Behavioral effects of acute glyphosate exposure in male and female Balb/c mice

Efeitos comportamentais da exposição aguda ao gliposato em camundongos Balb/c machos e fêmeas

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Abstract

We investigated the behavioral effects induced by an acute exposure to a commercial formulation of glyphosate (GF) in a dose that was about double the concentration of the no observed adverse effect level (NOAEL) in male and female BALB/c mice. The acute neurotoxicity induced by GF exposure was determined through analysis of general activity, the sensory system, the psychomotor system, the central nervous system and the autonomous nervous system in both male and female mice. The behavioral effects on exploration, anxiety and depression induced by GF exposure were determined with the open field, elevated plus maze and tail suspension tests, respectively. GF induced few signs of acute neurotoxicity. Locomotion in the open field was decreased in only female mice. No signs of anxiety were detected in the plus maze test in both sex, however, a reduced exploration was observed in male mice in this apparatus. In the tail suspension test, both male and female mice showed an increased immobility time. No interaction between sex and treatment was detected. In conclusion, GF exposure at about a dose twice that of the NOAEL induced few signs of neurotoxicity and no sexual dimorphism in all behavioral models employed.

Keywords: Animal behavior. Sexual dimorphism. Glyphosate. Neurotoxicity. Mice.

Resumo

Neste trabalho investigou-se em camundongos BALB/c, machos e fêmeas, os efeitos comportamentais da exposição aguda a uma formulação comercial do glifosato (GF) em uma dose duas vezes maior que a dose sem efeito observado (NOAEL). A neurotoxicidade aguda ao GF foi determinada por meio da análise da atividade geral, de parâmetros sensoriais, psicomotores, do sistema nervoso central e autônomo em machos e fêmeas. Os efeitos exploratório, de ansiedade e depressão induzidos pelo GF foram observados no campo aberto, labirinto em cruz elevado e no teste da suspensão da cauda, respectivamente. O GF promoveu poucos sinais de neurotoxicidade. A capacidade exploratória de fêmeas foi reduzida no campo aberto. Nenhum sinal de ansiedade foi detectado tanto em machos como em fêmeas no labirinto em cruz elevado porém, notou-se redução da exploratória neste aparelho. No teste de suspensão da cauda tanto as fêmeas como machos mostraram aumento no tempo de imobilidade. Não foi observado neste caso interação entre sexo e tratamento. Concluiu-se que a exposição ao dobro da dose da NOAEL do GF induziu poucos sinais de neurotoxicidade e poucos efeitos sexualmente dimórficos em camundongos machos e fêmeas.

Palavras-chave: Comportamento animal. Dimorfismo sexual. Glifosato. Neurotoxicidade. Camundongo.

Introduction

Glyphosate is a highly effective herbicide used to control weeds in several crops[1,2]. Oral and dermal LD50 values for glyphosate in rats are greater than 5000 mg/kg[3]. Using the acute toxicity classification system employed by the U.S. EPA, both glyphosate and its metabolite, AMPA are classified in the least toxic category, i.e., IV (EPA)[4]. These results show that

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the acute toxicity of glyphosate and AMPA is very low. However, acute studies have reported that products such as Roundup®, which contains glyphosate and polyoxyethyleneamine, may be more toxic than glyphosate alone[5]. Studies show that deaths have occurred 1–2 days after exposure to 8,300 mg/kg[5].

The main objective of the behavioral toxicology studies is predicted the effects of chemical exposures on human brain function by animal models to evaluate the neurotoxicity. Kamel and Hoppin[6] commented that neurotoxicity to acute high-level exposure to certain pesticides has well-known but findings from studies of acute exposure to moderate levels of pesticides are inconsistent. These authors point that most studies of moderate pesticide exposure have found increased prevalence of neurologic symptoms and changes in neurobehavioral performance, reflecting cognitive and psychomotor dysfunction and not deficits in sensory or motor function or peripheral nerve conduction. Thus, we employed a low glyphosate (GF) dose, i.e., approximately twice the NOAEL to mice an rats.

