



Data Report

Movento® 240SC (Spirotetramat) and Envidor® 240SC (Spirodiclofen) keto-enol insecticides induce DNA damage in *Drosophila melanogaster* ovaries

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ABSTRACT — Movento® 240SC and Envidor® 240SC are new insecticide derivatives of tetramic acid belonging to a keto-enol pesticide family. However, few studies have reported genotoxic effects in non-target organisms. In the present study, the genotoxic effects of Movento® 240SC and Envidor® 240SC on *Drosophila melanogaster* ovaries were analyzed using the alkaline comet assay. Simultaneously, we determined the LD₅₀ for both insecticides. Virgin females were exposed to food at three sublethal concentrations (11.2, 22.4, 37.3 mg/L) of Movento® 240SC and (12.3, 24.6, 41.1 mg/L) of Envidor® 240SC for 72 hr. As a negative control group, females were exposed to food without insecticides, and as a positive control group, females were exposed to 17.5 mg/L bleomycin under the same experimental conditions. We analyzed three genotoxic parameters, tail length, tail moment, and tail intensity, in ovarian cells. The results showed that 11.2 mg/L Movento® 240SC insecticide significantly increased the tail intensity mean in ovarian cells compared with the negative control. However, 22.4 and 37.3 mg/L Movento® 240SC significantly increased tail length and tail moment means compared with the negative control. Envidor® 240SC insecticide at 12.3, 24.6, 41.1 mg/L significantly increased the three genotoxic parameters in ovarian cells compared with the negative control. The LD₅₀ values of Movento® 240SC and Envidor® 240SC insecticides were 79.1 mg/L and 78.0 mg/L, respectively. The genotoxic response of the two keto-enol pesticides was dependent on the concentration of each pesticide. The results demonstrated that Movento® 240SC and Envidor® 240SC keto-enol insecticides are genotoxic agents in *D. melanogaster* ovaries.

Key words: Movento® 240SC, Envidor® 240SC, Keto-enol insecticides, DNA damage

INTRODUCTION

Epidemiologic studies have indicated that there is an association between pesticide exposure and the development of diverse reproductive disorders, allergies and cancer. Pesticides are also considered to be one of the main factors involved in environmental contamination (Zaller, 2020). Keto-enol insecticides are a new group of pesticides derived from tetrone and tetramic acids, were

developed by Bayer in the 1990s and contain three commercially available cyclic keto-enol compounds, i.e., spirodiclofen (Envidor® 240SC), spiromesifen (Oberon® 240SC) and spirotetramat (Movento® 240SC) (Sparks and Nauen, 2015) (Fig. 1A, B), which have been successfully registered in the United States, Brazil, Mexico, and other countries for the control of a broad spectrum of sucking insects, such as aphids, psyllids, mealybugs, whiteflies and oth-

