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Research Article

Behavioral and Laboratory Abnormalities in Rat Offspring Exposed to Fipronil during the Perinatal Period

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Abstract Perinatal exposure to pesticides was linked to changes in neurobehavioral development of rat offspring. This study aimed to evaluate the behavior of rat offspring exposed to fipronil during the perinatal period associated with laboratory findings. For this, pregnant Wistar rats were divided into five groups (n=15) namely: control (Ct) and exposed - gestation (G), lactation (L), gestation more lactation (G+L) 7-14, and gestation more lactation (G+L) 1-21. The behavioral parameters evaluated were anxiety, aggressiveness, motor coordination, exploration and locomotion. Parameters related to physical and sensory-motor development, organ/animal weight ratio, biochemistry, histology, blood cortisol levels and Fipronil and its metabolites (sulfone and dessulfinil) in offspring brains of different groups were also assessed. Results showed an increase in anxiety and aggression and a decrease in motor coordination. The negative effects caused by pesticides appear to be dependent on their presence in brain tissue. It was observed a decrease in time to eruption of incisors, an increased weight of the offspring liver; increased serum cortisol and histological changes in the liver; fipronil and fipronil sulfone were detected in the brain of offspring. In conclusion perinatal exposure to fipronil increased the aggression and anxiety, confirming its toxicity on neurodevelopment of rat offspring. Fipronil caused decreased motor coordination in the offspring, suggesting toxicity on motor nerves. The results confirm the toxicity of fipronil on rat offspring exposed during the perinatal period.
Keywords *Aggressiveness; Metabolites; Phenylpyrazole; Physical development; Sensory-motor development; Toxicity*

1. Introduction

It has been shown that pesticide toxicity in animals and humans is considerable and that indiscriminate use of these substances is associated with neurodevelopmental changes in children, specially when exposure occur in the perinatal period (Shafer et al., 2005; Bouchard et al., 2011; Shelton et al., 2014).

Exposure to pesticides for a short period of time can cause serious effects on the development of both animal and human fetuses and, after birth, sequelae such as learning disabilities, decreased reflexes, sterility, and increased susceptibility to neoplasias and other diseases might persist (Lyons, 2000).

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]- 4-[(trifluoro-methyl) sulfinyl] -1H-pyrazole -3-carbonitrile) is a pesticide, chemically derived from the phenylpyrazole or fiproles family and targets the gamma amino butyric acid receptor (GABA) (Udo et al., 2014; Magalhães et al., 2015). Due to its lipophilic properties, fipronil can diffuse to the nervous system during fetal development (Udo et al., 2014). Because it is widely commercialized and used in domestic environments, issues were raised about the adverse effects of fipronil on public health (Jennings et al., 2002; Tingle et al., 2003).

Since behavior is a neurological function affected by neurotoxic pesticides, understanding the effects of fipronil on behavior in animals and humans is necessary considering fipronil wide use. The aim of this study was to evaluate the effect of exposure to the pesticide fipronil on anxiety, aggressiveness, motor coordination, exploration, locomotion, developmental parameters and blood serum cortisol in in Wistar rats during different stages of the perinatal period.

2. Materials and Methods

This experiment was approved by the Ethics Committee for Animal Use of the Faculty of Veterinary Medicine (FMVZ), Universidade Estadual Paulista (UNESP) - protocol number 59/2012.

2.1. Animals

The animals used were female and male Wistar rats obtained from the colony of the Central Biotério of UNESP, Campus Botucatu-SP, removed from the Central Biotério after weaning at the age of 22 days (AGE 22) and allocated in the Biotério of the Center for Toxicological Assistance (CEATOX), under controlled temperature (24 ± 2 °C) and relative humidity (55 ± 5 %), 12 h light/dark cycle, continuous ventilation, water and feed *ad libitum* until reaching mating age. During the experiment, polypropylene cages were washed, sanitized and wood bedding replaced every 3 days.

2.2. Experimental Procedure

The animals (AGE 80) were mated in the cage with a ratio of 3 females to one male and remained in the cage until pregnancy confirmation or during a maximum period of 5 days.

Every 24 hours, pregnancy tests were carried out using cotton swab with physiological saline solution to collect vaginal secretion and subsequently, a smear was prepared and observed with a microscope. The presence of spermatozoa was considered a positive pregnancy.

Pregnant females were isolated in separate boxes and the day of separation was considered day zero of the gestation period. The pregnant rats were randomly assigned into 5 groups with 15 animals each namely, control (Ct), gestation (G), lactation (L), gest/lact 7-14 (G+L 7-14) and gest/lact 1 -21 (G+L 1-21), respectively. Pregnant rats were weighed every three days.

The commercial form of fipronil, Topline® (1% fipronil - Merial, Brazil), was the test article. The dose for exposure treatment was 1 $mg.Kg^{-1}$ per day applied topically, after trichotomy, in the epidermis of the dorsal region of the neck. Control animals were treated with 0.2 mL of corn oil during gestation and lactation periods from 7 to 14 days; Groups G, L and G+L 7-14 were treated with fipronil from 7 to 14 days of gestation and / or lactation; The G+L 1-21 group was treated with fipronil from 1 to 21 days of gestation and during lactation.

Immediately after birth the offspring were sexed and eight male offspring were selected from each rat dam to standardize the litter size. The offspring remained with their respective dams until AGE 21, when they were weaned.

One male per litter was selected and assigned to the treatment groups until completing 15 animals for each experimental group.

Tests of behaviour were filmed pending behavioral evaluation. At the end of each session with each animal the test equipment was sanitized with ethyl alcohol (5%) to eliminate animal traces from previous tests.