Our group employed several animals' models to evaluate the central nervous effects of organocloride[7], pyrethroid[8,9,10,11] and organophosphate[12] insecticides and heavy metals as lead[13], and cadmium[14]. In the present study, the acute neurotoxicity of a commercial formulation of glyphosate [GF] was evaluated by behavioral methods. To assess the acute neurotoxicity it was employed a battery of tests including the analysis of multiple parameters related to general activity, the sensory, psychomotor, the central nervous and the autonomous nervous systems as previously reported[15]. The open field behavior was used to evaluate the GF emotional /motor effects. The anxiety was observed in the plus maze and the depressive state by the tail suspension model. The BALB/c strain was used to avoid genetic interferences since these mice were genetically identical.

Material and Method

Male and female adult BALB/c mice (25-35 g, approximately 60 days old) provided by the Faculty of Medicine Veterinary, São Paulo University facilities, were used. At arrival, the animals were housed in individual microisolator cages with controlled-temperature (22-26°C) and humidity (65–70%) 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. The behavioral tests were performed after one week the arrival of mice. 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.

Experiments were completed using Roundup Transorb (Monsanto Co., St. Louis, MO; Monsanto of Brazil Ltda, São Paulo, Brazil). This formulation was composed of 480 g/L of GF, 648 g/L of isopropylamine salt and 594 g/L of inert ingredients.

The open field (OF) test was used to assess the effects of GF exposure on emotionality and exploration. A white, circular, wooden arena was built as previously published by[16] 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 case has an open portion in the front, covered by a curtain that provides observers complete visibility of the animals in the arena; however, the mice cannot see the observer. The apparatus was placed in a sound-proof room with dim light (55 lux at the open field 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 and rearing as well as immobility duration. A unit of locomotion was defined as the frequency of an animal entering with its four paws in one segment 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 of time, 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.

A total of two separate OF experiments were performed. The first consisted of the determination of the dose and the latency of GF effects in mice. We used 42 male mice that were divided into four groups: three experimental groups that received *per os*, 25, 50 and 100 mg/kg of GF (n=10/group) and a control group that received 1 mL/kg of water (n=12). In the OF, 3 min observations were made every 15 min between 15 and 120 min from oral administration. The doses of GF were chosen based on the NOAEL for GF, 50 mg/kg, according to[17]. In the second experiment, groups of male and female mice (n=8/group; n_{total}=32) were used to analyze the sexual dimorphic effects in the OF due to exposure to 100 mg/kg of GF *per os*. Animals in control groups received a saline solution (0.9% NaCl). Fifteen min after treatments, all mice were observed for 3 min.

Acute neurotoxicity was assessed based on a modified method proposed by Brito[18]. BALB/c mice were kept in laboratory conditions for eight days. Groups of male and female mice (n=5; n_{total}=20) were used to analyze effects of 100 mg/kg *per os*, of GF. Animals in the control group received a 0.9% saline solution. Mice were observed in a glass cage (20×10×15 cm) for the presence of GF-induced effects. Observations were filmed in 15 min intervals between 15 and 60 min after exposure. The scores of two observers during testing were correlated with the video recordings to validate our method. After that period, observations were completed every 24 h for seven consecutive days, including the day of exposure. Similar reactions to GF were observed in the control group. The animals were weighed at the beginning and end of the experiment. Parameters related to the general activity, the sensory system (vocal tremor, irritability, auricular reflex, corneal reflex, tail squeeze, response to touch), the psychomotor system (contortion, hindquarter fall, surface-righting reflex, body tone and grip reflex), the central nervous system (convulsions, ataxia, anesthesia, hypnosis, Straube tail, tremor and sedation) and the autonomous nervous system (lacrimation, breath, ptosis, piloerection, micturition, defecation, hypothermia and cyanosis) were accessed, and a score from 0 to 4 was counted, except for that of micturition and defecation. At the end of the experiment, all scores were summed for each mouse. The number of micturition events and fecal *boli* were summed at the end of each session.

The EPM, an apparatus first conceived by the British psychologist Sheila Handley, is a commonly used test to evaluate anxiety[19] (Our EPM was made of black-painted wood with two open arms and two closed arms (25x5x15 cm). The apparatus was elevated 55 cm above the ground and placed in a soundproof 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 and the total number of arm entries. The anxiety index (AI) was determined by the percentage of time spent in the open arms versus the time spent in both the closed and open arms. 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. Groups of male and female mice (n=8; n_{total}=32) were used to analyze effects of 100 mg/kg of GF or 0.9% saline solution *per os*. All mice were observed 15 min after treatment.

