) is among the most used and most valid for testing antidepressant drugs in which the animal's immobility time decreased significantly due to the action of antidepressant drugs. For the test, it was used a glass container measuring 22 cm

in diameter and 40cm height with 19 cm water, the average temperature of 23-24°C. Each animal was placed inside this container so that it swam for five minutes (training session) and is then withdrawn from the container and dried. Twenty-four hours later, the mouse was placed back in the container for five minutes (test session) being annotated the latency and time to float. The animal is considered immobile when moving only to avoid sinking. This behavior indicates a state of hopelessness after the animal has learned that escape is impossible (PORSOLT; LE PICHON; JALFRE, 1977).

Experimental design

Groups of male and female mice received daily, by oral route, 50 mg/kg of glyphosate or water from the PND23 TO PND 45. The behavioral observations were made ten days after the end of treatments. To open field t and plus maze tests 53 mice were used (male control group, n = 11; male experimental group, n = 14; female control and experimental groups; n=14 per group). To forced swimming behavior 8 mice per group were employed. The behavioral observations were made ten days after the end of treatments.

Statistical analysis

Homoscedasticity was verified the Bartlett's test. Normality was verified using a Kolmogorov-Smirnov test. The two-way ANOVA was used followed by a Bonferroni test and the results are expressed as the mean ± SEM. The number of mice in each behavioral method was estimated based in previous studies of our laboratory showing a minimal number of subjects in each experiment. All results were considered significant if $p < 0.05$. The tendency was considered when $p < 0.06$.

Results

OF studies

The figure 1 shows the effects of male and female mice treated daily, by oral route, 50 mg/kg of glyphosate or water from the PND23 to PND45 in the OF test.

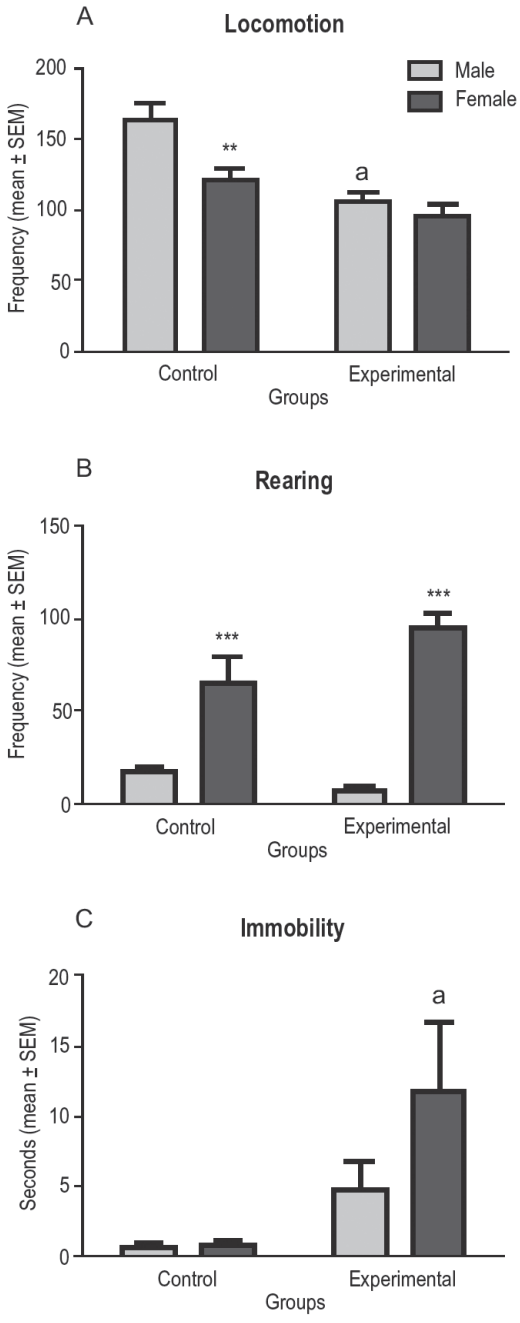

Figure 1 – Open field behavior of male and female mice treated with RT during the pre-pubertal period and observed ten days after the end of exposure. Graphic A- locomotion frequency; Graphic B- rearing frequency; Graphic C- immobility time (s). Data are presented as means ±SEM

**p < 0.01

*** p < 0.001 in relation to male rat of the same treatment

a p < 0.05 in relation to respective control group

Source: (JOAQUIM et al., 2014)

In locomotion frequency (Graphic A), the two-way ANOVA showed no interaction between factor

F1/49 = 3.72, p = 0.06). A significant difference between treatments (F1/49 = 22.52, p < 0.0001), sex (F1/49 = 9.18, p = 0.004) were observed. The Bonferroni test indicated a decreased locomotion frequency in female mice of control group relative to male mice of the same group (p < 0.01). Glyphosate administration reduced the locomotion of male mice (p = 0.0001) relative to male control group. No differences were observed between female groups.