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er sucking insect pests of agricultural crops (Ouyang *et al.*, 2012). Keto-enol insecticides interfere with lipid biosynthesis, are thought to act as inhibitors of acetyl-coenzyme A carboxylase (ACCase) (Bretschneider *et al.*, 2003; Nauen *et al.*, 2003) and are mainly effective against eggs and all developmental stages of spider mites, with limited acute toxicity in adults (Bretschneider *et al.*, 2003; Van Pottelberge *et al.*, 2009; Marčić *et al.*, 2010). Therefore, concern over keto-enol insecticides for environmental safety is increasing, and numerous studies have reported their toxicity to eco-environments and nontarget organisms. Previous investigations have demonstrated that larval and juvenile African catfish *Clarias gariepinus* decreased their hatching rates after exposure to spirotetramat, with alterations in larval swimming coordination (Agbohessi *et al.*, 2013). Spirotetramat also poses potential biochemical and genetic toxicity to earthworms (*Eisenia fetida*) (Zhang *et al.*, 2015). Movento commercial insecticide affected the expression of the FoxO1 and Vnn1 genes in granulose cells of BALB/c female mice (Kafshgiri *et al.*, 2016) and inhibited normal growth and reproduction in *Daphnia magna* (Chen *et al.*, 2018). This compound also causes biochemical, histopathological, and physiological changes in the ovaries of adult zebrafish (*Danio rerio*) (Wu *et al.*, 2018). In zebrafish embryos, it is a teratogen agent (Zhang *et al.*, 2019), affects lipid metabolism and causes mitochondrial lesions (Zhang *et al.*, 2020). Spirodiclofen is considered to be a risk factor for adrenal cortical vacillation, atrophy of male reproductive tract Leydig cell hyperplasia and uterine adenocarcinoma in rats (Yoshida *et al.*, 2015). Spirodiclofen was monitored in human Caco-2 cells (Shi *et al.*, 2017) and in human serum (Shin *et al.*, 2018) and produces defects in zebrafish embryonic development (Zhang *et al.*, 2019). Toxicity tests with different model organisms play an important role in evaluating environmental risk and in classifying the hazardous effects of pesticides on human health. *Drosophila melanogaster* is an established insect model organism for human diseases and toxicological research that has also been recommended by the European Centre for the Validation of Alternative Methods (ECVAM) for research and testing (Festing *et al.*, 1998). Recently, the term Drosophotoxicology has emerged due to the implementation of *D. melanogaster* in toxicology research in different stages (embryo, larvae, pupae, and adults) (Affleck and Walker, 2019) using different genetic assays, such as the comet alkaline assay to evaluate DNA damage by environmental pollutants, such metals (Mojica-Vázquez *et al.*, 2019), nanoparticles (Alaraby *et al.*, 2019) and pesticides (Misra *et al.*, 2014). The alka-

line comet assay is a genetic technique that is rapid and sensitive for detecting *in vitro* and *in vivo* genome strand breaks induced by agrochemicals (Ku-Centurión *et al.*, 2016). The ovaries of *D. melanogaster* have been used to elucidate the genetic and molecular mechanisms of xenobiotics in the cell cycle, death, signaling, migration, differentiation, oogenesis, and development of the female reproductive system (Velentzas *et al.*, 2015). In the present study, the genotoxic effects of the commercial keto-enol insecticides Movento® 240SC and Envidor® 240SC were analyzed on *D. melanogaster* ovaries using the alkaline comet assay, and the LD₅₀ of both pesticides in the same model organism was determined.

MATERIALS AND METHODS

Chemicals

For the present study, we used phosphate-buffered saline (PBS) obtained from Gibco (Carlsbad, CA, USA), and low melting point agarose (LMPA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). GelRed® was purchased from Biotium (Fremont, CA, USA). Normal melting point agarose (NMPA), Triton X-100, ethylenedi-aminetetraacetic acid (EDTA), sodium hydroxide (NaOH), sodium chloride (NaCl), Tris base, saccharose, yeast, agar and propionic acid were obtained from Amresco (Denver, CO, USA).

Fly strain

All treatments were performed in flies of the wild-type strain of *D. melanogaster* (Oregon-R) cultured at 24 ± 1°C on standard *Drosophila* food containing agar, corn meal, sugar, and yeast with 60% humidity.

Preparation of keto-enol insecticide yeast paste

The keto-enol insecticides Movento® 240SC (spirotetramat as the active substance (cis-4-(ethoxycarbonyloxy)-8-methoxy-3-(2,5-xylyl)-1-azaspiro [4.5] dec-3-en-2-one)), (RSCO-INAC-0103Z-301-409-015) and Envidor® 240SC (spirodiclofen as the active substance (3-(2,4-dichlorophenyl)-2-oxo-1-oxaspiro [4.5] dec-3-en-4-yl 2,2- dimethylbutyrate)), (RSCO-INAC-0103R-301-064-022) were donated by Bayer-Mexico. They were diluted with deionized water to final concentrations of (11.2, 22.4, 37.3 mg/L) of Movento® 240SC and (12.3, 24.6, 41.1 mg/L) of Envidor® 240SC. Two hundred microliters of each solution was mixed with 0.5 mg of inactivated dry yeast and deposited in the center of a layer of 1% agarose at the bottom of a *Drosophila* vial from Genesee Scientific (Wilford, Nottingham, U.K.). The concentrations of both pesticides that induced

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DNA damage in ovarian cells were determined from preliminary experiments and used in three independent experiments.