Evaluation of Physical and Sensory-motor Development

The offspring underwent a physical (weight gain, hair appearance, eye opening, eruption of the incisors, detachment of the ears, descent of the testicles) and sensory-motor evaluations (palmar, righting or postural gripping and negative geotaxia reflexes) after birth and during the lactation period.

Evaluation of Behavior

The general activity and the locomotor activity (3 min) of the young (AGE 25-30) and adult (AGE 75-80) offspring were evaluated using the Open Field Arena Test (OFT) (Trombini et al., 2001).

Motor coordination and exploration were evaluated using the Hole-board apparatus (HB) (Meyer & Caston, 2005).

The anxiety level was evaluated in young (AGE 25-30) and adult (AGE 75-80) offspring according to the methodology described by Trombini et al. (2001) using the Elevated Plus-Maze (EPM) constructed as specified by Pellow & Chopin (1985) and validated by Pellow & File (1986) during research of anxiolytic and anxiogenic drugs in rats.

Aggression behavior was evaluated in adult animals (AGE 80) using the resident / intruder mouse paradigm. Intruder animals received no treatment and remained during the pre-tested period grouped in cages, tested once a day and never returning to the same resident animal (control and fipronil).

When animals were 50 days of age (AGE 50), control and exposed offspring were separated in isolating boxes, remaining 30 days in them, subsequently, the aggression test was performed. During this period boxes were not washed, bedding was changed and the cages were not cleaned in the last week before the test.

In order to evaluate aggression, an intruder was placed in the resident's cage and observed 15 minutes to determine latency time for the first attack, total number of attacks and total time of attack episodes (including bite outbursts, side threats, domination of the other animal with paws or body and more intense cleaning).

Biochemical and Chromatographic Analyzes

After the behavioral tests, the animals were anesthetized (xylazine 10 $mg.Kg^{-1}$, plus ketamine - 80 $mg.Kg^{-1}$, intraperitoneally), blood samples were collected by cardiac puncture and subsequently the animals were euthanized (anesthetic overdose) for tissue sample collection (brain, liver, kidneys, spleen, and testes).

Blood samples were centrifuged (2500 rpm, 5 min) and cortisol (Cortisol - ELISA-DRG, USA) and the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Ultrospec 2000-Pharmacia Biotech, Biotécnica-Brasil kits) were measured in the resultant sera.

In order to validate the offspring exposure and its possible toxic effects, the dosage of fipronil levels and its main metabolites (sulfone and desulfinyl) in the brain was determined by chromatography (Shimadzy HPLC, Prominence model Type Triple quadrupole MS-MS AB Sciex, model 4500), using Sigma-Aldrich reference standards (Xavier et al., 2014). The detection limits for fipronil, fipronil sulfone and fipronil desulfinyl in ppb were 0.051, 0.013, 0.050 respectively.

Organ/animal Weight Ratio

Tissues collected from adult offspring, namely, brain, liver, kidneys, spleen, and testicles were weighed and their fresh weight-related weights of animal were calculated with the ratio body weight / animal weight (sample weight * 100 / animal weight).

Histopathological Evaluation

Liver samples were fixed with 10% buffered formalin solution for 3 days and then immersed in liquid paraffin and blocks were sectioned into 4 µm thickness samples, mounted on glass slides, stained with hematoxylin and eosin (HE) and evaluated with a microscope.

2.3. Statistical Analysis of Results

The data obtained were statistically analyzed using analysis of variance (One-Way ANOVA) using the analytic software Instat 3.0 (Graph Pad Software). Tukey-Kramer Multiple Comparisons test was used to identify differences between groups. The comparison between groups was considered significantly different when $p<0.05$ (Terçariol & Godinho, 2011).

3. Results

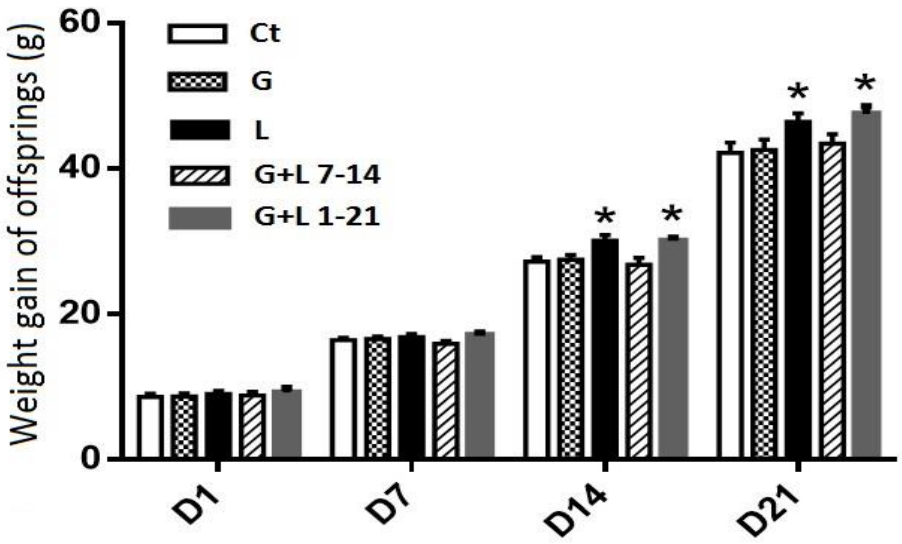

Figure 1: *Effect of fipronil on the weight gain of offspring of Wistar rats exposed to the pesticide (1 mg.Kg-1) in the perinatal period. D = day of evaluation of animal weight. Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21). Values represent the mean ± S.P.M. (N = 15). * p<0.05 in relation to Ct.*

3.1. Physical and Sensory-motor Development

Signs of fipronil intoxication were not observed in pregnant animals.

The weight gain of the offspring varied significantly ($p<0.05$) between treatments (Figure 1), but there was no difference in the total number of offspring born per rat and in the number of stillbirths ($p>0.05$).

