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Evaluation of the genotoxic effects of carbamate and organophosphate insecticides using the comet assay

Dr.Rajendraprasad, .N.Sravya, Jayaprad,Priyanka singh

SAMSKRUTI COLLEGE

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ABSTRACT

Context and Objectives: The pervasiveness of pesticides in the environment makes pesticide poisoning the leading occupational risk for agricultural workers in the emerging nations. Organophosphosphate and carbamate pesticides' cytotoxicity and DNA damaging properties were the focus of this investigation.

To determine the cytotoxicity of chlorpyrifos methyl, azinphos ethyl, aldicarb sulfone, ethiofencarb, and [(O-Ethyl O-(p-nitrophenyl) pheriylphosphonothioate)] (EPN), this research used the trypan blue dye exclusion assay. To determine if pesticides administered to human peripheral blood cells had any genotoxic effects, an alkaline comet test was carried out.

Conclusion: After 30 and 120 minutes of exposure at a concentration of 100 µg/mL, we showed that EPN had a cytotoxic impact. The in vitro comet test revealed considerably greater amounts of DNA damage after 120 minutes of exposure to chlorpyrifos-methyl and azinphos ethyl, even though these pesticides seem to be less harmful in terms of cytotoxicity than other pesticides. The pesticides were ranked according to their potential DNA-damaging effects: chlorpyrifos-methyl, aldicarb sulfone, EPN, and azinphos ethyl after 30 minutes of exposure, and again after 120 minutes: chlorpyrifosmethyl, azinphos ethyl, aldicarb sulfone, and EPN. Based on our findings, these herbicides have a tendency to cause a dose- and time-dependent increase in DNA damage.

In conclusion, the DNA-damaging potential of pesticides may lead to genotoxic risk and negative health impacts like cancer. These pesticides are used extensively and may have genotoxic effects.

Genetic toxicity, in vitro comet assay, carbamate insecticides, organophosphate pesticides.

INTRODUCTION

Pesticides are often used in agriculture to enhance food production efficiency, decrease food expenses, and guarantee high-quality produce. They are chemicals with physical, chemical, and biological activities. More than 40% of the world's food supply is wasted every year due to pests, illnesses, and weeds (Jamil, Shahboob, Krishna, & Krishna, 2004; Suratman, Edwards, & Babina, 2015). Furthermore, pesticides aid in limiting the transmission of contagious illnesses. Even while pesticides have their uses, there is concern that the chemicals left behind in our food, water, air, and soil might harm us and the ecosystem (van der Werf, 1996; Ahmed, 2001). This might lead to both short-term and long-term environmental damage, upsetting the delicate ecological balance. Workers in underdeveloped countries are also at risk of chemical exposure due to the nature of their jobs, which include tasks like spraying, handling, manufacturing, and packaging. Pesticides are effective in killing off the intended pests, but they may also harm non-target creatures, including people, beneficial insects, fish, birds, and plants (Mohanty, Mohanty, Jena, & Dutta, 2011).

Exposure to pesticides is thought to cause its harmful effects, which include bioaccumulation, biomagnification, chronic toxicity, hypersensitivity reactions, acute immunological response, mutagenic, carcinogenic, and teratogenic responses (Ecobichon, 2001).

Pesticides such as carbamates, organophosphates (OPs), and synthetic pyrethroids are used extensively to control insects in agriculture and homes; thus, these compounds are in direct touch with people. More than 100 OP compounds have been found and used globally since 1943, with OPs being developed specifically for this purpose (Suratman et al., 2015). Because of their capacity to inhibit the enzyme acetylcholinesterase (AChE), which is responsible for the breakdown of the neurotransmitter acetylcholine (ACh), OPs and carbamate insecticides are poisonous to both insects and mammals. Pesticides containing OP have an irreversible effect on the acetylcholinesterase enzyme, whereas those including carbamate have a reversible effect. Cholinergic neurons are overstimulated due to an excess of accumulated ACh caused by this inhibition. According to Munoz-Quezada et al. (2016), there are cases when pesticide concentrations are so high that death may happen within minutes.

