Genotoxic effect of flonicamid and etofenprox on mice.

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Flonicamid and etofenprox are two novel insecticides used in agriculture to protect the food and commercial products against the insect's pests. The study aimed to evaluate the genetic risk of the flonicamid and etofenprox on mice's genome as a monitor for detection the toxicity, mutagenicity and carcinogenic influence that human and livestock exposed. RAPD markers, DNA fragmentation and comet assay were used as molecular genetic tools for genotoxic evaluation of the two insecticides. The experimental animals divided into three groups. The first and second groups divided into three sub-groups of animals which were treated with different doses of each insecticide. The third group was the control groups. The results of the RAPD markers revealed that the high dose of flonicamid caused degraded in the DNA and severe genome damaged while etofenprox low dose was the most genetically safe on the genomes of the treated mice groups. The quantitation of DNA fragmentation and comet assay results showed that the two insecticides caused DNA damage in all treated groups compared to the control groups. Etofenprox low dose recorded the less percentage of DNA fragmentation compared to the other doses of flonicamid and etofenprox. The obtained results of the different molecular tools used to evaluate the genotoxic effect of flonicamid and etofenprox insecticides revealed that the two insecticides caused DNA damage and apoptosis which could be reflected as disorders in the biological functions of the body and/or serious diseases disorders according to the damaged level.

Keywords: Flonicamid, Etofenprox, Molecular markers, Genotoxic, DNA fragmentation, Comet assay.

INTRODUCTION

The 1.8 billion people worldwide are engaged in agriculture and most of them used the pesticides to protect the food and commercial products from the harmful insects (Larramendy et al., 2014). A serious diseases problem such as mutations, cancer and/or birth defects is reported when the human and livestock exposed to continuously or repeatedly different insecticides (Benedetti et al., 2014).

Flonicamid is a novel insecticide that has a selective effect on sucking pests. This insecticide acts by inhibiting the feeding activity of insect's pests without showing symptoms of neuronal intoxication (Morita et al., 2007). Roditakis et al., (2014) found that flonicamid was very effective against the whitefly, *Bemisia tabaci*.

Etofenprox is a pyrethroid ethyl used against many insect pests such as thrips, whitefly and aphids. Hwang et al., (2015) examined the residual effect of etofenprox in onion and found that the half-lives of etofenprox in spring onion were 9.5 and 7.9 days, at the single or double application rates, the washing processes remove...
21.6- 43.9 % of the insecticide residues. Benli (2015) found that etofenprox was very toxic against crayfish and non-target organisms.

Identifying the genetic risk factors is used as a monitor for detection toxicity, mutagenicity and carcinogetic material that human and livestock exposed and/ also to decrease the healthcare costs (Giacomini et al., 2007). Evolution of the genomic status is classified to general uses such as clinical response and differentiation; risk identification; dose selection guidance; susceptibility, resistance, and differential disease diagnosis; and polymorphic drug targets (Ahmed 2014). Evaluation of the genotoxic effects of insecticides at the molecular level offering careful assessment for the adverse effects of different insecticides compounds.

Randomly amplified polymorphic DNA (RAPD) markers are utilized to generate complex patterns of PCR to various random segments of genomic DNA. RAPD analysis has several applications such as the study of population genetics, molecular evolutionary genetics, plant and animal breeding and evaluation the hazard risk of natural and chemical compounds where the amplified patterns used to screen the genome for detecting the amplified DNA abnormalities (Kumar and Gurusubramanian 2011). DNA fragmentation is used to study the DNA degrade damage in the cells (Yokozawa et al., 2001, Ahmed 2014). Whereas Comet assay is a sensitive method for measuring the cells toxicity that extends to the deoxyribonucleic acid (DNA) strand breaks damage (Liao et al. 2009, McArt et al., 2009). The study aimed to evaluate the genetic risk of the flonicamid and etofenprox on mice’s genome using RAPD- PCR, DNA fragmentation and comet assay.

MATERIALS AND METHODS

Tested insecticides

Flonicamid is a systemic insecticide belongs to pyridinecarboxamide insecticide group. This insecticide is recommending to sucking insects such as aphids and whiteflies. The mode of action is different from neonicotinoids insecticides. It’s acting by inhibiting the feeding behavior of insects (Morita et al., 2007). The field rate of this insecticide is 80 g/400 l. This insecticide was obtained from Sumitomo, Cairo, Egypt.

Etofenprox is belonging to pyrethroid ethyl group, while other pyrethroids belong to pyrethroid esters. However, the mode of action of etofenprox is on the membrane of nerve cells. This insecticide is blocking the closure of the ion gates of the sodium channel during re-polarization. This acting disrupts the transmission of nervous impulses, causing spontaneous depolarization of the membranes or repetitive discharges. The field rate of this insecticide is 200 ml/ l. The insecticide obtained from StarChem Industrial Chemicals, Egypt.