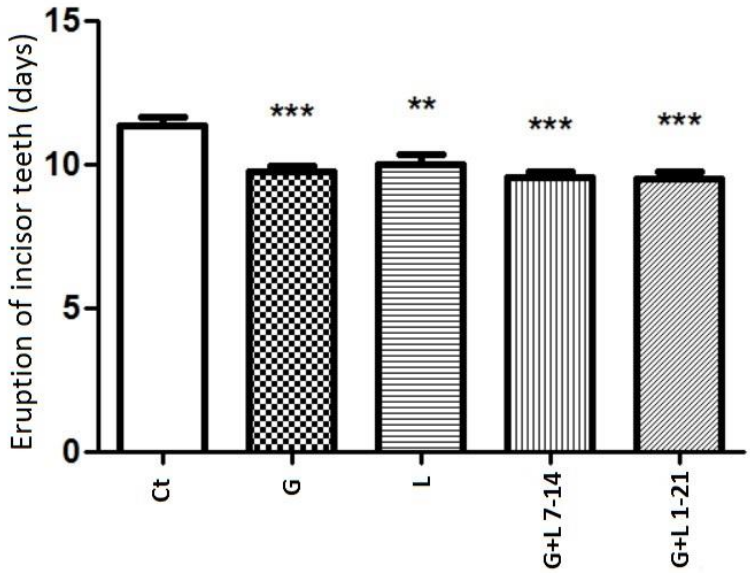

Figure 2: *Number of days for eruption of incisor teeth in offspring of Wistar rats exposed to fipronil (1 mg.Kg-1) in the perinatal period. Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21). Values represent the mean ± E.P.M. (N = 15). ** p<0.01 and *** p<0.001 with respect to Ct.*

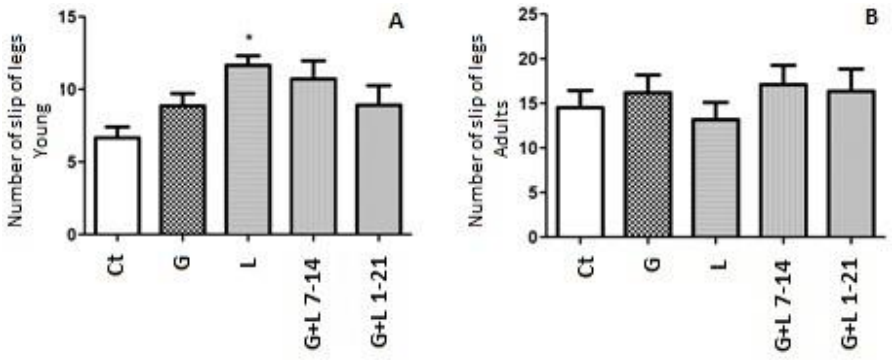

Figure 3: *Number of foot strings in the behavior of motor coordination in the Hole-Board apparatus by offspring of Wistar rats exposed to fipronil (1 mg.Kg-1) in the perinatal period. A: young offspring; B: adult offspring. Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21). Values represent the mean ± E.P.M. (N = 15). * p<0.05 in relation to Ct.*

There was no significant difference ($p>0.05$) in the offspring' physical development parameters namely: ear detachment, eye opening, testicle descent and hair appearance. In relation to the time for eruption of the incisor teeth, there was a significant decrease in gestation ($p<0.001$), lactation ($p<0.01$), Gest/Lact 7-14 ($p<0.001$) and Gest/Lact 1-21 ($p<0.001$) groups when compared to the control group (Figure 2).

The sensory-motor development parameters (acquisition of the postural reflex, loss of the palmar grip reflex and acquisition of the negative geotaxia reflex) showed no significant variation ($p>0.05$) due to treatment.

