Genotoxicity assessment of fipronil (frontline plus®) in *Canis familiaris*[1]

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ABSTRACT.- Ziliotto L., Luna S.P.L., Filho D.A.A., Resende L.O., Aun A.G. & Braz M.G. 2017. **Genotoxicity assessment of fipronil (frontline plus®) in *Canis familiaris.*** *Pesquisa Veterinária Brasileira 37(3):257-260.* Faculdade de Medicina de Botucatu, Universidade Estadual Paulista, Avenida. Prof. Mário Rubens Guimarães Montenegro s/n, Botucatu, SP 18618-687, Brazil. E-mail: mgbraz@hotmail.com

Fipronil is a pesticide widely used for controlling fleas and ticks in domestic animals, and its short-term exposure can lead to serious effects on animals. However, the possible genotoxic effect of this compound has not been investigated in target animals. Based on the hypothesis that fipronil can induce genotoxicity, this study evaluated the effect of fipronil on DNA damage in peripheral blood cells. For that purpose, ten dogs of both sexes were used in the study. The product (6.7mg/kg) was applied on the dorsal neck region of each animal. Peripheral blood samples were collected immediately prior to application of the product, and at 3, 8 and 24 hours after the application. Samples were processed for comet assay. No statistically significant differences were found among the four time points. The current study suggests for the first time that a single exposure to this pesticide does not induce systemic genotoxic effect in dogs.

INDEX TERMS: Fipronil, frontline plus®, pesticide, DNA damage, comet assay, dogs.

RESUMO.- [**Avaliação de genotoxicidade do fipronil (*frontline plus*®) in cães.**] O fipronil é um inseticida/herbicida amplamente utilizado para controle de pulgas e carrapatos em animais domésticos. Sua exposição a curto prazo tem acarretado efeitos deletérios em animais. Entretanto, o possível efeito genotóxico deste composto ainda não foi investigado em animais alvo. Baseando-se na hipótese de que o fipronil pode induzir genotoxicidade, o presente estudo avaliou o efeito deletério do fipronil no material genético de células de sangue periférico. Para isso, dez cães sadios, de ambos os sexos, foram utilizados neste estudo. O produto (6,7mg/kg) foi aplicado na região dorsal do pescoço de cada animal. As amostras de sangue foram coletadas imediatamente antes da aplicação do produto (controle) e após três, oito e 24 horas da aplicação. As amostras foram imediatamente processadas para condução do teste do cometa, a fim de se avaliar os danos basais no DNA. Não houve diferença significativa entre os quatro momentos de coleta em relação aos danos no material genético. O estudo sugere, pela primeira vez, que uma exposição única a este pesticida não induz efeito genotóxico sistêmico em cães.

TERMOS DE INDEXAÇÃO: Fipronil, frontline plus®, pesticida, dano ao DNA, ensaio cometa, cães.

INTRODUCTION

Frontline plus® is a pesticide formed by the compounds fipronil (active principle) and methoprene. It is a highly efficient second generation-pesticide and widely used in veterinary products for controlling agricultural pests and ectoparasites in domestic animals, including those resistant to pyrethroid, organophosphate, and carbamate insecticides (Kidd & James 1991). Fipronil is applied specifically in dogs and cats to control fleas and ticks (Tingle et al. 2003).

The control of ectoparasites is of fundamental importance because they are vectors of various protozoa, bacteria and viruses that cause diseases in animals and humans.

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Once applied, the product spreads through the body, is stored in the lipid layer of the skin and hair follicles and continues to be released by the skin and its hairy covering, which results in long-term activity (Jennings et al. 2002). Residues may still be present in significant amounts a week after the application of the product (Hugnet et al. 1999).

Short-term exposure to fipronil can lead to serious effects on fetal and postnatal development, such as learning disability, reflex reduction, sterility, and also increased susceptibility to many diseases, including cancer (Lyons 2000). Rats exposed to fipronil demonstrated a significant increase in thyroid cells, leading to tumor formation (Chodorowski & Anand 2004). In addition, this compound was capable of changing the values of thyroxine hormones (Hurley et al. 1998). Fipronil has been classified by the U.S. Environmental Protection Agency (EPA), as a possible human carcinogen (group C) based on an increase in thyroid follicular cell tumors in rats of both sexes (EPA 1996).

Since the advent of Genetic Toxicology, it has become possible to assess the toxicity of pesticides in the genetic material. Thus, it is relevant to evaluate whether these compounds have genotoxic/mutagenic effects and their possible mechanisms. Taking into account that the genotoxic effect of fipronil remains unknown and that there is a scarcity of data about the genotoxicity of pesticides evaluated in exposed mammals, the current study was designed to evaluate the genotoxic potential of fipronil by detecting systemic DNA damage in dogs.

