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Behavioral effects of acute exposure to the insecticide fipronil

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ABSTRACT

The effects of fipronil (Frontline® Top Spot) were investigated in 40 days old rats utilizing open field (OF), hole-board (HB) and elevated plus-maze (EPM) apparatus. Rats ($N = 15$) received topical application of fipronil (70, 140 and 280 mg/kg) in the neck region and behavior was tested 3 h after administration. Animals treated with corn oil (vehicle) were used as controls. In the OF test animals treated with fipronil at 140 mg/kg showed increased rearing, whereas animals exposed to 280 mg/kg showed increased freezing, grooming, and rearing. In the HB test fipronil at 280 mg/kg increased head-dip and head-dipping behaviors. In the EPM test the only observed effect was increased number of entries in both open and closed EPM arms in animals treated with 280 mg/kg. In conclusion, dermal exposure to fipronil causes effects related to emotionality, fear, and exploratory activity; results add strength to the growing concern that pirazole insecticides can be neurotoxic to humans.

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1. Introduction

In recent years there is a growing concern that pesticides and other chemicals substances might modify normal endocrine function of humans and wild animals. Hormonally active chemicals (also known as endocrine disruptors) can cause a variety of adverse effects including developmental, reproductive and behavioral hazards [1]. It is known that exposure to pesticides is neurotoxic to rodents and other mammals, including humans [2].

Fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl] – 4-[(trifluoromethyl) sulfinyl] -1H-pyrazole -3-carbonitrile) is a highly active, broad spectrum pesticide from the phenyl pyrazole family that targets the gamma-amino butyric acid (GABA) receptor [3–5]. Fipronil is used in the agriculture against pests in a wide variety of food crops [6–8]. It has also non-agricultural applications, including control of veterinary pests [9]. In addition, fipronil was designated by the Environmental Protection Agency (EPA) as one of the alternatives to the organophosphates for termites and fire-ants control. Concerns about fipronil adverse effects on public health have been raised because of its wide commercial and domestic uses [9,10]. Fipronil has higher toxicity to insects than mammals [11–13]. Its selectivity is due to its greater potency in blocking the insect isoform of GABA-gated chloride channels than their mammalian counterparts [12,14]. However, fipronil can bind to mammalian $GABA_C$ and $GABA_A$ receptors [15,16]. Its sulfone metabolite, as well as fipronil desulfinyl, a product of photodegradation, were reported to be more toxic to insects, mammals, fish and birds than the parent compound itself [17].

Although phenyl pyrazole neurotoxicity is well characterized and their mechanism of action in mammals is already known, the potential neurobehavioral effect of this class of insecticides in mammals is limited. Recently, a case report described fipronil-induced symptoms (headache, nausea, vertigo and weakness) in a patient intoxicated by accidental dermal and inhalation exposure [18]. This report suggests that second generation insecticides may also have severe effects on humans after chronic exposure. Since humans and animals are exposed to fipronil, either at low doses chronically or at an accidental single high dose, possible behavioral effects elicited by dermal exposure to these insecticides, such as can occur in in pet care and agricultural use, need to be fully evaluated. Therefore, the purpose of the present study was to elucidate whether fipronil poses behavioral hazards to adolescent male rats acutely exposed by topical administration of a formulated product, since topic application is the most popular form of therapeutic use of this pesticide.

2. Material and methods

2.1. Experimental design

The fipronil insecticide used was an available commercial formulation (FrontLine® Top Spot), containing 10% fipronil [(±)-5-amino-3-cyano-1-(2,6-dichloro-α- α - α –trifluoro-p-tolyl)-4-trifluoromethyl sulfinyl pyrazole-carbonitrile], obtained from Merial Saúde Animal Ltda (São Paulo/SP, Brazil). For the experiments,

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animals were obtained from the colony housed at the Sao Paulo State University. Animals were maintained under standard conditions (up to four rats per cage, temperature and humidity controlled, on a constant 12 h light/dark cycle starting at 6 a.m.). Standard rat pellet chow (BioBase®, Santa Catarina/SC, Brazil) and tap water were available *ad libitum*. All procedures were approved by the the Committee of Ethics in Animal Experimentation (CEEA) of the College of Veterinary Medicine and Zootecny, Sao Paulo State University at Botucatu.