The tail suspension (TS) test was carried out according to the method of[20]. Briefly, mice were suspended 5 cm above the floor with an adhesive tape placed approximately 1 cm from the tip of the tail. The total duration of immobility (seconds) was quantified during a test period of 6 min. Mice were considered immobile when they were completely motionless. Groups of male and female mice (n=9-10/group; n_{total}=39) were used to analyze effects of 100 mg/kg of GF *per os*. Animals in control groups received 0.9% saline solution. All mice were observed 15 min after treatment.

Homoscedasticity was verified using an F test or Bartlett's test. Normality was verified using a Kolmogorov-Smirnov test. For the neurotoxicity signs scores analysis, a Kruskal-Wallis test was used followed by a Dunn test, but data are presented as mean± SEM. For the remaining data, a 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 were estimated based in previous studies of our laboratory showing a minimal number of subjects in each experiment. All results were considered significant if $p < 0.05$.

Results

In terms of locomotion frequency (Figure 1), differences were observed between treatments ($F_{5/228}$=3.84; p<0.0001) and sessions ($F_{3/228}$=9.12; p< 0.0001), and there was an interaction between factors ($F_{15/228}$=2.0; p<0.05). When compared with the control group, all doses in the first session (15 min after exposure), 50 mg/kg in the second session (30 min), and 25 mg/kg in the last session (120 min) show a decrease in locomotion frequency. Immobility duration was significantly different between treatments ($F_{5/228}$=48.48; p<0.0001) and sessions ($F_{3/228}$=15.87; p<0.0001), and there was an interaction between factors ($F_{15/228}$=3.21; p<0.0001). When compared with the control group, exposure mice display a decrease in immobility time from the third to the last session (25 mg/kg dose) and in the second session (50 mg/kg dose). The 100 mg/kg dose significantly increased the immobility time in mice during the second session. In relation to rearing behavior, differences were observed between treatments ($F_{5/228}$=8.25; p<0.0001), but not between sessions ($F_{3/228}$=2.01; p=0.11), and no interaction was detected between factors ($F_{15/228}$=1.50; p=0.11). When compared with the control group, a reduced rearing frequency in the first session was observed in mice exposed to a dose of 25 mg/kg. Based on these data, we chose to use a dose of 100 mg/kg and a latency period of 15 min after exposure to perform the OF, EPM and TS tasks. This dose and time period showed no gross motor effect. An immobility increase is a motor impairment that could interfere with the behavioral response of the mice in the various tasks.

There were few differences between control and experimental groups in the acute signs of neurotoxicity induced by 100 mg/kg of GF in mice. When compared with the control group, an increase in hindquarter fall was detected in both male and female GF-treated mice, and male mice presented with increased tremors (Table 1). No deaths occurred over the seven days after treatment.

As depicted in figure 2, 100 mg/kg of GF reduced locomotion frequency ($F_{1/28}$=10.06; p<0.01) in female mice; however, there was no effect in male mice ($F_{1/28}$=0.38; p=0.54) and no interaction between factors ($F_{1/28}$= 0.77; p=0.386). The immobility time