Determination of the lethal dose (LD₅₀)

The lethal dose 50 (LD₅₀) was determined from several preliminary experiments (data not shown). *D. melanogaster* virgin females were exposed orally to 79.1 mg/L Movento® 240SC and 78.0 mg/L Envidor® 240SC mixed with yeast plus a negative control (food without pesticide) for 24, 48 and 72 hr. Three independent experiments were performed.

Experimental design for keto-enol insecticide oral exposure

Fifty *D. melanogaster* Oregon-R virgin females were collected in a single 24-hr period. Then, 5 groups of 10 females were incubated in fly vials with a layer of 1% agarose and fed with a mixture of yeast paste mixed with (11.2, 22.4, 37.3 mg/L) final concentration of Movento® 240SC and (12.3, 24.6, 41.1 mg/L) of Envidor® 240SC keto-enol insecticides for 72 hr at 25 ± 2°C. As a negative control, 10 females were fed without pesticides (0.0 mg/L) and as a positive control, 10 females were fed 17.5 mg/L bleomycin under the same conditions as the experimental groups. After 72 hr of exposure, the females from all experimental and control groups were sacrificed for ovarian dissection. Twenty ovaries were obtained (2 per organism) for each experimental and control group, which were placed in tubes with 50 µL of cold 1X PBS and immediately mixed with an automatic pipette to disperse the cells. Tissue fragments were removed, and the cellular suspension was used to perform an alkaline comet assay. Three independent experiments were performed for each treatment.

Alkaline comet assay

A modified alkaline comet assay was developed for the *in vivo* detection of DNA damage in *D. melanogaster* ovaries from an original *D. melanogaster* modified protocol by Mojica-Vázquez *et al.* (2019). Then, a 100 µL cellular suspension was added to a microtube plus 100 µL of LMPA (0.5%, 28.5°C) and mixed gently. One hundred microliters of the mixture was used on a slide (Fisher Scientific, Waltham, MA, USA) (two slides per concentration of insecticide). The slides were stored at 4°C for 5 min to allow the agarose to solidify. The coverslip was then carefully removed, and the slides were immersed in a staining jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% Triton X-100,

pH = 10) at 4°C for 1 hr. The slides were placed in a horizontal electrophoresis chamber (Owl A5; Lab System, Inc., Waltham, MA, USA) containing freshly prepared cold electrophoresis alkaline buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 min to unwind the DNA. Electrophoresis was carried out at 25 V (0.7 V/cm) and 300 mA for 20 min in the dark to prevent additional DNA damage. The slides were washed three times with freshly prepared neutralization buffer (0.4 M Tris, pH 7.5) for 5 min, fixed with cold absolute methanol for 5 min, and air-dried at room temperature. Next, 60 µL of GelRed® (4 µg/mL) was added to each slide to stain the DNA. The slides were labeled with a code that was unfamiliar to the viewer and examined with a Nikon Eclipse NI fluorescence microscope equipped with an excitation filter (515–560 nm) and a barrier filter (590 nm). To visualize DNA damage, slides were observed at 40 × magnification using Comet IV software (Perceptive Instruments, Great Shelford, Cambridge, U.K.). Three parameters were used to determine genotoxicity: (a) tail length (distance between the head and last DNA fragment), (b) tail moment (defined as the tail length weighted by the percentage of tail DNA), and (c) tail intensity (percentage of DNA in the tail, as most relevant) in 75 randomly selected nuclei on each slide (two slides per concentration) for a total of 150 nuclei per experiment for each concentration (Fig. 1C, D). Comets with completely fragmented DNA (Hedgehog-like figures with no apparent head) were excluded from the evaluation (Calderón-Segura *et al.*, 2015).