The genotoxic effects of pesticides on humans have been a major worry over the last ten years. Genotoxicity studies in both laboratory and living organisms have shown that oxidative stress and DNA damage are the fundamental reasons why pesticides like carbamates and OPs are harmful (Muniz et al., 2008; Mohanty et al., 2011). The genotoxicity of these substances was evaluated in vitro using micronucleus (MN) tests, sister chromatid exchange (SCE), chromosomal aberrations (CA), and the alkaline comet assay (single cell gel electrophoresis assay, SCGE). Genotoxicity assays may be used to determine DNA damage at the individual cell level. One well-known technique is the comet assay. This method has been used by Singh, McCoy, Tice, and Schneider (1988) and McKelvey-Martin et al. (1993) to detect DNA strand breaks, alkali-labile spots, and cells with insufficient excision repair pathways. These features allow for the assessment of oxidative DNA damage levels that are important to the body's physiological processes. There is a lack of data on the genotoxic effects of pesticides on human peripheral blood cells in vitro. The target pesticides in this investigation were organophosphate pesticides (azinphos ethyl, chlorpyrifos methyl, EPN) and carbamate pesticides (aldicarb sulfone, ethiofencarb).

MATERIAL AND METHODS

Encounters with pesticides

Two healthy female volunteers, ranging in age from 30 to 32, who did not smoke, had their heparinized blood samples taken, each of which weighed five milliliters. Members of the public gave their informed permission. All procedures followed in the research were compliant with the guidelines laid forth in the Declaration of Helsinki. We immediately put the samples to work in our trypan blue dye exclusion test to determine viability and our comet assay to determine DNA damage.

Our exploratory investigation used Maden-Darby Canine Kidney (MDCK) cell lines and human blood lymphocytes to assess the genotoxic effects of certain carbamates, including aminocarb, carbaryl, methiocarb, promecarb, and propoxur. The MDCK cell lines were treated with pesticides at varying doses. Based on our preliminary investigation, the concentrations of the pesticides and the incubation duration used in the present study were determined. Isolated lymphocytes were washed with PBS after being separated with Histopaque 1077. We made sure the cell concentration in the buffer was around 2x105 cells/mL. For 30 and 120 minutes at 37°C, isolated lymphocytes were exposed to pesticide doses of 10, 50, and 100 µg/mL (aldicarb sulfone, azinphos ethyl, chlorpyrifos methyl, ethiofencarb, EPN). By incubating lymphocytes with the solvent DMSO at a final concentration of 1% at the same temperature and exposure time as the pesticide standards, negative controls were set up in parallel. The cells were incubated at 37°C for 5 minutes after being treated with 30% H2O2 at 100 µM, serving as a positive control. In triplicate testing, blood samples were collected from the same donor at various intervals. In each experiment, both positive and negative controls were used.

Proliferation test for cells

A technique called trypan blue dye exclusion was used to determine the cell viability. Following exposure to the herbicide, the lymphocytes were rinsed in PBS. Afterwards, a 1:1 ratio was used to combine the cell suspension with the 0.04% trypan blue solution. Living cells were then counted manually using a hemacytometer twice.

A mean proportion of viable cells was determined.

Evaluate comets

In this study, the comet test was performed using an alkaline version that was slightly adjusted from the technique described by Singh et al. (1988). After the incubation period ended, specimens treated with pesticides and those left untreated were combined with 0.7% low melting agarose (LMA) and then spread out on microscope slides that had been previously coated with 0.7% normal melting agarose. After being immersed in a lysing solution (10 mM Tris, 100 mM Na2EDTA, 2.5 M NaCl pH 10, with 10% DMSO and 1% Triton X-100), the slides were left at +4 °C for at least 1 hour.

Now the LMA was completely set. After dipping the slides into electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH 13) for 20 minutes, DNA could be unwound and alkali labile damage could be seen. Electrophoresis was then performed on the DNA for 30 minutes at 300 mA and 15 V. I used 50 µL of ethidium bromide (EtBr - 20 µg/mL) to stain the cells after neutralizing them with 0.4 M Tris buffer, pH 7.5. A fluorescent microscope (Olympus BX51, Tokyo, Japan) with a 200x objective was used to analyze the stained DNA pictures. For the purpose of determining DNA damage, one hundred randomly selected cells from each sample were visually inspected for comet appearance. By visual inspection, five distinct levels of DNA movement were identified in the cells. Collins (2014) categorized the results into five groups, with the lowest representing no DNA damage and the highest representing the most extensive damage.

The DNA damage assessment was conducted using the metric total comet score (TCS). The formula for total comet score (TCS) was then used, where "n" denoted the number of cells in each class, and it was expressed as TCS= 0 (n)+1(n)+2(n)+3(n)+4(n).