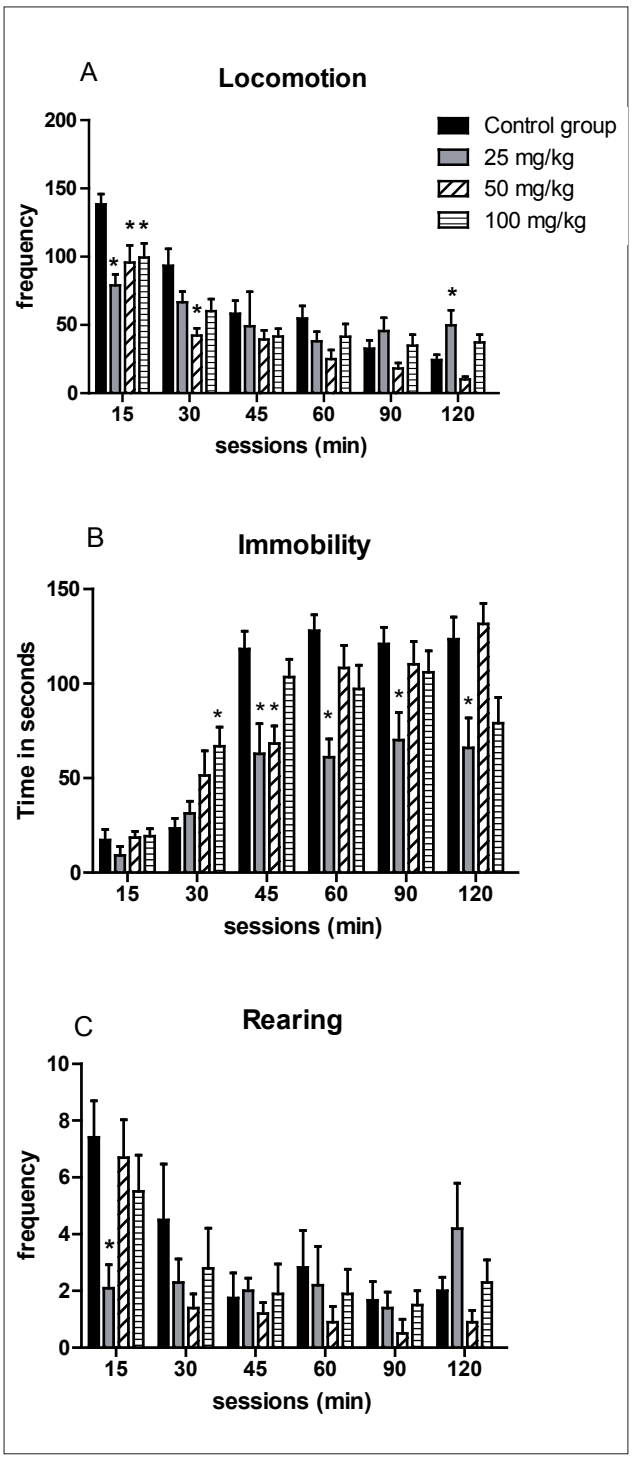

Figure 1 - Effects of 25, 50 and 100 mg/kg doses of a glyphosate formulation observed at 15, 30, 45, 60, 90 and 120 min after treatment of male mice observed in the open field. A- locomotion frequency; B-immobility duration in seconds; C-rearing frequency. Data are presented as means ± SEM. N= 10-12 /group, $n_{total} = 42$. Two way ANOVA followed by the Bonferroni test. * p< 0.05 in relation to control group of the same sex

[treatment - $F_{1/28}$= 0.44, p=0.514, sex- $F_{1/28}$= 0.63, p=0.434, interaction- $F_{1/28}$=2.03, p= 0.515] and rearing frequency [treatment- $F_{1/28}$=0.02,p=0.875, sex- $F_{1/28}$= 0.20, p=0.660, interaction- $F_{1/28}$=1.33, p=0.258] were not modified by treatment or sex.

There were no differences in the AI (Figure 3) between control and experimental groups ($F_{1/28}$=0.13, p=0.719) or between male and female mice ($F_{1/28}$=0.20; p=0.655). However, the number of entries in the closed arms in GF-exposed male mice was significantly reduced when compared with the control group ($F_{1/28}$=11.0; p<0.01). No effects on entries into the closed arms were related to sex ($F_{1/28}$=0.83; p=0.369) or interactions between factors ($F_{1/28}$=1.21; p=0.288). Significant differences were observed in the center crossing between treatments ($F_{1/28}$=5.54, p<0.05) and sex ($F_{1/28}$=18.9; p<0.01) and interaction was observed between factors ($F_{1/28}$=5.63; p=0.024). Relative to the respective control group, male rats treated with GF showed a decreased center crosses. Moreover, comparison between female and male center crosses indicates that female rats presented higher levels of activity than males.

GF exposure (Figure 4) increased immobility time in both male and female mice ($F_{1/35}$=17.73; p<0.001) with no interference of sex ($F_{1/35}$=2.10; p=0.156) or interactions between sex and treatment ($F_{1/35}$=0.23; p=0.633).

Discussion

The present results show that acute toxicity to 100 mg/kg GF dose induced by behavior effects reduced signs of neurotoxicity in male and female mice. In behavioral tests a decreased exploration in open field and an increased immobility time in the tail suspension test were observed. No signs of anxiety was detected in the plus maze apparatus. The statistical analysis did not reveal a sexual dimorphism in all behavioral models.