Statistical analysis

The results obtained from the tail length, tail moment and tail intensity analyses are reported as the mean ± standard error of three independent experiments for each concentration of insecticide and for negative and positive controls. The data were analyzed with analysis of variance (ANOVA) and Tukey's multiple comparison test to determine significant differences between the experimental groups and the respective control groups at ($p < 0.001$). To determine the LD₅₀, a Gehan-Breslow-Wilcoxon test was performed with GraphPad Prism version 8 software. The relationship between DNA damage and Movento® 240SC and Envidor® 240SC keto-enol insecticide concentrations was determined using linear regression analysis.

RESULTS

Determination of the LD₅₀ of the keto-enol insecticides Movento® 240SC and Envidor® 240SC

The LD₅₀ for Movento® 240SC was 79.1 mg/L and

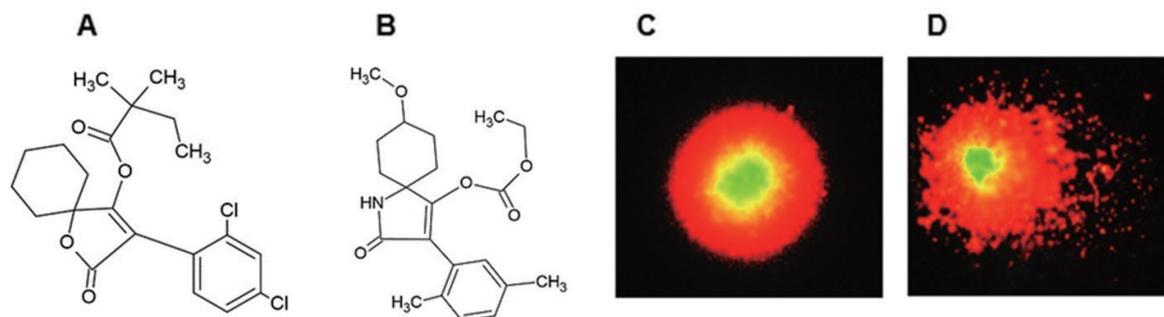


Fig. 1. Chemical structures are shown of the active ingredients of (A) Spirotetramat (Movento® 240SC) and (B) Spirodiclofen (Envidor® 240SC). (C) An image is shown of *Drosophila melanogaster* germinal cell nuclei without DNA damage (without comet). (D) Germinal cell nuclei with DNA damage (with comet) are shown.

78.0 mg/L for 72 hr for Envidor® 240SC. The mortality percentage from three independent experiments was 50% for the experimental *D. melanogaster* groups compared with the negative control group according to the Gehan-Breslow-Wilcoxon statistical test ($p < 0.001$).

DNA damage in *D. melanogaster* ovaries induced by Movento® 240SC and Envidor® 240SC keto-enol insecticides

The results of three independent experiments showed that at 11.2 mg/L Movento® 240SC, there were no significant differences in the means of tail length and tail moment compared with those of the negative control group (0.0 mg/L) ($p < 0.001$) (Fig. 2A and 2C), but this concentration induced a significant increase in the mean tail intensity (Fig. 2E). At 22.4 and 37.3 mg/L Movento® 240SC, the means of the three genotoxic parameters in the experimental group were significantly increased compared with the means of the negative control group ($p < 0.001$) (Fig. 2A, C, E). The regression plots showed a concentration-dependent effect of Movento® 240SC on tail intensity ($r^2 = 0.987$) and tail moment ($r^2 = 0.933$) but not tail length ($r^2 = 0.57$) (Fig. 2B, D, F). With Envidor® 240SC insecticide, significant increments were observed in the three genotoxic parameters (tail length, tail moment and tail intensity) of the three experimental group (12.3, 24.6 and 41.1 mg/L) with the negative control group (0.0 mg/L) ($p < 0.001$) (Fig. 2A, C, E). The regression plots showed a concentration-dependent effect on tail length ($r^2 = 0.99$), tail intensity ($r^2 = 0.98$) and tail moment ($r^2 = 0.98$) (Fig. 2B, D, F).