MATERIALS AND METHODS

Ethics statement. The Animal Experimentation Ethics Committee of the local institution approved the study, under the protocol number 154/2014 in adherence to the Principles of the National Council for the Control of Animal Experimentation, from the Ministry of Science and Technology.

Animal preparation. Ten healthy adult crossbred dogs of both sexes (5 male and 5 female), weighing between 15kg and 20kg were used in the study. The animals were considered healthy after clinical, hematological, biochemical, serological, urinalysis and fecal routine examination. Before the beginning of the study the animals had been vaccinated and vermifuged on a regular and conventional basis, and were housed in pairs in each kennel, where they were given commercial food and drinking water *ad libitum*. The animals were acclimated for a period of 30 days before the start of the experiment.

Study design. The fipronil (Frontline plus®) was applied on the dorsal neck region of each animal at a dose of 6.7mg/kg, according to the manufacturer's recommendation for the control of ectoparasites in dogs and cats in the routine.

Blood samples were collected by venipuncture (cephalic vein) in heparinized syringes immediately prior to application of the product (T0 - control), then at 3 hours (T1), 8 hours (T2) and 24 hours (T3) after the application. The samples were coded and blindly analyzed.

Genotoxicity assay. The comet assay was performed in duplicate to evaluate DNA damage in peripheral blood cells by following the guidelines (Singh et al. 1988) with some modifications (Tice et al. 1991, Braz & Salvadori 2007). Every step was carried out under indirect light. A volume of 5µl of fresh blood was added to 100µl of 0.5% low-melting-point agarose at 37°C, layered onto a pre-coated slide with 1.5% normal agarose, covered with a cover slip, and left for 5 min at 4°C to solidify the agarose. Afterwards, the cover slip was carefully removed and slides were immersed overnight into a cold lysis solution. Slides were washed in phosphate buffered saline (PBS) for 5 min, and immersed in a freshly prepared alkaline buffer (pH>13) in a horizontal electrophoresis tank. After 20 min of DNA unwinding period, electrophoresis was conducted at 25 V and 300mA for 30 min. Following 15 min of neutralization, slides were fixed with absolute ethanol for 5 min, and stored at 4°C. Prior to analysis, the slides were stained with 50µl of ethidium bromide and scored in a fluorescent microscope at 400 x magnification. Images from 50 nucleoids (25 from each replicate slide) were analyzed using the Comet Assay II image system (Perceptive Instruments, UK). Two parameters (tail intensity and tail moment) were used to estimate DNA damage. As tail moment gave similar results, only tail intensity (percentage of tail DNA) values were presented.

Statistics. Since the data presented a normal distribution, the four time points were compared by repeated measures analysis of variance; p-value less than 0.05 were considered statistically significant.

RESULTS

Results are presented in Figure 1. Despite a slight increase of DNA damage at 3 hours and 8 hours after exposure, no significant differences were found among the four time points (p-value = 0.52), showing that a single exposure to fipronil (frontline plus®) does not produce short-term genotoxicity in dogs.

DISCUSSION

There are many reports showing that pesticides can produce toxic effects in directly and indirectly exposed non-target organisms, including humans (Dulot et al. 1985). Therefore, the genotoxicity assessment of pesticides is of great importance. Since pesticides are widely used worldwide and information on the possible toxic effect of fipronil is limited, this study was designed to evaluate the ability of fipronil to induce DNA damage in whole blood cells. To the best of our knowledge, our findings suggest for the first time that this pesticide does not induce genotoxic effect in dogs.

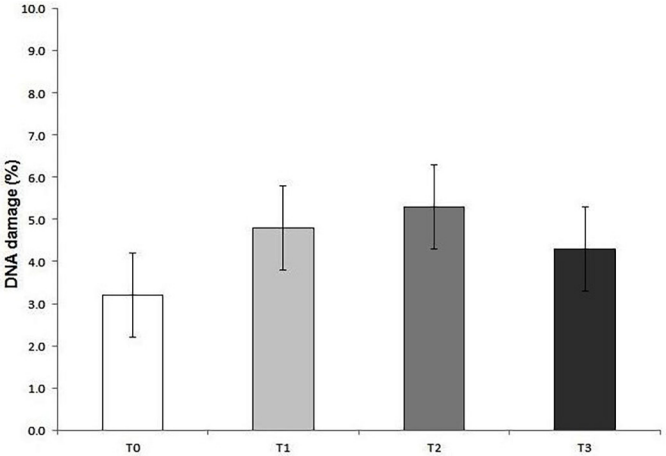

Fig.1. DNA damage (tail intensity) detected by comet assay in whole blood cells of dogs unexposed (T0) or exposed to fipronil after 3 (T1), 8 (T2) and 24 hours (T3). Data are expressed as X ± SE; p-value >0.05.