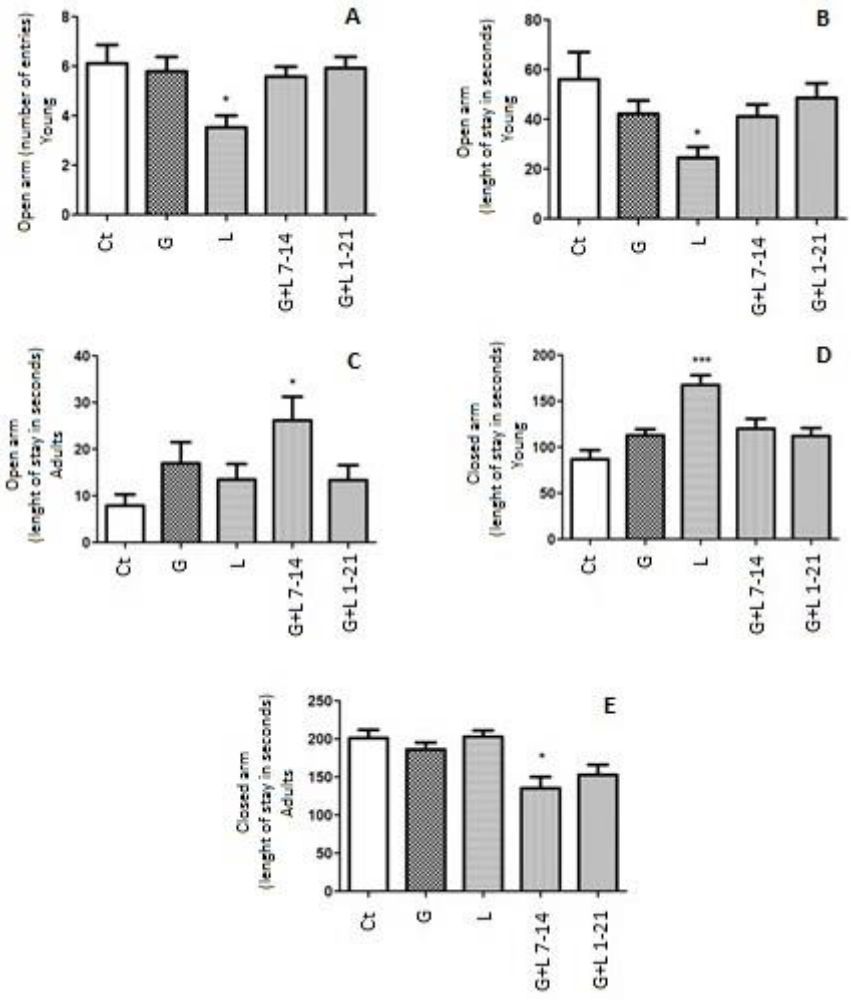

Figure 4: *Activity of the young and adult offspring of Wistar rats exposed to fipronil (1 mg.Kg-1) in the perinatal period in the Elevated Plus-Maze test. A: Number of entries of young offspring in open arms; B: Length of stay of young offspring in open arms; C: Length of stay of adult offspring in open arms; D: Length of stay of the young offspring in the closed arms; E: Length of stay of adult offspring in closed arms. Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21). Values represent the mean ± E.P.M. (N = 15). * p<0.05 in relation to Ct and *** p <0.001 in relation to Ct.*

3.2. Behavior

The OFT did not reveal any statistically significant differences (p>0.05) regarding locomotion, lifting, cleaning and freezing parameters.

The evaluation of motor coordination behavior in HB indicated that the young offspring of the lactation group presented a significant increase (p<0.05) in the number of paws in relation to the control group (Figure 3).

Young offspring exposed to fipronil during the lactation period presented a significant decrease (p<0.05) in the number of entries in the open arms of the EPM in relation to the control animals (Figure 4).

In relation to the length of stay in the open arms of the EPM, there was a significant decrease (p<0.05) in the young offspring of the lactation group when compared to the control group. The adult offspring of the G+L 7-14 had a significant increase (p<0.05) in the open arms of the EPM, in relation to the control group. The young offspring of the lactation group significantly increased (p<0.001) the

length of stay in the closed arms of the elevated plus-maze in relation to the control offspring. The G+L 7-14 adult offspring remained significantly less ($p<0.05$) in the closed arms of the elevated plus-maze when compared to control group (Figure 4).

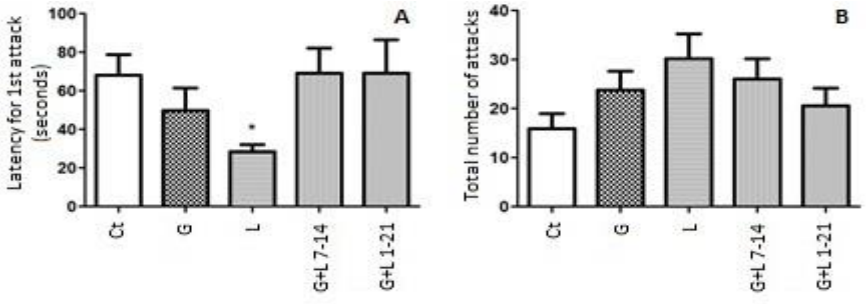

Figure 5: *Aggressiveness of the offspring of rats exposed to fipronil (1 mg.Kg-1) in the perinatal period. A: Latency time for the first attack; B: Total number of attacks. Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21). Values represent the mean ± E.P.M. (N = 15). * p<0.05 in relation to Ct.*

During the evaluation of aggressiveness, the offspring of the lactation group showed a significant decrease ($p<0.05$) in the latency time for the first attack, in relation to the control group (Figure 5).

Table 1: *Dosage of fipronil (µg/g tissue) and its metabolite fipronil sulfone, in the brains of the young offspring of rats exposed to the pesticide (1 mg.Kg-1) in the perinatal period*

Treatment	Ct	G	L	G+L 7-14	G+L 1-21
Fipronil	0,00	0,33±0,18*	0,65±1,30*	0,15±0,08*	2,67±0,24*
Fipronil Sulfona	0,00	314,3±14,00*	331,4±36,90*	560,6±95,00*	1.105,4±119,2*

Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21).
Values represent the mean ± E.P.M. (N = 15).
* p <0.05 in relation to Ct.

Table 2: *Histopathological changes in the liver of offspring (young offspring - 1A and adults offspring - 1B) of Wistar rats exposed to fipronil (1 mg.Kg^{-1}) in the perinatal period.*

1A – Young offspring					
Pathological Alteration	Control	Gestation	Lactation	Gest/Lact 7-14	Gest/Lact 1-21
Vascular congestion	++	+++	s.a.	+++	+++
Hidropic degeneration	++++	++++	++++	++++	++++
Lymphocytic infiltrate	s.a.	+	+	s.a.	s.a.
1B – Adults offspring					
Pathological Alteration	Control	Gestation	Lactation	Gest/Lact 7-14	Gest/Lact 1-21
Vascular congestion	++	+++	s.a.	+++	++++
Greasy degeneration	s.a.	s.a.	+	+	s.a.
Hidropic degeneration	+++	++++	++++	++++	++++
Lymphocytic infiltrate	+	+	++	+++	++