Bleomycin, as a positive control (17.5 mg/L), significantly increased the three genotoxic parameters (tail length, tail moment and tail intensity) in cell nuclei from the experimental group of *D. melanogaster* compared with

the negative control group ($p < 0.001$) (Fig. 2A, C, E).

DISCUSSION

In the present study, we analyzed DNA damage in the ovaries of *D. melanogaster*. After exposure to (11.2, 22.4, 37.3 mg/L) of Movento® 240SC and (12.3, 24.6, 41.1 mg/L) of Envidor® 240SC keto-enol insecticides for 72 hr, through an alkaline comet assay, we obtained the LD₅₀ of both pesticides. The results showed that exposure to sublethal concentrations of both pesticides significantly increased DNA damage in the ovaries, as evidenced by a significant increase in the mean of the three genotoxic parameters in the experimental group compared with the negative control group (0.0 mg/L) ($p < 0.001$). Except at 11.2 mg/L Movento® 240SC, only the mean tail intensity increased. Our results are consistent with a study reported by Zhang *et al.* (2015), who found that spirotetramat (the active ingredient of Movento® 240SC) induces DNA damage and increases the activity of the antioxidants SOD, CAT and GSH in *Eisenia fetida* coelomocytes. Many investigations have demonstrated that oxidative stress is a mechanism of pesticide-induced DNA damage. Yin *et al.* (2014) found that spirotetramat (Movento® 240SC) keto-enol insecticide induces oxidative stress and lipid peroxidation and is potentially toxic to tadpoles of the amphibian *Bufo bufo gargarinzas*. Another study, conducted by Chenlei *et al.* (2014), discovered a significant increase in oxidative stress biomarkers (CAT, GSH-Px) and lipid peroxidation in female zebrafish (*Danio rerio*) exposed to 0, 36, 360 and 1800 µg/L spirotetramat for 28 days. Oral administration of Movento® 240SC significantly decreased ovarian weight and the number of primary, secondary, and antral follicles in female mice and induced apoptosis of granulosa cells. The relative tran-