Adolescent male Wistar rats (40 days old) were divided into four experimental groups ($N = 15$) that received fipronil (70, 140, and 280 mg/kg) topically (dermal route). Control animals received corn oil (vehicle) topically. These doses correspond to one, two and four-fold the highest dose recommended by the manufacturers. The dose of 280 mg/kg for fipronil was utilized as a reference dose in this study because it has been recognized as sufficient to cause adverse reproductive effects in Wistar rats [19]. Topical applications of vehicle or fipronil were performed in the neck region to prevent licking of the insecticide. After application, rats were housed one per cage to prevent them from licking each other.

Behavioral tests were performed 3 h after fipronil administration. This time period was chosen based on the results of a pilot study using 280 mg/kg fipronil that evaluated (1) the time for disappearance of stress effects caused by handling of the animals, which could cause bias in the behavioral assessment; and (2) the better time to assess behavior after fipronil application.

2.2. Behavioral tests

Behavioral evaluations of rats were performed using open field, hole-board, and elevated plus maze apparatus tests in which the animals were tested once without prior habituation. These experimental models were chosen for behavioural evaluation because they are used to demonstrate drug-induced central nervous system effects [20,21] and risk assessment [22]. The room for the behavioral assessment was sound-proof, temperature-controlled and, illuminated by dim red lights. The period of behavioral observation was defined between 9 a.m. and 11 p.m. To prevent observational bias the testers were blind to the treatment group.

2.2.1. Open field test

The open field behaviour was assessed using a wooden box measuring 97 × 32.5 cm (diameter × height), as described previously [23]. The box was divided into three concentric circles, which were subdivided by painted black lines into 18 similar spaces. For open field observations, each rat was placed in the center of the arena and for the next 3 min was scored on the following parameters: ambulation frequency (number of floor units entered with the four paws), rearing frequency (number of times the animals stood on its hind legs), freezing duration (total time the animal was in an immobile state, often in a crouching posture with wide open eyes and irregular respiration, after it had remained motionless for at least 1 s), and grooming duration (total time used by the animal for grooming). The following grooming behaviours were considered: forepaw vibration, paw licking, washing of nose, face and head, body licking, genital grooming, scratching, and head-shaking. The open field was cleaned with 5% ethanol before each animal was introduced.

2.2.2. Holeboard test

The hole-board (HB) apparatus was an open field arena similar to that described previously [23] with four equidistant holes (3 cm diameter × 2 cm depth) in the floor. Each rat was placed at one corner of the board. Only one rat was tested each time. The rat was allowed to move around and dip its head into the holes. Poking the nose into a hole is a normal behaviour of the rat indicating curiosity and was utilized as a measure of exploratory behavior [24]. The head dip count and head dipping time duration (seconds) for five minutes (time allowed for curiosity behavior) was recorded and a head dip was scored if both eyes disappeared into the hole. The HB was carefully cleaned with 5% ethanol before each animal was introduced.

2.2.3. Elevated plus-maze test

The elevated plus-maze (EPM) behaviour was conducted as described previously [25] and was assessed using an apparatus consisting of two open and two enclosed arms of equal length and width (50 × 10 cm). The open arms had a 1 cm high Plexiglas edge while the enclosed arms are not entirely enclosed, but rather have walls that extend 40 cm high. The EPM was elevated 50 cm above the floor. Each rat was placed in the centre of the elevated plus-maze facing one of the open arms, and the number of entries with the four paws, and time spent (seconds) in the open or closed arms were recorded during a 3 min test period. The EPM test is based on the principle that exposure to an elevated and open arm maze leads to an approach conflict that is considerably stronger than that evoked by exposure to an enclosed maze arm. Thus, the total entries and time spent in both open and closed arms provide a measure of anxiety or fear-induced inhibition of normal exploratory activity [25,26]. In this test the number of entries in the closed arms is utilized as an assessment of locomotor activity (for a review see [27]. The EPM was carefully cleaned with 5% ethanol before each animal was introduced.

2.3. Statistical analysis

Data were analyzed by One-Way Analysis of Variance (ANOVA) using the Instat 3.0 software (Graph Pad Software). The post hoc Tukey–Kramer multiple comparisons test was used to identify differences between groups if means were considered significantly different at $P < 0.05$ [28].

3. Results

3.1. General observations

No mortality was observed in any of the animal's exposure to the various doses of fipronil.

3.2. Open field behavioral assessment

The effects of fipronil in the open field behavior are summarized in Table 1. Animals exposed to 70 mg/kg fipronil had no changes in OF behavior. Animals treated with 140 mg/kg fipronil showed a significant increase in rearing behavior ($p < 0.05$) when compared to control animals. The dose of 280 mg/kg significantly increased rearing ($p < 0.001$), freezing ($p < 0.001$), and grooming ($p < 0.01$) behaviors compared to controls. In addition, at 280 mg/kg fipronil significantly increased freezing and grooming behaviors then the doses of 70 and 140 mg/kg. Rearing behavior was not different between animals treated with140 and 280 mg/kg of fipronil. In the OF, locomotion behavior of animals was not altered by any of the three fipronil doses studied.