Table 1 - Effects of the administration of 100 mg/kg of glyphosate in the spontaneous behavior of male and female mice observed during 60 min. Data are presented as mean ±SEM

Parameter	Control male	Experimental male	Control female	Experimental female
General activity	5.8 ±2.3	5.0±0,8	5.6±2.9	4.4±1.1
Vocal tremor	0	1.6±0.9	0	2.0±1.5
Irritability	0.8±0.8	11.0±2.8	6.4±2.0	1.6±1.6
Auricular reflex	14.4±0.6	15.4±0.2	15.0±0.7	15.2±.5
Tail squeeze	7.4±1.3	8.6±2.5	4.5±1.1	2.8±0.8
Corneal reflex	12.2±1.3	14.2±1.1	12.2±1.3	14.6±1.2
Response to touch	10.0±1.7	8.0±3.1	10.8±2.7	7.6±2.0
Contortion	0	1.6±0.9	0	1.6±1.6
Hindquarter fall	0	3.8±1.8*	0	3.4±0.6*
Surface-righting reflex	6.4±3.9	9.6±3.9	3.2±3.2	0
Body tone	12.8±2.3	6.4±2.7	9.6±3..9	12.0±2.5
Grip reflex	1.6±0.6	1.8±0.7	1.0±0.7	0.2±0.2
Tremors	0	2.4±1.6*	0	0.8±0.8
Ptosis	0.2±0.2	0.6±0.4	1.8±1.8	2.6±1.8
Micturition	1.8±0.5	1.4±0.2	1.0±0.3	1.0±0.5
Defecation	5.0±1.7	5.8±0.8	3.0±0.8	4.2±1.4

Kruskal-Wallis test followed by the Duns test. *$p<0.05$ in relation to the respective control group. N=5/group, $n_{total}=20$

Within this context, there are several publications showing a low acute neurotoxicity for GF[5,21,22]. After exposure to 100 mg/kg of GF, the hindquarter fall was increased in both male and female mice. In addition, male mice showed an increase in tremor. Hindquarter fall is involved with psychomotor systems, while tremors are better related to a neurotoxic effect.

General activity is an index for evaluating behavioral changes in animals induced by not only physiological and genetic manipulation but also by toxicological interference[23,24]. As shown GF exposure decreased the locomotion behavior of female mice in the OF, but it had no effect on male mice, but statistical analysis did not reveled an interaction between sex and treatment. Immobility duration, rearing and defecation frequencies were not affected by sex or GF exposure.

Among the techniques used to assess general activity, OF enables the measurement of multiple parameters, including emotional, exploratory and motor behavior[25,26]. Defecation and locomotion represent variations in the emotional state of the animal; for example, an increase in defecation and a reduction in the duration of locomotion would imply an increased emotionality. Under this premise, the exposure of mice to intense light and a noisy environment during the OF testing could have adverse effects. On the other hand, when an animal is exposed to less aggressive condition, such as low luminosity and absence of noise, the duration of locomotion may be used to assess changes in exploratory and motor behavior. 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, data from this experiment should be represent more an exploratory OF interference than emotional effect.

In addition, several studies have shown that female rodents explore a new environment more than males[23,25]. More specifically, several data show the sexual dimorphism in OF behavior, with females being more active