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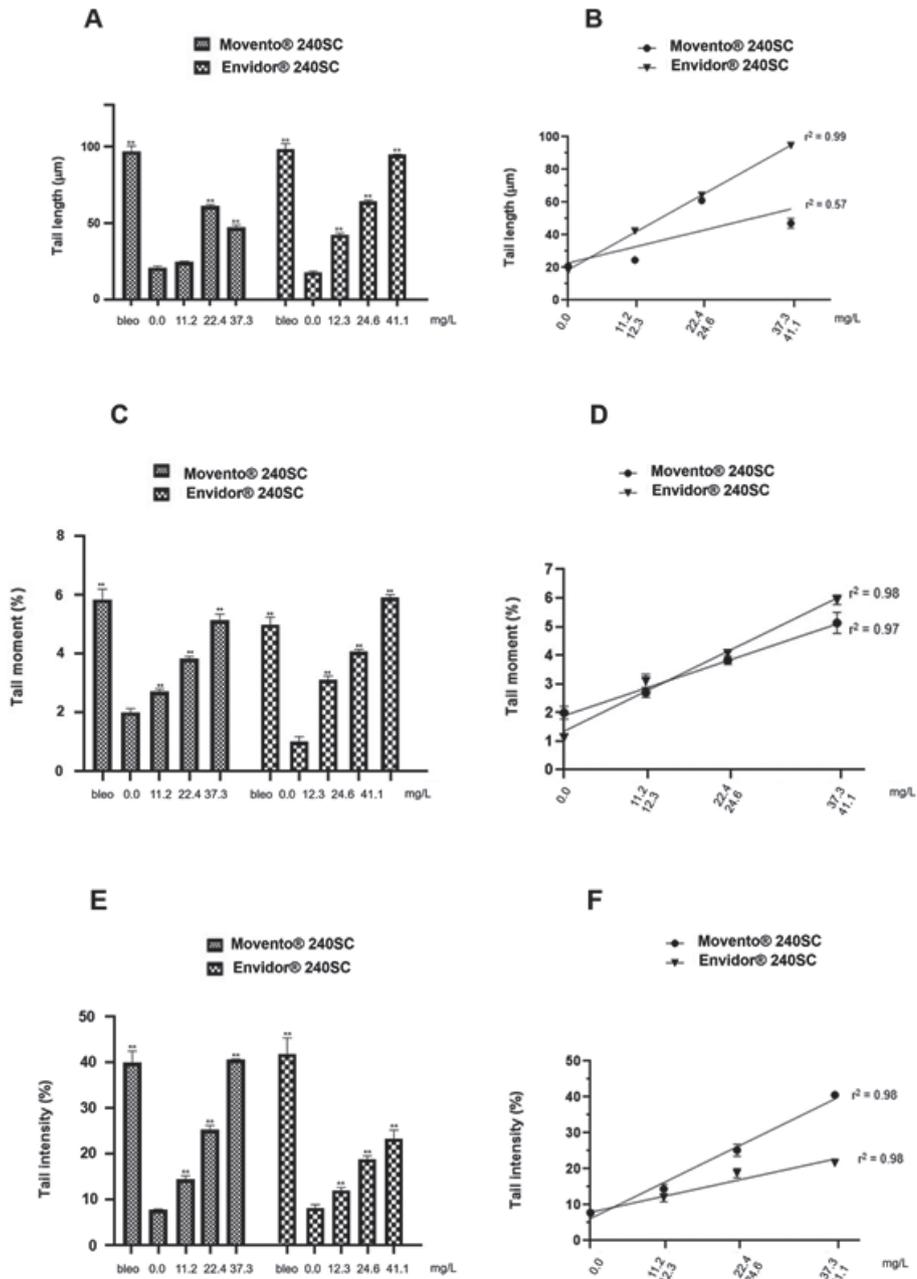


Fig. 2. Genotoxic parameters were assessed in ovaries using Movento® 240SC (11.2, 22.4, 37.3 mg/L) Envidor® 240SC (12.3, 24.6, 41.1 mg/L), a negative control (0.0 mg/L) and bleomycin as a positive control (17.5 mg/L). (A) When compared with the negative control (0.0 mg/L), exposure to Movento® 240SC (22.4, 37.3 mg/L) and Envidor® 240SC (12.3, 24.6, 41.1 mg/L) the keto-enol insecticides significantly increased tail length. (B) A regression plot for tail length shows a concentration effect on tail length with Envidor® 240SC ($r^2 = 0.99$) but not with Movento® 240SC ($r^2 = 0.57$). (C) Tail moment was also used to assess DNA damage, and it also increased in ovaries following treatment with either Movento® 240SC (22.4, 37.3 mg/L), Envidor® 240SC (12.3, 24.6, 41.1 mg/L) or bleomycin. (D) The regression plot for tail moment shows the concentration effect of both Movento® 240SC ($r^2 = 0.97$) and Envidor® 240SC ($r^2 = 0.98$) insecticides. (E) The most sensitive parameter, tail intensity, showed an increase in DNA damage in the ovaries of *Drosophila* treated with the three concentrations of either Movento® 240SC (11.2, 22.4, 37.3 mg/L), Envidor® 240SC (12.3, 24.6, 41.1 mg/L) or bleomycin with respect to the negative control group (0.0 mg/L). (F) The regression plot for tail intensity showed a concentration-dependent effect of the Movento® 240SC ($r^2 = 0.98$) and Envidor® 240SC ($r^2 = 0.98$) insecticides. Significant differences are denoted by $** (p < 0.001)$.

script level of the *vnn1* gene was downregulated in granulosa cells, and the transcript level of the *foxo1* gene was upregulated (Kafshgiri *et al.*, 2016). In zebrafish, spirotetramat induced the downregulation of the *fshr* gene and the upregulation of the *lhr* gene, oxidative stress, and lipid peroxidation and delayed oogenesis and oocyte maturation and decreased the size of the ovaries (Wu *et al.*, 2018).