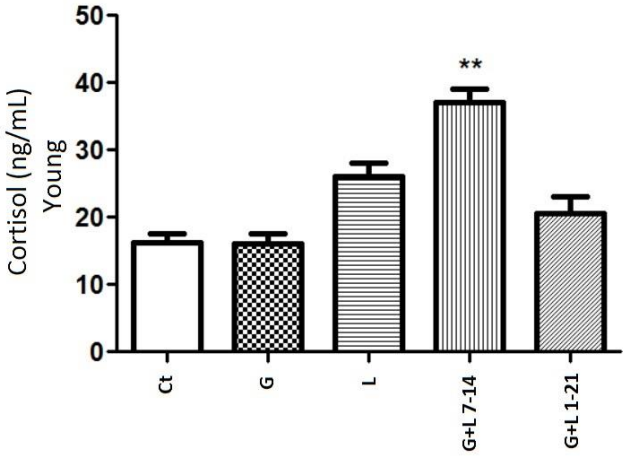

Figure 6: *Serum cortisol (ng/mL) dosage in the young offspring of rats exposed to fipronil (1 mg.Kg-1) in the perinatal period. Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21). Values represent the mean ± E.P.M. (N = 15). ** p<0.01 with respect to Ct.*

3.3. Biochemical and Chromatographic Evaluation

ALT and AST did not differ in treated young and adult offspring. Serum cortisol was significantly elevated ($p<0.01$) in offspring of group Gest/Lact 7-14 (Figure 6).

Table 1 shows values of fipronil and its metabolite fipronil sulfone in the brains of the offspring of the experimental groups. There was a significant ($p<0.05$) increase in the level of fipronil and fipronil sulfone in the offspring brains from fipronil treated animals in relation to the control animals, which is related to the exposure period and confirms the offspring exposure. In adult offspring the presence of fipronil and its metabolite was not detected. The desulfinyl metabolite was not detected in the brains of young and adult offspring.

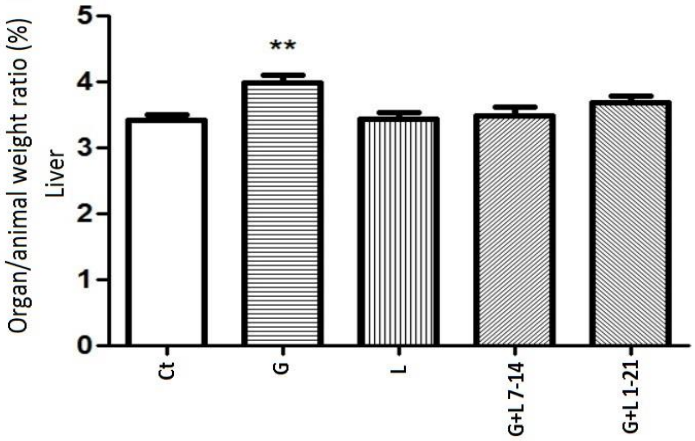

Figure 7: *Organ / animal weight ratio (%) to the liver of the adult offspring of Wistar rats exposed to fipronil (1 mg.Kg-1) in the perinatal period. Groups: control (Ct), gestation (G), lactation (L), gest / lact 7-14 (G+L 7-14) and gest / lact 1-21 (G+L 1-21). Values represent the mean ± E.P.M. (N = 15). ** p<0.01 with respect to Ct.*

3.4. Organ/Animal Weight Ratio

There was no statistically significant difference ($p>0.05$) in the organ / animal weight ratio in adult animals, among treatments for brain, kidneys, spleen and testes. The ratio of the calculated weight to the liver showed a significant increase ($p<0.01$) in the gestation group in relation to the control group (Figure 7).

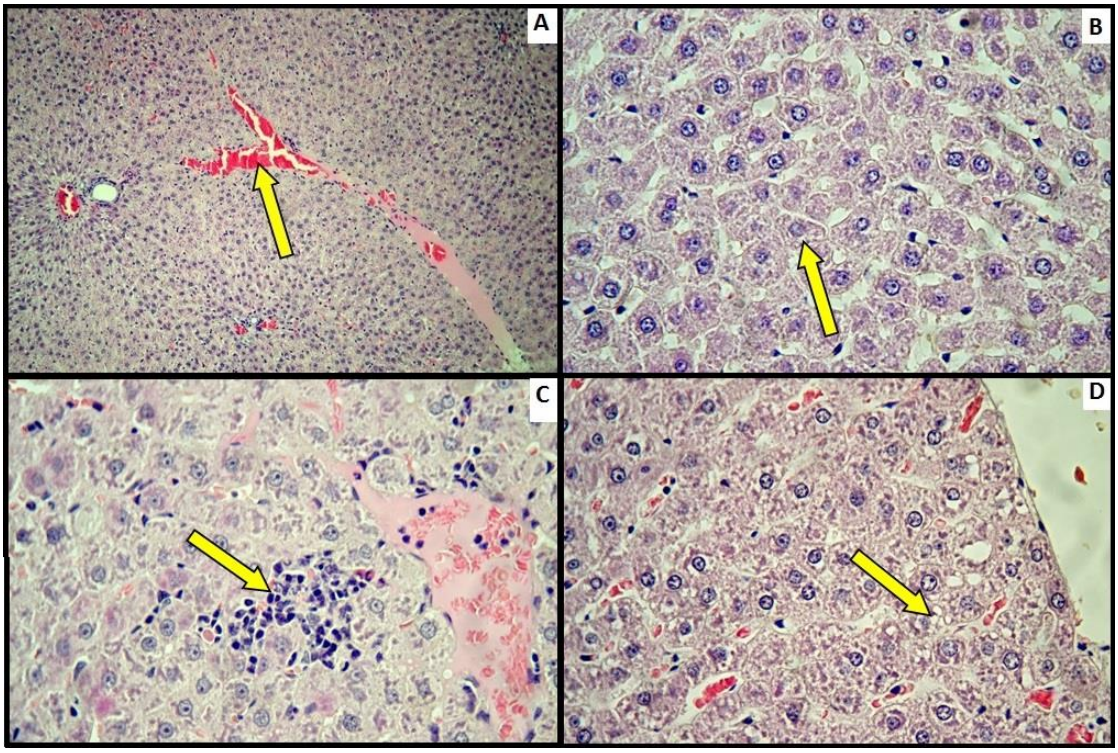

Figure 8: *Histopathological changes in the liver of offspring of Wistar rats exposed to fipronil (1 mg.Kg-1) in the perinatal period. A: Vascular congestion. 100x magnification; B: Hydrophobic degeneration. 400x magnification; C: Lymphocytic infiltrate. 400x magnification; D: Greasy degeneration. 400x magnification.*

3.5. Histopathological Evaluation

Histopathological evaluation of the liver showed changes in vascular congestion, hydropic degeneration and lymphocytic infiltrate in young rats. In adults, vascular congestion, fatty degeneration, hydropic degeneration and lymphocytic infiltrate were observed (Figure 8; Table 2).