The dose investigated appeared to be the ideal as it is the dose clinically utilized by pet owners according to indication of the laboratory and veterinarians. In addition, we investigated the genotoxic potential at three time points based on the guidelines for *in vivo* genetic toxicology after a single acute treatment (Sasaki et al. 2000, Tice et al. 2000).

The comet assay is a relatively simple, rapid and low-cost test used for detection in DNA of single- and double-strand breaks, alkali-labile sites and oxidative lesions (Tice et al. 2000). It is also known as the single-cell gel electrophoresis (SCGE) assay, which consists of immersing the eukaryotic cells in agarose gel to lyse the cell membrane by detergents and alkaline salts, and detecting damaged DNA by differential migration of the nuclear material when subjected to electrophoresis (Singh et al. 1988). This assay is an important tool to evaluate DNA damage in environmental monitoring, genetic toxicology, ecotoxicology, and clinical studies. Thus, the use of biomarkers as a measure of biological responses in affected organisms is a very important factor for simplification and cost reduction of biological monitoring.

Fipronil induced *in vitro* dose-dependent genetic lesion in lymphocytes exposed to 0.1, 0.3 and 0.7µg/ml (Çelik et al. 2014). Male rats orally given fipronil (2.5, 5.0 and 10mg/kg/day) for 28 days showed increased DNA damage when sperm comet assay was evaluated (Khan et al. 2015). There are indications of carcinogenic action of this agent in rats at 300ppm, but not in female mice at doses of 30ppm (Tingle et al. 2003). On the other hand, the use of topical on-spot flea and tick products containing fipronil does not increase the risk of transitional cell carcinoma (TCC) of the urinary bladder in Scottish Terriers (Raghavan et al. 2004).

Our findings are in agreement with other reports regarding the genotoxic/mutagenic potential of fipronil. The pesticide was found negative in bacterial mutagenic test and showed no clastogenic effect when exposed to human lymphocytes at doses of 75, 150 or 300µg/ml with or without metabolic activation (EPA 1997). None of the tested doses of fipronil (0.05 up to 0.23µg/l) was sufficient to modify DNA integrity in the gill cells evaluated by comet assay in the neotropical fish *Rhamdia quelen* (Ghisi et al. 2011). In addition, fipronil did not show genotoxic effect 24 hours after exposure or mutagenic effect when evaluated 24 hours to 72 hours after exposure at 15mg/kg or 25mg/kg in peripheral blood of mice (De Oliveira et al. 2012). These authors observed that only the highest dose tested (50mg/kg - LD_{50}) induced DNA damage and micronuclei 24 hours after the exposure.

Pesticides are noxious chemicals widely used in agriculture, either in isolation or combined with other substances and may affect long-term health (WHO 1992). The levels of fipronil residues on gloves worn after Frontline application in dogs peaked 24 hours after exposure and were undetectable after 5 weeks. Repeated exposure to such contamination might lead to human health risks (Jennings et al. 2002). In fact, patients with fipronil self-poisoning show vomiting, agitation, and seizures, usually with a favorable outcome (Mohamed et al. 2004). Several insecticides, including fipronil, were shown to be genotoxic to mucosal epithelial cells taken from human tonsil tissue whereas inhalation of these substances can damage epithelial cells of the upper aerodigestive tract (Tisch et al. 2007).

Fipronil may impair the normal functioning of the endocrine system and cause adverse reproductive effects in female rats (Ohi et al. 2004). This molecule is quickly metabolized and its residues are distributed, especially in fatty tissues under the skin and hair follicles (Jennings et al. 2002). The major metabolite of fipronil is fipronil sulfone. Although less selective, fipronil sulfone is more persistent in relation to GABA receptors and it is found in many organs of mice and other vertebrates after exposure, playing an important role in the adverse effects of the pesticide (Hainzl & Casida 1996, Hainzl et al. 1998). The increased power of fipronil sulfone to sensitize GABA receptors intensifies the concern about the toxicity of this pesticide, especially in mammals. The metabolites fipronil sulfone and desulfinylfipronil are more toxic to aquatic organisms than fipronil. The U.S. EPA states that fipronil is highly toxic to fishes and aquatic invertebrates but relatively less toxic to mammals and birds (EPA 1996).

CONCLUSION

From the results obtained in the present study, it can be concluded that a single exposure to the clinically recommended dose of fipronil (frontline plus®) does not induce short-term systemic DNA damage when topically applied in dogs. Further studies should be conducted to evaluate the possible genotoxic effects in other tissues and/or after chronic exposure to fipronil.

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