When comparing the genotoxic effects of the two keto-enol insecticides in ovaries from *D. melanogaster*, we observed that at low concentrations (11.2 mg/L), Movento® 240SC only increased tail intensity, which confirmed that this genotoxic parameter is very important to detect DNA damage in response to low concentrations of pesticide exposure (Ku-Centurión *et al.*, 2016). Both high concentrations of Movento® 240SC (22.4 and 37.3 mg/L) produced more DNA damage. However, we observed that the Envidor® 240SC insecticide was more genotoxic than Movento® 240SC; hence, Envidor® 240SC induced a higher number of DNA strand breaks than Movento® 240SC. The genotoxic results indicate that the induced DNA damage was not repaired and possibly was caused by either excessive reactive oxygen species (ROS), free radical production, or interaction with intermediate metabolites that interfered with repair mechanisms in the ovaries of *D. melanogaster*, resulting in DNA strand breaks that were visualized through comet formation. ROS affect different signaling pathways, such as oocyte maturation, folliculogenesis, ovarian steroidogenesis and luteolysis and embryonic development (Agarwal and Allamaneni, 2004; Agarwal *et al.*, 2005; Wells *et al.*, 2010). ROS can alter the implantation and fertilization of eggs (Sharma and Agarwal, 2004) and influence early embryo development by modifying key transcription factors, thereby modifying gene expression (Dennerly, 2004). In addition, there is a clear association between oxidative DNA damage and poor-quality oocytes, lower fertilization rates, and embryos with teratogenic effects (Seino *et al.*, 2002; Jameel *et al.*, 2020). In *D. melanogaster*, damage to the genetic material is related to cell death in the ovarian chambers, delays in the stages of oogenesis, a decrease in the size of the ovaries, a decrease in the number of eggs laid by the female and a decrease in the hatching rate (Panagopoulos *et al.*, 2004; Panagopoulos *et al.*, 2007; Panagopoulos, 2012). Therefore, the DNA repair mechanisms in female and male germ cells are required for controlling the quality of the germ line, maintaining genetic integrity, avoiding negative effects on fertility, and ensuring offspring survival (Drummond-Barbosa, 2019; Stringer *et al.*, 2020).

On the other hand, we used bleomycin, which we corroborated as an excellent positive control that significant-

ly increased DNA strand breaks in ovaries of *D. melanogaster* in comparison to the negative control group. This compound is a very well-known genotoxic agent that binds to DNA by intercalation or through interactions with the minor groove and induces genome fragmentation, point mutations, genetic recombination, and chromosomal aberrations in microorganisms and mammals (Povirk, 1996; Hoffmann *et al.*, 2001).

Finally, we obtained LD₅₀ values for Movento® 240SC (79.1 mg/L) and Envidor® 240SC (78.0 mg/L) keto-enol pesticides in *D. melanogaster*. After 72 hr of exposure, we found that Envidor® 240SC was more toxic than Movento® 240SC and more toxic at lower concentrations than in other organisms. For bees (*Apis mellifera*), LD₅₀ > 107 µg/bee were reported for Movento® 100SC (Sverdrup *et al.*, 2013) and LD₅₀ > 196 µg/bee was reported for Envidor (Sverdrup *et al.*, 2011).

Because keto-enol pesticides induce alterations in the female reproductive system, it is of great relevance to perform genotoxic studies in germ and somatic cells because the long-term survival of any multicellular species depends on the success of its germ line in the production of quality gametes and the survival of the offspring (Drummond-Barbosa, 2019).

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Conflict of interest---- The authors declare that there is no conflict of interest.

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