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# "Exposure to the insecticides permethrin and malathion induces leukemia and lymphoma-associated gene aberrations *in vitro*"

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ABSTRACT

Epidemiological studies have associated the exposure to permethrin and malathion with increased risk of leukemia and lymphoma. The aim of this study was to evaluate whether *in vitro* exposure to permethrin and malathion induces aberrations in genes involved in the etiology of these hematological malignancies. Genetic abnormalities in the *IGH*, *KMT2A* (*MLL*), *ETV6* and *RUNX1* genes, and aneuploidy induced by the *in vitro* exposure to permethrin and malathion (200 μM, 24 h), were analyzed by FISH in peripheral blood mononuclear cells (PBMCs). The gene fusions *IGH-BCL2*, *KMT2A-AFF1* and *ETV6-RUNX1* were further analyzed with nested RT-PCR in PBMCs, and in K562 cells exposed to acute and chronic treatments (0.1 μM, 24 h or every third day for two weeks) of insecticides. FISH analysis revealed that permethrin induces aneuploidy and structural alterations in *IGH* and *KMT2A* genes, and malathion induces breaks in *KMT2A*. RT-PCR detected *ETV6-RUNX1* fusion in PBMCs acutely exposed to permethrin. Permethrin also induced *ETV6-RUNX1* and *IGH-BCL2* fusions in K562 cells, and malathion induced *KMT2A-AFF1* and *ETV6-RUNX1* fusions. Overall, we identified that both insecticides induce breaks and fusions in the studied genes, and permethrin induces aneuploidy. This study presents evidence of damage in cancer genes caused by these insecticides.

## 1. Introduction

Each year, thousands of tons of insecticides are produced and used in numerous human activities worldwide [Matthews, 2008; Ding et al., 2012]. Several types of insecticides are essential for agriculture and are applied to a large number of different crops [Agopian et al., 2009]. In addition, insecticides such as pyrethroids and organophosphates are commonly used in household products [Menegaux et al., 2006; Ding et al., 2012; Lu et al., 2015]. These insecticides are also important for public health, given that they are used to control several vector-borne diseases, and are also applied directly to children to control head lice [Menegaux et al., 2006; Roberts et al., 2012].

Permethrin and malathion are among the most commonly used insecticides, and almost any individual may be exposed to these compounds through different routes [Quirós-Alcalá et al., 2011; Morgan, 2012; Ferland et al., 2015]. Permethrin is a pyrethroid, and malathion belongs to the organophosphate insecticide family [Krieger, 2010]. These insecticides became extensively used because they are considered to have low toxicity to humans compared to other insecticides (*e.g.*, organochlorines) [Matthews, 2008]. The main pathways of exposure in humans include dermal, inhalation and oral routes [Morgan, 2012]. Occupational and non-occupational exposures to these insecticides have been reported in adults and children [Barr et al., 2010; Egeghy et al., 2011; Ding et al., 2012; Morgan, 2012; Ferland et al., 2015]. Because almost any individual can be exposed to these insecticides, there is concern regarding the potential adverse effects of these agents.

Epidemiological studies suggest that pyrethroids and organophosphates may be associated with hematological cancer [Turner et al., 2011; Alavanja and Bonner, 2012; Ding et al., 2012; Schinasi and Leon, 2014]. Permethrin has been associated with an increased risk of leukemia in children [Menegaux et al., 2006; Ding et al., 2012; Hernández and Menéndez, 2016]. Higher levels of pyrethroid metabolites were detected in childhood leukemia patients compared to controls [Ding et al., 2012]. Furthermore, *in utero* environmental exposures to insecticides have been associated with the development of childhood leukemia [Armstrong and Look, 2005; Roberts et al., 2012], and traces

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of several insecticides have been detected in the blood, urine and hair of pregnant women [Berkowitz et al., 2003; Berman et al., 2011; Watkins et al., 2016]. Regarding adults, permethrin has been associated to multiple myeloma risk [Alavanja et al., 2014; Rusiecki et al.,2009], and malathion has been related to lymphoma risk in case-control studies [McDuffie et al., 2001]. However, the associations of malathion with lymphoma have not been replicated in longitudinal studies [Alavanja et al., 2014]. Epidemiological studies have limitations because they are often questionnaire-based, making it difficult to accurately determine the time and level of exposure as well as the insecticide or combination of insecticides used [Turner et al., 2011; Ding et al., 2012; Deziel et al., 2015; Hernández and Menéndez, 2016]. Furthermore, there is limited biological evidence supporting the epidemiological associations [Fuscoe et al., 1996; Lafiura et al., 2007].

Biological studies demonstrating that permethrin and malathion have the potential to induce breakages in cancer-associated genes are of great importance. These studies would help to understand the possible relationship of these insecticides with leukemia and lymphoma.

The genotoxicity of permethrin and malathion has been studied, and it is suggested that these chemicals induce DNA breaks (comet assay), micronuclei, chromosome aberrations, and sister chromatid exchanges. However, opposing results have been found in some cases, making the conclusions controversial, and therefore more investigations are needed [Titenko-Holland et al., 1997; Garry et al., 1990; Błasiak et al., 1999; Surrallés et al., 1995; Herrera et al., 1992; Barrueco et al., 1994; Turkez and Aydin, 2012; Ramos-Chavez et al., 2015]. Furthermore, most of these studies analyze the global damage in the DNA or at chromosome level. However, there are scarce studies assessing the potential of these insecticides to induce breakages or rearrangements in the specific genes involved in the alterations relevant in the development of leukemia and lymphoma.