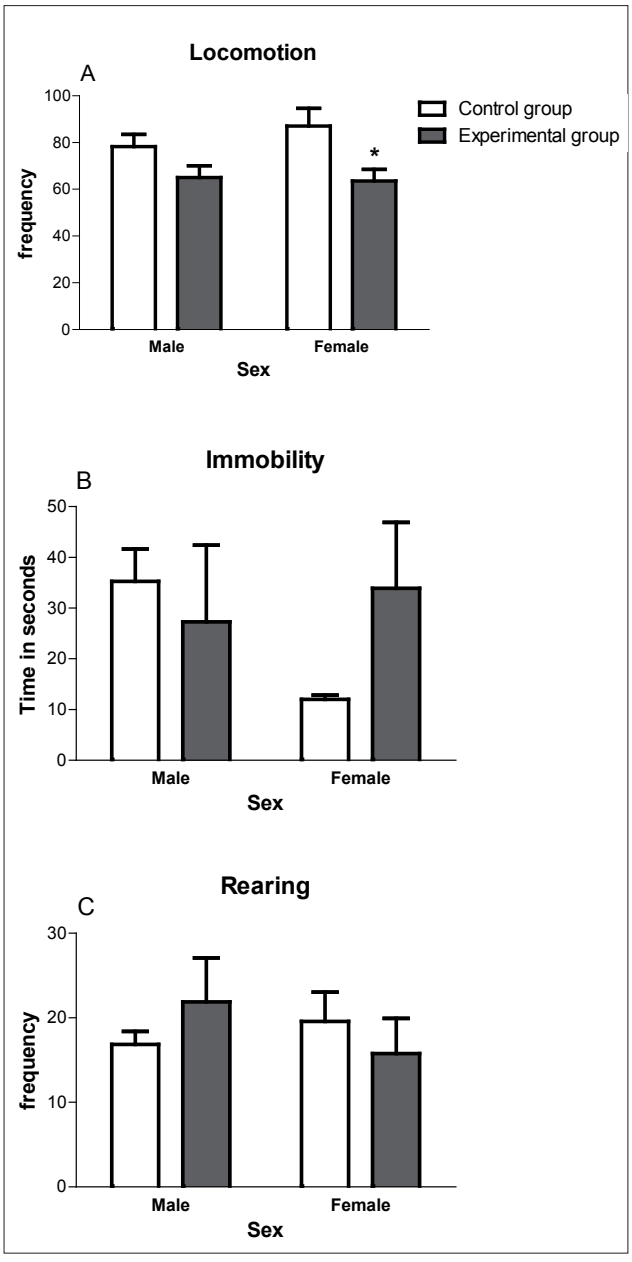

Figure 2 - Behavior of male and female mice in the open field exposed to 100 mg/kg dose of a glyphosate formulation and observed 15 min after treatment. A- locomotion frequency; B-immobility duration in seconds; C-rearing frequency . Data are presented as means ± SEM. N= 8 /group, n_{total} =32. Two way ANOVA followed by the Bonferroni test. * p< 0.05 in relation to respective control group of the same sex

than male rodents. Nonetheless, we did not observe significant differences between control male and female mice, although there was a tendency for females to explore more of the OF arena than males. However, only female mice exposed to GF showed a reduced locomotion frequency without interference of immobility duration. Moreover, the increased hindquarter fall observed in the acute neurotoxicity study occurs in both sexes 30 min after GF exposure, suggesting that this effect is not involved with the decreased locomotion behavior in female mice.

The anxiety level, a response to a situation in which behavior is influenced by two opposing motivational forces (e.g., a natural curiosity to explore unexplored or novel areas versus an aversion to open areas) is operationally inferred as suggested by Pellow et al[27]. The number of entries or the time spent by rodents in the open arms in the EPM is commonly used to indicate decreased levels of anxiety[27,28,29]. Anxiogenic drugs are reported to increase the time spent in the closed arms and to decrease the time in the open arms and central zones[29,30].

The reduced locomotor activity could be a result of anxiety-related reductions in motor or exploration activity[31]. To clarify this, animals were monitored in the EPM test. Male and female mice treated with 100 mg/kg of GF did not present any changes in the percentage of entries into open arms versus closed arms or the percentage of time spent in the open arms versus closed arms. Thus, the reduced open field activity was not consequence of an anxiogenic effect induced by the GF.

Depressive states also lead to decreases in locomotion behavior. The TS test, a method often used to evaluate antidepressant drugs, was used to investigate if the decreased locomotion in female rats treated with GF was related to a depressive state[20]. The immobility time in the TS is considered to be due to an inability or reluctance to maintain effort rather than generalized hypoactivity. Our present results show that both male and female mice had an increased immobility time in TS. Thus, the decrease in locomotion behav-