Animals

Seventy male adult Swiss albino mice weighing between 20-25 grams were used in this study. These animals were obtained from the animal house of the National Research Center, Cairo, in Egypt. The animals were housed in plastic cages, ten per cage, and maintained on standard laboratory diet and water.

Experimental design:

Animals were divided into two groups both of them had thirty animals and other ten animals as a control.
First group (FG) divided into three sub-groups each was ten animals and treated orally with flonicamid as followed:
1- FG A treated by one tenth of field rate at 10 mg/kg body weight.
2- FG B treated by 5 mg/kg body weight.
3- FG C treated by 2.5 mg/kg body weight.
Second group (EG) divided into three sub-groups each was ten animals and treated orally with etofenprox as followed:
1- EG A treated by one tenth of field rate at 5 mg/kg body weight.
2- EG B treated by 2.5 mg/kg body weight.
3- EG C treated by 1.5 mg/kg body weight.

The control animals were treated orally by distilled water only.

Genotoxic Assay.

DNA extraction:

The DNA of tested animals was extracted from the liver according to the Sharma et al. (2012). The concentration of DNA and its relative purity were determined using a spectrophotometer based on absorbance at 260 and 280 nm, respectively. The integrity of extracted genomic DNA was checked by electrophoresis in 1 % agarose gel.

RAPD-PCR electrophoresis:

The RAPD primers codes and sequences were listed in Table 1. The polymerase chain reaction (PCR) solution in total volume 25 μl. A
PCR cocktail consists of 0.2 μM primer and 2x power Taq PCR master mix (Intron Biotechnology) were placed into tubes with 50 ng of DNA. The reaction ran in a Coy Temp Cycler II (Coy Corporation, Grass Lake, USA). The PCR cycles condition were cycled for five minutes at 94°C, followed by 45 cycles at 94°C for one minute, 37°C one minute and 72°C for two minutes. Final extinction was at 72°C for five minutes. The used ladder was 50-bp (Fast Gene). PCR products were subjected to electrophoresis on 1 % agarose gels containing 6 μl of read safe nucleic acid staining solution (Intron Biotechnology). The polymorphic bands of RAPD were scored on the basis of the band mobility; clear bands were scored using GelDoc (Ingenious 3, USA). The Gel analyses was (1) for presence and (0) for absence in a binary data form, the unclear unidentified bands were excluded.

**Estimation of DNA Damages.**

**Quantitation of DNA Fragmentation:**
The liver cells of all investigated groups were used to quantitation of the DNA fragmentation using diphenylamine (DPA) as described by Paradones et al. (1993). The DNA absorbance was measured at 600 nm using a UV double beam spectrophotometer (Shimadzu 160A). The percentage of DNA fragmentation was taken as the ratio of DNA absorbance reading in the supernatant to the total amount of DNA in pellet and supernatant. T-test was used to evaluation of the results of the DNA fragmentation between the treated sub-groups and the control group.

**Comet Assay**
Liver from all experimental groups were minced and homogenized using a potter-type homogenizer. The minced liver cells were washed in cold buffer (NaCl: 75 mmol/l, EDTA-2Na; 24 mmol/l, PH 7.5). The cell suspensions were centrifuged at 4°C, 700 xg for 10 min. The slides were prepared according to Singh et al. (Liao et al. 2009) and examined at 100 magnifications under a fluorescence microscope using a FITC filter. The DNA damage was evaluated by determent the frequency of Untailed % (DNA non damaged cells), Tailed % (DNA damaged cells) and Tail Length. The ratio of tailing was assessed by counting the tailing DNA in 100 cells per sample. The numbers of comet were calculated using TriTekCometScoreTM Freeware version 1.5.

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Primers Sequences (5 → 3)</th>
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<tbody>
<tr>
<td>B10</td>
<td>CTG CTG GGA C</td>
</tr>
<tr>
<td>C02</td>
<td>GTG AGG CGT C</td>
</tr>
<tr>
<td>C05</td>
<td>GAT GAC CGC C</td>
</tr>
<tr>
<td>C06</td>
<td>GAA CGG ACT C</td>
</tr>
<tr>
<td>E04</td>
<td>AGG GGT CTT G</td>
</tr>
<tr>
<td>A04</td>
<td>AAT CGG GCT G</td>
</tr>
<tr>
<td>A16</td>
<td>AGC CAG CGA A</td>
</tr>
<tr>
<td>A20</td>
<td>GTT GCG ATC C</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**
Eight RAPD markers were used to screen the DNA of mice genome in control and treated animals groups by flonicamid and etofenprox. Evaluation of the adverse effectsof the two insecticides on the genetic materials of the mice using RAPD-PCR markers were measured by detected the amplified pattern numbers and molecular size.