Although the etiology of leukemia has not been completely elucidated, it is known that certain driver mutations potentially transform normal cells into leukemic cells [Pui, 2009]. The most common abnormalities in leukemia include numerical (aneuploidy) and structural chromosome aberrations, such as translocations, which give rise to gene fusions. Among the most common leukemia gene fusions in children are *ETV6-RUNX1*, *KMT2A-AFF1* (*MLL-AF4*), *BCR-ABL1* and *IGH-CRLF2* [Pui et al., 2008; Mrózek et al., 2009; Mullighan, 2012]. Lymphoma-specific genetic aberrations have also been defined. It has been demonstrated that the gene fusion *IGH-BCL2*, has the potential to drive the development of B cell non-Hodgkin lymphoma [Fuscoe et al., 1996; Roulland et al., 2004].

The first events that occur before the formation of these gene fusions are double strand breaks (DSB), which are subsequently mis-repaired, giving rise to genetic aberrations [Greaves and Wiemels, 2003; Hernández and Menéndez, 2016]. Although it has been suggested that exposure to environmental factors, such as insecticides, could induce the formation of these cancer-associated gene rearrangements, more investigation is needed. [Borkhardt et al., 2003; Roulland et al., 2004; Lafiura et al., 2007; Lu et al., 2015; Hernández and Menéndez, 2016]. In the present study we investigated whether permethrin and malathion induce *in vitro* gene breakages in the specific breakpoints of genes involved in rearrangements present in leukemia and lymphoma. This study offers biological evidence that helps to understand the relationship between insecticides and the formation of genetic rearrangements associated with hematopoietic malignancies.

## 2. Materials and methods

In order to compare the results obtained in this study with those referred in the literature, we included the same cellular model used in previous genotoxicity studies testing these insecticides [Herrera et al., 1992; Barrueco et al., 1994; Surrallés et al., 1995; Titenko-Holland et al., 1997; Błasiak et al., 1999; Undeğer and Başaran, 2005]. We analyzed human peripheral blood mononuclear cells (PBMCs) stimulated with phytohemagglutinin (PHA), for assessing the effect of both insecticides *in vitro*. The treatment (24 h, 200 μM) was selected according to references in the literature, as well as pilot experiments. The exposure time and/or concentration are below to those reported as genotoxic in assays that analyzed whole genomes using the same cellular model [Garry et al., 1990; Barrueco et al., 1994; Surrallés et al., 1995; Pluth et al., 1996; Titenko-Holland et al., 1997; Błasiak et al., 1999; Undeğer and Başaran, 2005; Turkez and Aydin, 2012]. Although the acute dose included is higher than the reported in exposed populations, the lower concentration included in the chronic model has been reported as biologically relevant [Błasiak et al., 1999; Ramos-Chavez et al., 2015].

We performed fluorescence *in situ* hybridization (FISH) for detecting gene breakages. Based on the positive results obtained in these experiments, we achieved nested polymerase chain reaction (nested-PCR) assays to detect rearrangements of the *IGH*, *KMT2A (MLL)* and *ETV6-RUNX1* genes induced by insecticides in PBMCs, applying the same acute exposure model, and in the K562 cell line, using lower concentration of insecticides in an acute and chronic exposure models. These gene breakpoints and fusions are biologically significant in the etiology of leukemia and lymphoma [Pui et al., 2008; Roulland et al., 2004]. We also assessed whether permethrin and malathion induce aneuploidy, since numerical chromosome aberrations are common events in hematological cancer [Vučković et al., 1995; Mullighan, 2012].

### 2.1. Chemicals

Analytical grade malathion (CAS No. 121-75-5) and permethrin (mixture of 27% cis and 71% trans isomers, CAS No.52645–53-11) were purchased from Sigma, USA. Stock solutions of malathion and permethrin were prepared in ethanol (Merck, USA) (0.2% v/v, final concentration in RPMI 1640 medium, Gibco, USA) and DMSO (Sigma-Aldrich, USA) (0.4% *v/v final concentration* in medium), respectively.

### 2.2. Cell cultures and exposure to insecticides

a) Peripheral blood mononuclear cells (PBMCs) analyzed by FISH (structural and numerical chromosome gene aberrations) and nested PCR analysis (gene fusions).

Peripheral blood samples from young healthy male non-smoker volunteers were obtained. Informed consent was obtained according to the recommendations of the Helsinki Declaration. Mononuclear cells from peripheral blood were isolated using a density gradient medium (Lymphoprep, Nycomed, Norway). Cells were cultured at a final density of 5–7 $\times$ $10^5$ cells/mL in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco,USA), 1% non-essential amino acids (Gibco, USA), 1% sodium pyruvate (Gibco, USA), 1% L-glutamine (Gibco, USA) and 2% PHA (Gibco, USA).

The initial cell viability was consistently > 98%. Cells were treated with the insecticides after 48 h of culture. The final concentration of both insecticides was 200 μM. Cells exposed to ethanol (0.2%) and DMSO (0.4%) were included as negative controls for malathion and permethrin respectively. Cells exposed to 10 μM of etoposide dissolved in DMSO were included as a positive control [Libura et al., 2008, Lu et al., 2015]. In addition, untreated cells were also included for comparison against cultures exposed to solvents (DMSO and ethanol). Each treatment condition was performed in triplicate for each individual included (2 volunteers). After treatment, the cells were incubated for another 24 h. After 72 h, cell viability was determined, and cells were harvested by centrifugation at 126 $\times$ *g* for 15 min. Cells were incubated with a hypotonic solution (KCl, Merck, Germany 0.075 M) at 37 °C for 20 min. The cells were then fixed with Carnoy's fixative solution (3:1 methanol:acetic acid, Merck, Germany). Cell suspensions were kept at 4 °C until slide preparation.

b) K562 cells exposed to chronic treatment and analyzed by nested