3.3. Hole-board behavioral assessment

The effects of fipronil in the HB behavior are summarized in the Fig. 1. Animals exposed to 70 mg/kg fipronil had no changes in HB behavior compared to controls. Animals of the 140 and 280 mg/kg fipronil treated group had a significant increase ($p < 0.05$ and $p < 0.001$ respectively) in the number of head-dip and in the

Table 1
Open field behavior of rats exposed to the insecticide fipronil insecticide. Values are mean ± SE, $n = 15$.

Treatment	Locomotion	Rearing	Freezing	Grooming
Control	52,15 ± 6,47a	1,62 ± 0,31a	2,79 ± 0,21a	6,69 ± 0,61a
Fipronil 70 mg/kg	53,22 ± 6,01a	1,74 ± 0,27a	2,74 ± 0,36a	7,07 ± 0,82a
Fipronil 140 mg/kg	47,05 ± 5,77a	3,55 ± 0,35a	3,07 ± 0,39a	7,79 ± 0,87a
Fipronil 280 mg/kg	49,50 ± 4,16a	5,07 ± 0,64cb	6,34 ± 0,47b	13,06 ± 2,14b

Different letters in the same column represent different statistical significant differences.

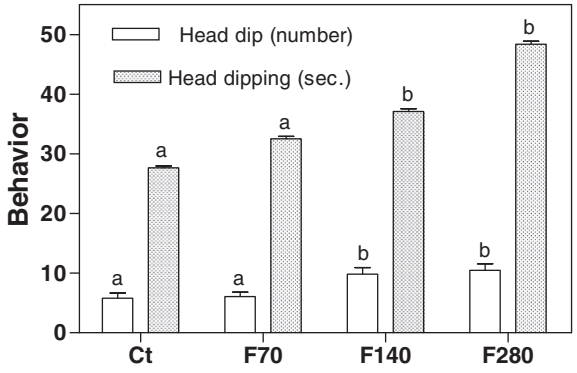

Fig. 1. Hole board behavior of rats exposed to the insecticide fipronil. Values are mean ± SE, $n = 15$. Ct = control; F70 = fipronil 70 mg/kg; F140 = fipronil 140 mg/kg; F280 = fipronil 280 mg/kg. Different letters in the same assessment type represent different statistical significant differences.

head-dipping duration when compared to control animals, without differences between these two doses.

3.4. Elevated plus-maze behavioral assessment

The effects of fipronil in the EPM behavior are summarized in Table 2. Animals exposed to 70 and 140 mg/kg fipronil had no changes in EPM behavior. Rats exposed to 280 mg/kg fipronil had a significantly increased number of open and closed arms entries ($p < 0.05$) than controls. The permanency time in both open and closed arms of the EPM was not changed by fipronil treatment.

4. Discussion

The present study shows strong experimental evidence that a single, large dose of fipronil may influence mammalian neuronal excitability using behavioral investigation. Although it has been demonstrated that the new generation of insecticides shows greater affinity to invertebrates than to mammalian receptors [29,30],the data obtained here with fipronil insecticide exposure suggests that their effects in vertebrate's central nervous system cannot be excluded.

In the present experiment adolescent rats were chosen because a great preoccupation exists on exposure of infants and children. These individuals are more sensitive to effects of some pesticides [31]. There is a growing concern that exposure to neurotoxicants during development might result in acceleration of age-related decline in central nervous system function. Thus, it has been speculated that small effects during development can have a profound social impact when amortized across the entire population and across the life span of humans. It is important to stress that the adolescence is a critical period for the deleterious effects of drugs, including insecticides, which act as endocrine disruptors [32].

The test of open field is considered an indicator of the emotional state of the animal and is commonly used for pharmacological selection of drugs that act on the central nervous system [33]. In this test, locomotion and rearing behaviors are considered indicators of locomotor and exploratory activities, respectively, whereas grooming and freezing are positively correlated with fear or emotionality ([33–37]. In the present study, animals receiving fipronil presented increased freezing, grooming and rearing behaviors, suggesting that the insecticide increases emotionality and exploratory activities without modifying locomotor activity despite the fact that locomotion can also be related to exploration [35].