An interaction between factors was observed in rearing behavior (F1/49 = 5.43, p = 0.024, Graphic B). The Bonferroni test indicated that female presented higher rearing frequency relative to male mice.

No interaction between factors was observed in the immobility time (F1/49 = 1.47, p = 0.23, Graphic C). The treatments modify the results (F1/49 = 7.11, p = 0.01) but not sex (F1/49 = 1.59, p = 0.21). The Bonferroni test indicates that the parameter was higher experimental group (p < 0.05) that the respective control group.

No interaction between factors was observed in defecation (F1/49 = 0.03, p = 0.80, data not show). Also, no significant differences were observed in defecation frequency induced by treatments (F1/49 = 0.92) or sex (F1/49 = 1.14, p = 0.29).

EPM studies

A significant interaction was observed between the factors in the time in the open arms (F1/49 = 7.45, p = 0.0008, Figure 2, Graphic A) but not in the time in closed arms (F1/49 = 1.76, p = 0.19, Figure 2, Graphic C). Treatments did not affected the time in the closed arms (F1/49= 0.27, p = 0.60) in male and female controls and experimental mice. Also, no differences were observed by sex in these parameters (time in closed arms - F1/42 = 0.40, p = 0.57).

An interaction was observed in the number of open arms entries (F1/49 = 12.60, p = 0.0009, Figure 2, Graphic B). A decreased in the number of entries in male experimental mice was observed relative to female experimental mice (p < 0.001). No differences were observed between both female control and experimental mice.

In relation to the number entries in the closed arms an interaction was observed (F1/49 = 4.30, p = 0.04, Figure 2, Graphic D). Experimental female mice presented an increased number of entries relative to the experimental male mice.

No interaction between factors was observed in the time of center (F1/49 = 0.49, p = 0.49, Figure 2, Graphic E). Relative to time in the center the treatment did not affected the results (F1/49 = 0.80, p = 0.37) but sex interfered (F1/49 = 11.42, p = 0.001). The Bonferroni test indicates that female control mice had an increased time in the center in relation to control male mice.

An interaction between factors was observed in relation to the number of crossings the EPM center (F1/49 = 4.41, p = 0.04, Figure 2, Graphic F). A decreased in male experimental mice was observed in this parameter relative to the respective control group.

FWT studies

No interaction was observed between factors in the latency to float (F1/28 = 18.62, p = 0.0002, Figure 3, Graphic A). The latency to float of female control mice was higher than male controls. No interaction was observed in the time of floating (F1/28 = 0.03, p = 0.80, Figure 3, Graphic B). The time of float in female control mice was smaller than those of male control mice.