The results of the OPB-10 marker in control and the treated sub-groups at the different doses showed polymorphic patterns differences in numbers and molecular sizes (Figure 1, A1& B1). The PCR products of OPB-10 marker for the sub-group (FG1) treated by one tenth of field rate (10 mg/kg body weight) showed smear without recognized pattern while animals in sub-groups FG2 and FG3 recorded 3 to 5 and 3 to 4 amplified DNA fragments, respectively, at different molecular sizes compared to the band numbers and sizes observed in control group. Figure 1-B1, illustrates the genotoxic effects of etofenprox at the molecular level, the treated sub-groups animals showed similar PCR patterns as the control group.

OPC-2 marker was observed with polymorphic patterns difference in numbers and molecular sizes for the different doses in the treated sub-groups and controls (Figure 1, A2& B2). The high dose of flonicamid showed smear without recognized pattern while animals in subgroups FG (5 mg/kg) and FG (2.5 mg/kg) recorded 2 to 6 and 5 amplified DNA fragments, respectively, at different molecular sizes compared to the bands number and sizes recorded for the control group. The polymorphic
patterns of etofenprox in all treated sub-groups determined similar PCR patterns as the control group (Figure 1-B²).

RAPD-PCR products of the OPC-5 marker showed polymorphic patterns difference in numbers and molecular sizes at the different doses of the treated sub-groups compared to the controls (Figure 1, A³ & B³). The high dose of flonicamid (10 mg/kg body weight) showed smear without recognized pattern while animals in sub-groups FG⁸ (5 mg/kg) and FG² (2.5 mg/kg) recorded 6 to 7 and 5 polymorphic patterns, respectively at different molecular sizes than that those observed in control group (5 polymorphic patterns). The polymorphic patterns of etofenprox in treated sub-groups EG⁴ and EG⁸ showed 4 polymorphic patterns compared to 5 polymorphic patterns for the control and EG² sub-group which was treated with 1.5 mg/kg of etofenprox (Figure 1-B³).

Figure 1, A⁴ and B⁴ illustrates that thePCR products of OPC-6 marker, FG⁸ sub-group showed smear without recognized pattern compared to the controls and the other sub-groups treated with flonicamid and etofenprox. The results of OPC-6 marker in the control groups and the other sub-groups treated with both insecticides revealed that the PCR patterns for sub-groups FG³, EG⁴ and EG⁸ were at different molecular sizes and various fragments numbers compared to the PCR patterns in sub-groups FG² and EG² which were similar to the control groups.

OPC-4 marker was observed with polymorphic patterns difference in numbers and molecular sizes in controls and treated sub-groups for the different doses (Figure 1, A⁵ & B⁵). The high dose of flonicamid showed smear without recognized pattern while the results of the other sub-groups treated by the both insecticides revealed that the PCR patterns for sub-groups FG³ and EG⁴ were at different molecular sizes and various fragments numbers compared to the PCR patterns in sub-groups FG², EG⁸ and EG² which were similar to the control groups.

Evaluation of the genotoxic effects of flonicamid and etofenprox by OPA-4 and OPA-16 markers illustrated that the control groups were monomorphic with one pattern but at different molecular sizes (Figure 2, A⁶ & B⁶ and A⁷ & B⁷). The sub-group FG⁴ showed smear without recognized pattern while the PCR products for FG², FG³, EQ⁴, EQ³ and EQ² were polymorphic patterns. The EG² was the only sub-group similar to the controls.

OPA-20 marker was observed with polymorphic patterns difference in numbers and molecular sizes between controls and the two insecticides treated sub-groups (Figure 1, A⁶ & B⁶). The high dose of flonicamid showed smear without recognized pattern while the results of the other sub-groups treated by the both insecticides showed differences in the molecular sizes and various fragments numbers compared to the control groups. The EG² was the only sub-group similar to the controls.

The achieved results investigated that the genome screening fingerprint resulted from RAPD markers could reflect the genome alteration from single base changes to complex rearrangements as the disappearance of bands result from the presence of DNA photoproducts (pyrimidine dimers), which could reduce the DNA polymerization in the PCR reactions (Nelson et al., 1996) while the incidence of new fragments could be attributed to mutations and/or large rearrangements take place in genomic DNA (Williams et al., 1990, Atienzar et al. 2002). The etofenprox at 1.5 mg/kg dose was the only sub-group showed the similar RAPD markers patterns of the control group which could be indicated that the low dose of etofenprox (1.5 mg/kg) was genetically safe on the genomes of the treated mice while the high dose of flonicamid caused complete DNA degradation and severe genome damage, and the other different doses of flonicamid and etofenprox had adverse effect on the mice’s genome.