The data from the OF test indicates a dissociation between locomotor activity and rearing behaviors in animals exposed to fipronil. These are in contrast with results of others authors that reported that ambulation and rearing are positively correlated behaviors [38,39]. However, the study of Szegedi [40] on fipronil behavioral effects, which used the oral route of exposition (intragastrically), draws attention to the fact that problems in handling the animals during treatment can cause discomfort and increase in excitation, which may negatively influence the outcomes. For example, improper handling causing changes of mood enhances animal's escape activity, including rearing. This is not the case here, since maximum care was taken to prevent the influence of handling on animal's behavior. The fact that fipronil doses of 70 and 140 mg/kg increased rearing behavior and that the 280 mg/kg fipronil dose caused a further increase in the rearing behavior suggests that this effect might be dose-dependent.

Due to the problems in interpreting the behavioral measures from the open field, the use of a specific test of anxiety conditions in animals is strongly advised and the holeboard test is recommended as a test that can provide independent measures of exploration and motor activity ([41,42]. In our experiment, animals exposed to fipronil at 140 and 280 mg/kg showed a significant increase in the head dip and head dipping behaviors, suggesting a stimulatory effect of this insecticide in their central nervous system [43]. According to Adzu [44], decreases in exploratory activity by reduction in head dip is a measurement of depression of central nervous system activity, whereas an increase is a measurement of stimulation of CNS activity. Therefore, our data from the HB test

Table 2
Elevated Plus Maze behavior of rats exposed to the insecticide fipronil. Values are mean ± SE, n = 15. Different letters in the same column represent different statistical significant differences.

Treatment	Open arms		Closed arms	
	Number of entries	Time spent (sec.)	Number fo entries	Time spent (sec.)
Control	4,51 ± 0,61a	94,46 ± 8,48a	7,76 ± 0,73a	177,63 ± 20,12a
Fipronil 70 mg/kg	5,33 ± 0,73ab	97,42 ± 1,07a	8,33 ± 0,81ab	180,28 ± 20,37a
Fipronil 140 mg/kg	5,55 ± 0,69ab	101,88 ± 10,89a	8,42 ± 0,96ab	187,32 ± 19,98a
Fipronil 280 mg/kg	7,65 ± 0,81b	102,74 ± 11,66a	11,57 ± 1,17b	190,15 ± 21,74a

further suggests a dissociation between locomotion and exploratory activity, as observed in the OF test.

On the other hand, the EPM test is considered an indicator of the animal anxiety state ([25,26] and a higher entry number in the closed arms of the apparatus, together with an increase in permanence time, reflect augmentation in the animal's anxiety. In the EPM test, fipronil did not alter open and closed arms entry or the permanence time in both arms in animals exposed to 70 and 140 mg/kg. However, fipronil at 280 mg/kg caused an increase in the number of entries in both open and closed arms. These effects should not be considered solely as indicators of altered anxiety because increased entry might be influenced by the increased locomotor and exploratory activities [27]. The results obtained in the EPM are consistent with the data from the OF and HB assessments, suggesting that fipronil apparently stimulates the animal's sense of exploration without altering locomotion. Although anxiety can be considered a component of the emotional state [45], the present findings suggest that fipronil is capable of affecting emotionality without changing the animal's anxiety.

The behavioral effects discussed here occurred in animals exposed through a dermal route. This route of exposure is the most commonly observed due to the agricultural and therapeutically uses of this insecticide [19]. Importantly, compounds absorbed *via* a dermal route do not undergo first pass metabolism by the liver. As consequence the compound is distributed to the tissues prior and after its metabolism in the liver. As fipronil metabolite is more toxic than the parent compound [17], the slow rise in circulating levels of the metabolite, depending on the dermal dose, might attenuate the effects of fipronil on the brain neurochemistry. The fact that the dermal LD50 of fipronil is higher than 2000 mg/kg bw [46] agrees with this observation. This kinetic profile might help to explain the three hours onset of behavioral effects observed in our pilot studies. As opposed to fipronil, others pesticides act in mammals in their original molecular form and have their effects diminished after metabolism. Thus, future research is important to study the implications of kinetic parameters on risk assessment for neurotoxicity by these compounds.

In conclusion, since non-target organisms are evidently exposed to the insecticides because of colocalization, it is important to have more information about their undeliverable effects. The present study confirmed that the insecticide fipronil has central behavioral effects in rats. Further studies with pirazole insecticides, including fipronil, are necessary to verify their neurotoxic potential in humans because of accidental and professional exposure.

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