4. Discussion

The results show that the exposure to fipronil in the perinatal period causes changes in aggression and anxiety of young and adult offspring. These results suggest that the neurobehavioral development of the offspring is altered by in-uterus exposure, especially during lactation.

One of the signs of maternal toxicity is weight loss (Chernoff et al. 2008) and changes in maternal homeostasis, which could indirectly interfere with the offspring's development (Salvatori et al. 2004). The absence of signs of maternal toxicity in the present study leads to suppose that any change that occurred with the offspring was therefore, exclusively due to exposure to fipronil.

Previous literature indicates that female exposure to agrochemicals during pregnancy and lactation can cause physical and behavioral changes depending on the animal development stage (Chelonis et al. 2004, Costa et al. 2004). Perinatal exposure may involve latent effects that might be observed later, even in exposure at lower doses than those promoting effects in adults, since the developing body does not have the same mechanisms of defense as adults.

4.1. Physical and Sensory-Motor Development

According to Jackson et al. (2009), rats receiving single administration of fipronil by gavage at doses of 0.5, 2.5, 7.5 and 25 mg.Kg^{-1} had a decrease in weight gain 7 days after administration of the single

dose of 7.5 mg.Kg^{-1}. Gill & Dumka (2013), exposing buffalo calves to an oral fipronil dose of 0.5 mg.Kg^{-1} per day for 98 days, found that fipronil induced a decrease in body weight gain in animals and clinical signs of intoxication. Udo et al. (2014) did not observe significant differences in the physical development of rats exposed to 0.1 mg.Kg^{-1} per day of fipronil in the prenatal period.

Contrary to that data, the study presented herein, showed, during the initial development period of the offspring, increased weight gains in the 14th and 21st days of life in the lactation and gestation plus lactation 1-21 (G+L 1-21) groups and a decrease in the time for the appearance of the incisors in all groups exposed to fipronil. Post-natal effects mediated by chemical agents found in breast milk may cause altered physical development in offspring (Salvatori et al., 2004). It is probable that the body level of the pesticide in the offspring, transferred from the mothers, could cause alterations on the homeostasis and maturation of their organism.

In addition, the effects of the pesticide on the hypothalamic-pituitary-adrenal axis and on glucocorticoid secretion could cause changes during the development of the offspring (Salvatori et al., 2004; Seckl & Holmes, 2007). Supporting this hypothesis, the fact that serum cortisol concentration in young offspring was increased, could suggest the possibility of an endocrine disrupting effect. In addition, the study of endocrine disrupting caused by pesticides present in the environment has been detected and discussed previously (Bila & Dezotti, 2007), including fipronil (Ferreira et al., 2012).

4.2. Behavior

In this study the OFT, a behavioral test commonly used for pharmacological screening of drugs that act on the central nervous system (Eidman et al., 1990), did not show alterations in animals treated with fipronil. These results are different from those observed by Terçariol & Godinho (2011), which demonstrated that fipronil, at higher doses, can interfere with the emotional, fear and exploratory activities of the animals in OFT. This difference could be attributed to the acute exposure of adult rats to high doses used by these authors.

Regarding the behavior of locomotion in OFT, there was no significant difference among groups of offspring, both young and adult. This agrees with observations of Terçariol & Godinho (2011), who did not observe alteration of the locomotion behavior of animals exposed to fipronil as well.

Magalhães et al. (2015), when exposing rats to fipronil in the prenatal period, did not observe significant differences in locomotion, cleaning and freezing. However, in their studies, the animals presented a high frequency of activity, when compared to the control group.

Regarding motor coordination, the young rats of the lactation group presented a significant increase in the number of leg paws. There was no significant difference among groups when adult rats were evaluated. This result is similar to that obtained by Terçariol & Godinho (2011), who studied the neurobehavioral activity of adult rats exposed to fipronil (0, 70, 140 and 280 mg.Kg^{-1}, dermal). Treatment with 70 mg.Kg^{-1} showed no alterations in motor coordination, whereas treatment with 140 and 280 mg.Kg^{-1}, caused a decrease in the motor coordination of the animals, suggesting a dose-dependent effect of fipronil on neural motor command.

Fipronil also caused motor defects in embryos and larvae of fish, increasing the range of organisms susceptible to the motor effects of fipronil (Stehr et al., 2006).

Godinho et al. (2014) studying motor coordination of adult animals exposed to the pyrethroid permethrin, a GABA antagonist similar to fipronil, observed motor incoordination due to the effect of the pesticide, an effect attributed to the action of the pyrethroid on the channels of calcium that control the cellular movement. This mechanism is very different from the GABA-ergic antagonistic action (Alviña & Khodakhah, 2008).

The reduction or increase of head thrusts in the HB apparatus are related to a depression or stimulation of the central nervous system activity, respectively (Adzu et al., 2002). In the experiments reported herein, both young and adult offspring, exposed to fipronil during the perinatal period, had no alterations in the exploratory behavior in the HB apparatus, suggesting that fipronil had no effect on general stimulation of the central nervous system. However, it cannot be excluded that the brain or any other specific neuronal mechanism of the offspring could be affected by the offspring exposure to fipronil through their mothers.