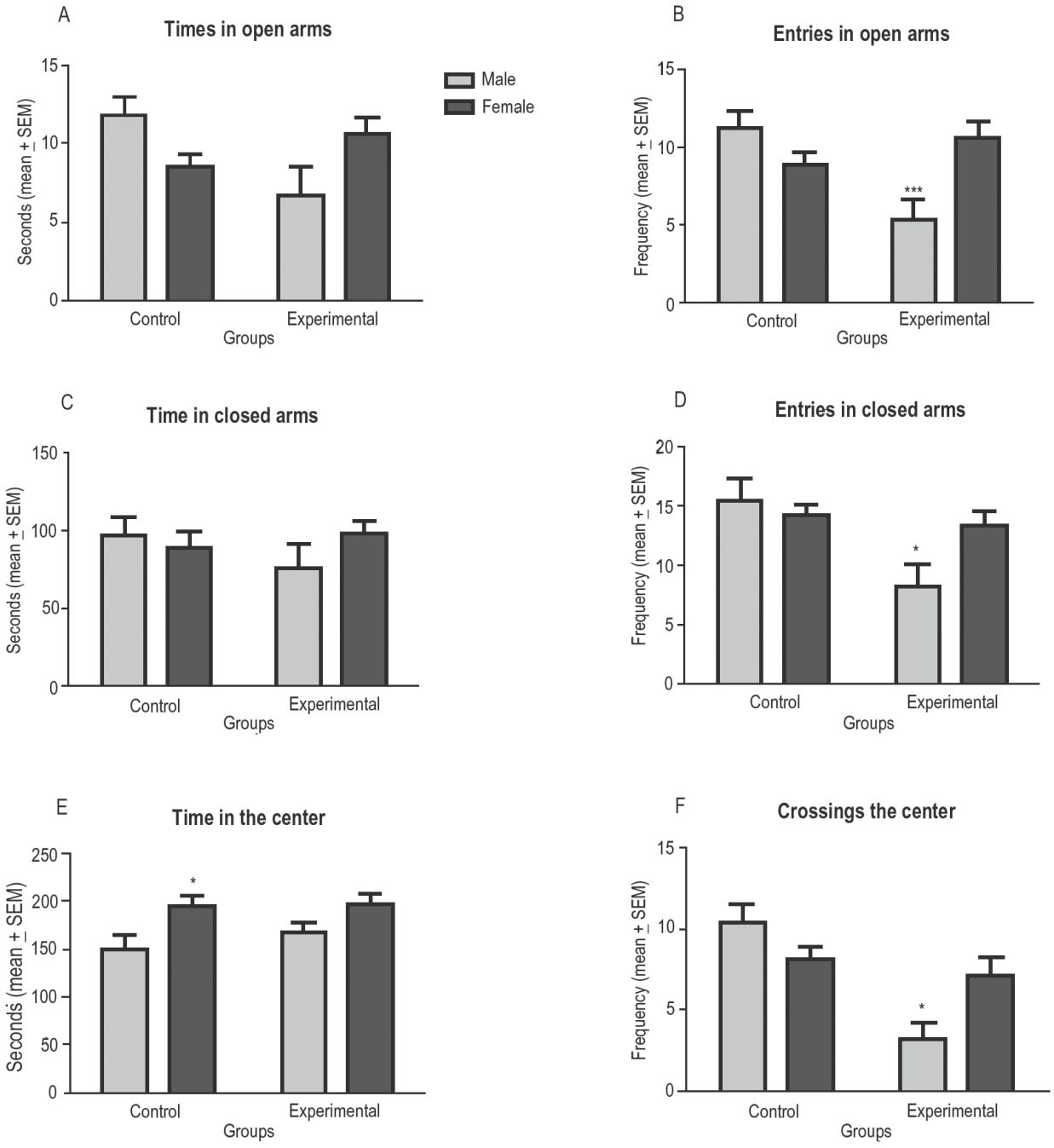

Figure 2 – Elevated plus maze behavior of male and female mice treated with RT during the pre-pubertal period and observed ten days after the end of exposure. Graphic A- time in open arms (s); Graphic B- entries in open arms; Graphic C- time in closed arms (s); Graphic D- entries in closed arms; Graphic E – time in the EPM center (s); Graphic F- crossings the EPM center. Data are presented as means ±SEM

* $p < 0.05$ in relation to male rat of the same treatment

*** $p < 0.001$ in relation to male rat of the same treatment

Source: (JOAQUIM et al., 2014)

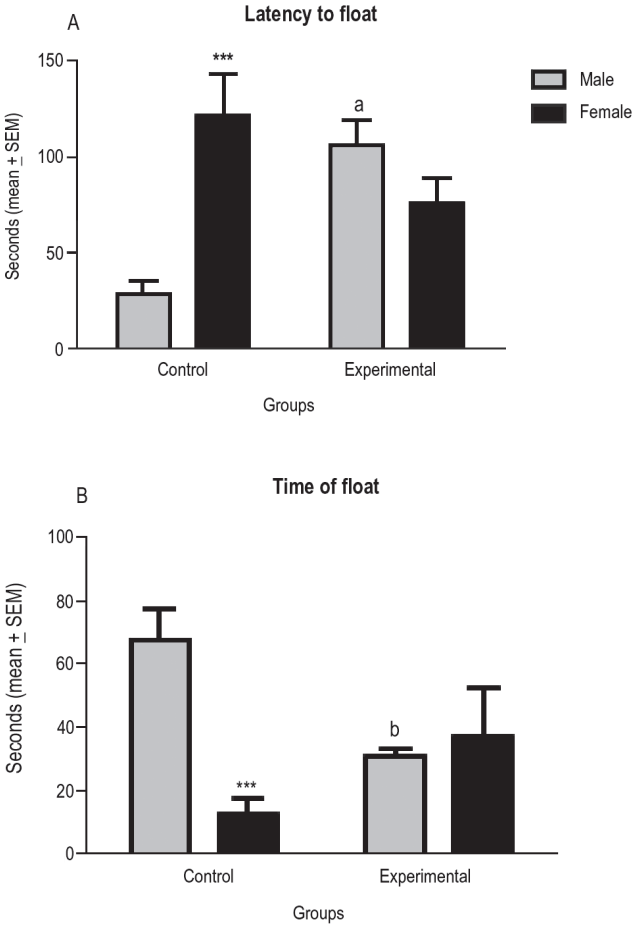

Figure 3 – Forced swim behavior of male and female mice treated with RT during the pre-pubertal period and observed ten days after the end of exposure. Graphic A- latency to float (s); Graphic B- time to float (s). Data are presented as means ±SEM
*** $p < 0.001$ in relation to male rat of the same treatment
a $p < 0.01$ in relation to respective control group
b $p < 0.05$ in relation to respective control group
Source: (JOAQUIM et al., 2014)