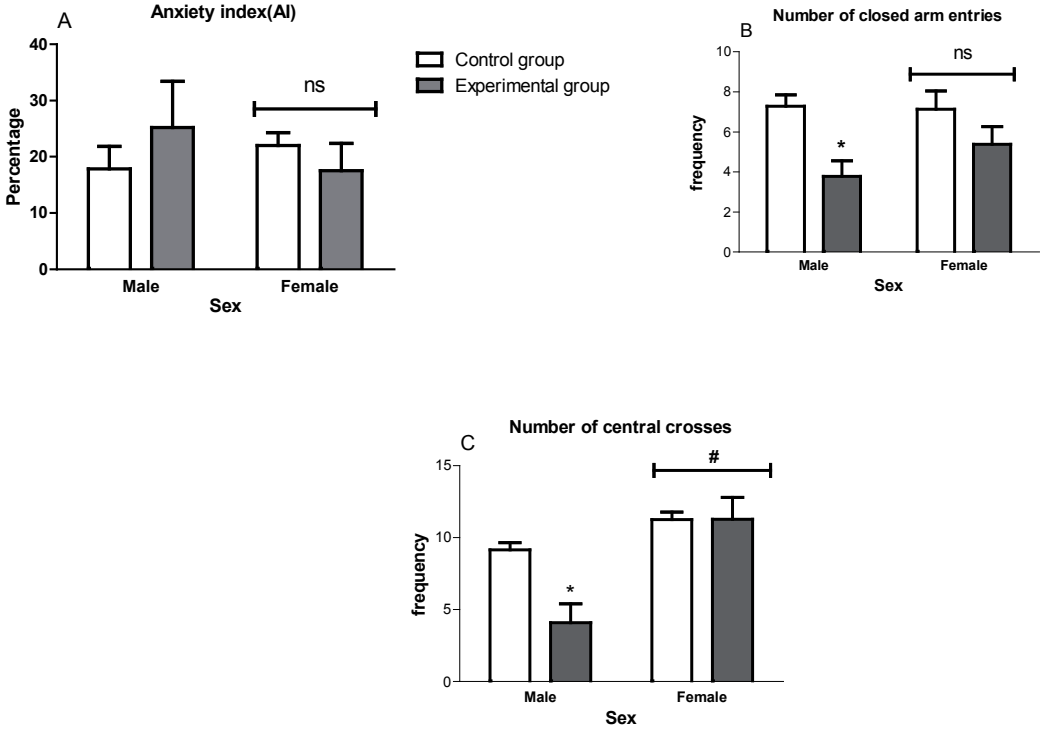

Figure 3 - Behavior in the elevated plus maze of male and female mice exposed to 100 mg/kg dose of a glyphosate formulation and observed 15 min after treatment. A- anxiety index; B-number of entries in the closed arms; C-number of central crossings. Data are presented as means ± SEM. N= 8/group, n_{total} =32. Two way ANOVA followed by the Bonferroni test. * p< 0.05 in relation to respective control group of the same sex.# p< 0.05 in relation to sex, ns = no statistical difference

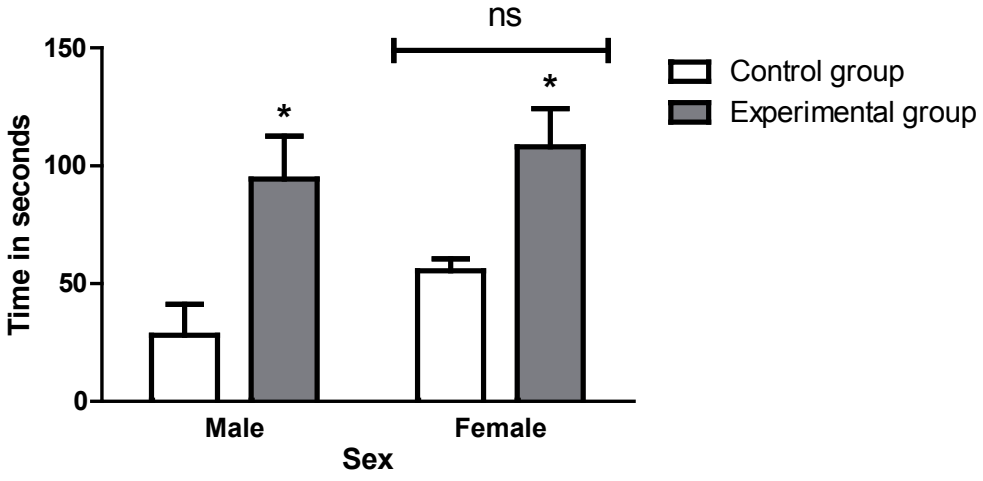

Figure 4 - Data of tail suspension test of male and female mice exposed to 100 mg/kg dose of a glyphosate formulation and observed 15 min after treatment. Data are presented as means ± SEM. N= 9-10/ group, n_{total} = 39. Two way ANOVA followed by the Bonferroni test. * p< 0.05 in relation to respective control group of same sex. ns = no statistical difference

ior observed only in female mice was not a result of a depressive state.

Conclusion

In conclusion, 100 mg/kg of GF administered by oral route, twice the glyphosate NOAEL, induced few signs of acute neurotoxicity by behavioral tests, reduced the capacity of exploration in the open field test and increased the immobility time in the tail suspension test. Finally, the GF acute treatment did not produced a sexual dimorphic response in all behavioral models here employed.

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