A major function of the cerebellum is to coordinate movement and maintain body posture and body balance (Ito, 1984). Anatomically, the cerebellum consists of two distinct structures: the cortex and the deep nuclei. The cerebellar circuits of the cortex receive and integrate a large amount of sensory and cortical information whereas the neurons of the deep cerebellar nuclei encode the cerebellar signals required for the coordination of movement (Raman et al., 2000). Changes in the intrinsic activity of deep cerebellar nuclei neurons adversely affect cerebellar function causing ataxia (Shakkottai et al., 2004). The lack of motor coordination caused by fipronil in animals could be related to a toxic action of the pesticide on cerebellar function. Further research should be carried out to test this hypothesis.

The effect of fipronil motor incoordination was observed only in young rats and not in adults, and fipronil active metabolite sulfone, was detected only in the young offspring, suggesting that the motor effect caused by fipronil in the animals was dependent on the presence of the chemical agent in the brain. This hypothesis could be tested with dosage of the agent and active metabolites in nervous structures potentially implicated in the motor effect observed here, especially the cerebellum.

The results of the motor coordination tested in the HB could be influenced by the locomotor activity of the animals (Meyer & Caston, 2005), but the fact that, in the open field arena test, no alteration in the locomotion activity was observed reinforces the toxic influence of fipronil on motor nerves. Thus, the results obtained in this study confirm that fipronil exposure in the perinatal period alters the motor nerve activity in offspring, without modifying their exploratory activity or locomotion.

The behavior of the animal in the EPM is an experimental model of anxiety and an excellent model to evaluate drugs with GABAergic action (Carobrez & Bertoglio, 2005). GABA is the major inhibitory neurotransmitter and has been associated with a broad spectrum of physiological and pathological functions including anxiety (Narvaes & Almeida, 2014). In the EPM test, young offspring exposed to fipronil during the lactation period entered less frequently and spent less time in the open arms and remained longer in the closed arms. As aversion to the open arms demonstrates an anxiogenic effect (Terçariol et al., 2011), it is possible that exposure to fipronil during lactation increased anxiety of young offspring.

The adult offspring of the group G+L 7-14 remained longer time in the open arms than in the closed arms of the EPM, suggesting that perinatal exposure to fipronil may have influenced the offspring differently than in adulthood. This biphasic effect has not been described yet in the literature for fipronil class agents and there is no documented plausible explanation for it. Hypothetically, the presence of fipronil and its sulfone metabolite in the brain of young offspring and the absence of them in adult animals could be responsible for this reversal effect. In the adult would function as a sequel or imprinting due to the previous presence of the chemical agent in the brain tissue.

Exposure of adult male Wistar rats to fipronil (oral - gavage) for 28 days at doses of 0.1; 1; and 10 $mg.Kg^{-1}$ BW, did not cause significant differences between the control and treated groups in relation to the number of entries in the closed and open arms of the EPM (Silva, 2008). Differences with the present results may be due to the dose, commercial product, and route of administration used. Oral administration, due to the first pass effect, renders a different kinetics of the chemical agent.

Our results agree with those of Udo (2012), who exposed Wistar rats, at doses of 0.1; 1.0 and 10.0 $mg.Kg^{-1}$ per gavage from the 6^{th} to the 20^{th} day of gestation and demonstrated, when the offspring were AGE 75 and exposed to 10 $mg.Kg^{-1}$ of fipronil decreased the number of entries in the open arms. Male offspring exposed to any of the doses, decreased their permanence in the open arms. Furthermore, Wistar rats exposed to 280 $mg.Kg^{-1}$ of fipronil topically, did not show any significant difference among groups when evaluating the number of entries and permanence in open and closed arms, but showed a difference in emotionality, fear and locomotor activity. The difference could be attributed to the experimental protocol and dose used, since the exposure route was the same (Terçariol & Godinho, 2011).

The results of the present study suggest that fipronil may influence the anxiety state of the animals, corroborating results obtained in previous studies developed by U.S. EPA (1996).

Rats, fed with one of the metabolites of fipronil, fipronil-desulfinil, at doses of 0, 0.025, 0.098 and 0.050 $mg.Kg^{-1}$ per day for males and 0, 0.032, 0.130 and 0.550 $mg.Kg^{-1}$ per day for females, showed an increase in the incidence of aggression at the highest dose tested (Jackson et al., 2009). The results presented here also demonstrate the influence of fipronil on aggressiveness, since the offspring exposed in the lactation period presented a significant decrease in the latency time for the first attack. Currently, there are no publications describing the effect of the perinatal exposure of fipronil on aggressive behavior of rat offspring.

Magalhães et al. (2015) exposed rats at 0.1 $mg.Kg^{-1}$ of fipronil and observed an increase in latency time for the first attack compared to the control group. The same authors, when they tested a fipronil dose of 10.0 $mg.Kg^{-1}$ observed a higher frequency of attacks to the intruder animal when compared to the control group.

From the results obtained in the present study, and those obtained by Magalhães et al. (2015), it can be suggested that the aggressiveness resulting from the action of fipronil is dose dependent, where higher doses tend to cause greater aggressive behavior in rats.

The offensive and defensive behaviors of rats have common features with aggressiveness in humans, and are used in neurophysiology and neuropharmacology studies of aggression, as well as models for psychiatric disorders (Coccaro & Kavoussi, 1997). According to Beiderbeck et al. (2012), the highest levels of aggression in laboratory male rats are accompanied by excessive neuronal activity within the paraventricular nucleus. They can also be induced by stimulation of the hypothalamus, lateral hypothalamus or lesions of the olfactory bulb, amygdaloid nucleus and septal region. The effects of the lesions on aggressive behaviors vary according to the location of the lesion (HO et al., 2004). The GABAergic blocking effect caused by fipronil could be translated, in this region, in a stimulating effect and thus cause a greater state of aggressiveness.