Discussion

In this study, we examined the male and female sexual dimorphic behaviors in mice exposed to glyphosate during peripuberal development and observed in adult age, since alteration in the process of hypothalamic sexual differentiation, if present, generally are perceived only at puberty or in adult reproductive life (PIFFER; PEREIRA, 2004). These effects were observed ten days after the end of treatments to investigate the glyphosate effects as endocrine disruptors and not by the herbicide direct effect. Thus, exposure to glyphosate during infantile and part of peripuberal periods of postnatal development, modify the sexual dimorphism in animals models of exploration and depression. Relative to sex, few effects were observed in both male and female mice in the anxiety behavioral model.

The sexual dimorphism in the OF behavior was attributed to differences on gonadal hormones (BLIZARD; LIPPMAN; CHEN, 1975). It is known that male mice were more active than female mice (PALANZA et al., 2002). We observed a sexual dimorphism in locomotion frequency of control male and female mice. However, in rearing behavior, a vertical exploratory behavior, female mice showed high levels in relation to male mice similar to previous reported by Rubin et al. (2006).

Relative to locomotion behavior, the behavioral dimorphism disappears in mice treated with glyphosate. This way, male mice exposed to glyphosate showed similar locomotion frequency as female control and experimental mice. These data clearly show a sexual dimorphic effect induced by the peripuberal exposure to glyphosate on exploratory behavior of male and female mice.

Clair et al. (2011) investigated the effects of glyphosate on fresh testicular cells in a range in some human urine and in environment to agricultural levels. Glyphosate damages the Leydig and Sertolli cells even in no toxic low doses reducing in 35% in testosterone levels. Previously, we observed that glyphosate is a potent endocrine disruptor *in vivo,* causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period (ROMANO et al., 2010). Despite we did not investigate the testicular glyphosate effects, in the present experiment, it is possible that a damage in the testicular cells had occurred in our mice, critical to male brain masculinization. We suggest that glyphosate exposure during pre-pubertal period had a demasculinizing effect in male mice. By the way, testosterone administration increases and

gonadectomy decreases the activity in the central area of an OF, place involved with anxiety (JUSTEL et al., 2012). Thus, a reduced in the testosterone levels by a testicular damage could be responsible by the decreased locomotion frequency of male mice observed in the OF test.

In our conditions, i.e., low levels of light and absence of noise, the locomotion frequency may be used to assess changes in exploratory and motor behavior in the OF. Importantly, the emotional component does not disappear in such conditions; rather, it becomes less relevant. In our present conditions, the mice were observed under the laboratory light, i.e., in less aggressive conditions. Thus, the decreased male locomotor activity could represent an interference of glyphosate on motor or exploratory activities and not related to anxiety. To clarify this aspect, animals were monitored in the EPM test. The number of entries or the time spent by rodents in the open arms in the EPM is commonly used to indicate decreased or increased levels of anxiety (LISTER, 1987; PELLOW et al., 1985). In the present study the time in open and closed arms were not modified either by sex or treatment indicating a lack of effects in anxiety. However, a sexual dimorphism was observed in response to glyphosate pre-pubertal treatment. Male mice entered less in the open and closed arms and showing less crossing in the EPM center. These data is in agreement with the reduced locomotion frequency in the OF and the suggestion that glyphosate pre-puberty exposure induces sexual brain demasculinization.

Our results show that control male mice had a decreased latency to float and a decreased time of floating in FWT. Otherwise, female mice presented increased time to float and a reduce time to floating. Thus in this behavioral model female control mice was less depressive that control male mice. After pre-pubertal glyphosate exposure the sexual differences disappear. These results also are in agreement with our suggestion that the herbicide exposure during pre-pubertal had a demasculinization effect in male mice.

Conclusions

We concluded that pre-pubertal exposure to glyphosate reduced in male mice the capacity of exploration in the OF and EPM tests suggesting that the herbicide interfered with the central mechanism related to brain masculinization of exploratory and anxiety behavioral models. In the FWT it was observed a decreased depressive response in male mice while in female an increased response was detected.

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