Quantitation of DNA fragmentation and comet assay, two techniques were used to measure the toxicity of the insecticides on the mice’s liver cells and the quantitation of the DNA damage. The results of DNA fragmentation revealed that the three sub-groups treated by flonicamid showed significant increases in DNA fragmentation compared to the control group, the difference between sub-groups FG⁴ and FG⁸ was non-significant while it was significant between FG⁴ and FG² and FG⁸ and FG². The percentage of the DNA fragmentation between the three sub-groups treated by etofenprox was significant difference. Comparative significant difference was observed between the sub-groups of flonicamid and etofenprox (Table 2 and Figure 3).
Figure 1: RAPD – PCR pattern of primers OPB-10, OPC-2, OPC-5, OPC-6 & OPE-4 in all experimental groups. M: 50-bp ladder. A: treated subgroups by flonicamid. B: treated subgroups by etofenprox.
Figure 2: RAPD – PCR pattern of primers OPA-4, OPA-16 in all experimental groups. M: 50-bp ladder and primer OPA-20 in all experimental groups. M: 100-bp ladder. A: treated subgroups by flonicamid. B: treated subgroups by etofenprox.
Table 2: DNA fragmentation detected in liver tissues of mice in treated and control groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>DNA Fragmentation %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean± SE</td>
</tr>
<tr>
<td>FG³ 23-25</td>
<td>23.3±1.09*,* ##</td>
</tr>
<tr>
<td>FG² 19-25</td>
<td>21.8±1.04* ##</td>
</tr>
<tr>
<td>FG¹ 17-20</td>
<td>18.5±0.65* ##</td>
</tr>
<tr>
<td>EG¹ 17-19</td>
<td>17.6±0.41* ##</td>
</tr>
<tr>
<td>EG² 14-17</td>
<td>15.4±0.52* ##</td>
</tr>
<tr>
<td>EG³ 11-13</td>
<td>11.8±0.50* ##</td>
</tr>
<tr>
<td>CG 5-7</td>
<td>6.3±0.48</td>
</tr>
</tbody>
</table>

The significant at p < .05, * is the significant between treated sub-groups and control group. ● or ♦ is the significant between treated sub-groups for each insecticide. # is the significant between treated high doses for flonicamid and etofenprox. ### is the significant between treated medium doses for flonicamid and etofenprox. #### is the significant between treated low doses for flonicamid and etofenprox.

Figure 3: Histogram of the DNA fragmentation percentage in liver tissues of the treated and control groups of mice.

The obtained results indicated that the two insecticides responsible for DNA damage in all treated groups compared to the control groups. Comet assay is a method used for measuring the substances cytotoxicity and the extent of their damage at the molecular level. The results of the treated groups by flonicamid and etofenprox showed the extent of the DNA damage compared to the control groups. The damaged was assessed from the length of DNA migration derived by subtracting the diameter of the nucleus from the total length of the image. The grading was as follows: grade I: tailing length/diameter of the nucleus<1; grade II: tailing length/diameter of the nucleus<2; grade III: tailing length/diameter of the nucleus=2. Grades I and II indicate generic rupture of the DNA chain while Grade III indicates apoptosis, presenting a small comet head and a large, bright tail that looks like a broom. The results showed that all treated sub-groups by flonicamid and etofenprox had DNA damage and apoptosis.

The DNA damaged cells in sub-groups treated by etofenprox were less numbers compared to the sub-groups treated by flonicamid (Table 4).

The results of RAPD markers, quantitation of the DNA fragmentation were confirmed by comet assay. Those results suggested that the variation in the composition of DNA (loss nuclease bases and/or fragmentation) could cause abnormalities of many genes along the genome which could be resulting as a disturbance in the functions of these genes according to which part of genetic material lost and/or nucleotides rearrangement. The disturbances in genes functions are reflected in the growth, development, and functions of the body’s systems (Ahmed 2014). Accordingly, the variations could be fatal (large genomic damage), serious diseases disorders (medium genomic damage).

CONCLUSION
In conclusion, the obtained results of the molecular tools used to evaluate the genotoxic effect of flonicamid and etofenprox insecticides revealed that the two insecticides at different doses caused DNA damage and apoptosis which could be reflected as disorders in the biological functions of the body and/or serious diseases disorders according to the damaged level.

CONFLICT OF INTEREST
The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS
Al-kazafy H. Sabry, insecticides treated to mice, collecting data, participating in putting the idea. Publishing; Lamiaa M. Salem, genotoxicity evaluation, collecting the data; Narea I Ali, genotoxicity evaluation, collecting the data; Sahar Ahmed, genotoxicity evaluation, participating in putting the idea, writing the paper. Data analysis

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