According to the serotoninergic hypothesis of aggression proposed by Nelson & Chiavegato (2001), the neurotransmitter serotonin may be the main central endogenous mediator involved in aggressive behavior. In the present study it cannot be ruled out that fipronil may influence neurophysiological mechanisms that control serotonin in the central nervous system. This could be the aim of future studies.

On the other hand, Adams et al. (1993) demonstrated that the GABA antagonist, picrotoxin causes aggression when injected in the same hypothalamic area where the electrical stimulation promotes attacking behavior in rats, suggesting a role of this neurotransmitter in the behavior of animal aggression. In addition, Greg & Siegel (2001) discussing the brain structures and neurotransmitters involved in aggressive behavior, suggested a role of GABA in this behavior. More recently, Lee & Gammie (2010) discussed the involvement of GABA in maternal aggression in mice as well.

As fipronil acts as an antagonist to GABA, it is not possible to exclude an effect of fipronil on this central mediator that might result in the increased aggressiveness observed here. As the toxic action of fipronil occurs by competitive GABA blockade, a deregulation of the balance between excitation and inhibition of the central nervous system and related neurotransmitters could result on significant changes in aggressiveness.

The role of the GABA transmitter in aggressive behavior is still controversial. Studies directly manipulating GABA levels point to an inverse correlation with aggressive behavior (Miczek et al., 2003); However, studies using GABA positive allosteric modulators, such as alcohol, have reported increased aggressive behavior (Almeida et al., 2005).

Dietrich et al. (2013) indicated that higher levels of cortisol in children are related to anxiety and lower levels of cortisol are associated with aggression. This is not supported by the results obtained in the present study, where animals with high cortisol levels (young offspring of the G+L 7-14 group) were not more anxious. The animals that were most anxious (young offspring of the lactation group), did not present increased levels of cortisol; animals that presented more aggressive behaviour (lactation) did not show decreased levels of cortisol; and in less anxious animals (adult offspring of G+L 7-14 group), no decrease in cortisol was observed.

The facts that fipronil or fipronil metabolites are not found in the adult offspring, while some behavioral changes remain, indicate that the presence of the chemical agent or its metabolites in the perinatal period may leave imprinting on the neurodevelopment of the offspring, which warrants further research.

4.3. Biochemical Evaluation

ALT and AST enzymes are commonly used in the diagnosis of hepatic diseases in rodents; however, there is not much information available regarding fipronil toxicity in biochemical responses (Gupta et al. 2014). In the present study, the quantification of marker enzymes to liver injury showed normal levels in offspring exposed to fipronil. Gupta et al. (2014), exposed carps (*Cyprinus carpio*) to fipronil at different concentrations (0.01, 0.1, 1.0 and 10.0 $mg.L^{-1}$), and observed that AST activities in both liver and muscle ratio were significantly higher in fish exposed to fipronil than in control. However, they did not find significant changes in the level of ALT in the hepatic tissue. The differences observed may be due to differences in the experimental models used.

4.4. Organ/animal Weight Ratio

The ability of chemicals to induce metabolic enzymes, including cytochrome p450, has been considered one of the biochemical cellular responses most sensitive to toxic aggression, since many occur at much lower doses of the chemical than those known to cause toxic effects and lethality (Hernández et al. 2013). The organ / animal weight ratio calculated for the liver of the offspring of the gestation group was increased by fipronil suggesting an inducing effect of the hepatic enzymatic system. This result supports previous data presented by Tang et al. (2004) and Das et al. (2006), suggesting that fipronil increases the activity of microsomal liver enzymes.

4.5. Histopathological Evaluation

Rats exposed to fipronil presented more intense vascular congestion in the liver than rats in the control group. Hydropic degeneration was identified in all groups of animals. Other histopathological changes in the liver were diagnosed in the groups exposed to fipronil, such as lymphocytic infiltrate and fatty degeneration. When the animals in the control group had these alterations, there was a tendency of these animals to be less intense; suggesting that exposure to fipronil may have exacerbated already existing alterations.

Hydropic degeneration is the most common and fundamental expression of cell injury. It is manifested as an increase in cell size and volume from a water overload caused by cell failure and lack of maintenance of normal homeostasis in regulating the inflow and outflow of water. It is accompanied by the modification and degeneration of the organelles (Myers et al. 2012). As one of the probable organelles affected by fipronil in the liver may be the smooth endoplasmic reticulum, further research is needed to confirm this suggestion.

5. Conclusion

The present results, overall, suggest that perinatal exposure of Wistar rats to fipronil, especially during the lactation period, increases aggressiveness and anxiety behaviors in offspring, specifically during the young phase of life. It is suggested that this effect occurs due to the presence of fipronil and the interference of fipronil and its sulfone metabolite in the activity of the brain. The increased level of serum cortisol observed in the young offspring may not be related to the greater aggressiveness observed since it occurred in the adult offspring when cortisol level was normal. The fact that some behavioral changes have been observed in adult rats in the absence of fipronil or its sulfone metabolite suggests an imprinting effect, which needs to be investigated further.

Despite the normality of hepatic enzymes, histology of the liver indicates negative effects of exposure to fipronil, which could interfere with the homeostasis of the organ and modify its metabolic capacity. Together, alteration of these parameters during treatment confirms the toxicity of perinatal exposure of rats to fipronil.

Finally, the perinatal exposure of rats to fipronil caused a decrease in the motor coordination in young offspring, suggesting toxicity on the motor nerves. The motor incoordination effect seems to be dependent on the presence of fipronil and its active metabolite sulfone.

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