Data analysis using statistics

Using SPSS (version 22.0). The statistical analysis was conducted using SPSS (Chicago, SPSS, Inc.). After completing the non-parametric Kruskal-Wallis test and the post hoc examination of differences between the groups, the findings were compared statistically using the Mann Whitney U test. The findings were presented as the mean±standard deviation, and statistical significance was defined as a p-value below 0.05.

RESULTS

The trypan blue dye exclusion technique was used to test the cytotoxic effects of the pesticides on the cells. After the pesticides were incubated, the cytotoxicity increased in a concentration- and time-dependent way, according to results from three independent studies. After 30 minutes of incubation, the concentrations of 50 and 100 µg/mL of EPN showed the maximum toxicity ($p>0.05$). Compared to the other pesticides examined, chlorpyrifos methyl had a significantly greater cell viability percentage of lymphocytes ($p>0.05$). The toxicity of EPN at a dosage of 100 µg/mL was found to be statistically significant ($p>0.05$) after the tested compounds were incubated for 120 minutes.

At 50 µg/mL concentrations, chlorpyrifos methyl and azinphos ethyl were discovered to be less harmful than the other pesticides that were examined. On the other hand, lymphocytes treated with 100 µg/mL of azinphos ethyl showed cell viability percentages exceeding 60%. Lymphocyte cell viability percentages after 30 and 120 minutes of exposure to increasing pesticide doses are shown in Figures 2 and 3, respectively.

Comet tail length, an estimate of DNA damage for each cell, was obtained after seeing the comet tail using a fluorescent microscope. This length is based on the DNA movement in the comet tail. Researchers found that when pesticide concentrations rose, the comet tail lengthened. While all quantities of pesticides resulted in DNA damage as compared to the negative control, the reactions to this damage differed. The peak

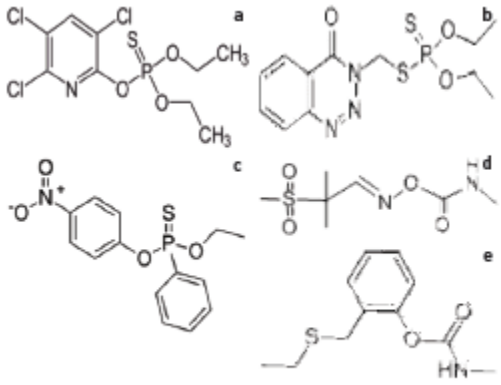

Figure 1. Chemical structures of tested pesticides (a. chlorpyrifos methyl; b. azinphos ethyl; c. EPN; d. aldicarb sulfone; e. ethiofencarb)

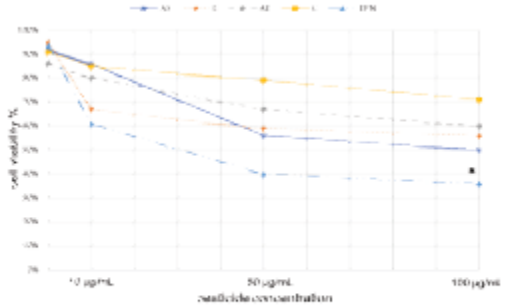

Figure 2. Cell viability percentage in lymphocytes after exposures to increasing concentrations of pesticides for 30 min (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

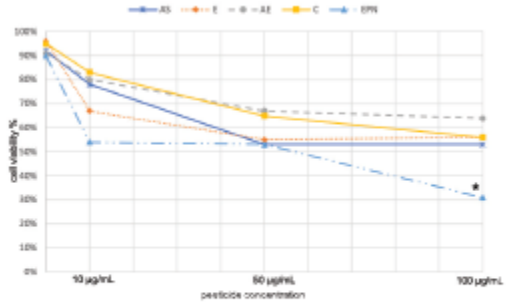

Figure 3. Cell viability percentage in lymphocytes after exposures to increasing concentrations of pesticides for 120 min. (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

The most harmful effect of chlorpyrifos methyl was seen after 120 minutes of incubation when exposed to 100 µg/mL (TCS: 222±85, $p<0.05$). There was a statistically significant increase in DNA damage after exposure to OPs (chlorpyrifos methyl, azinphos ethyl and EPN) at a dose of 50 µg/mL and carbamates (aldicarb sulfone) at a concentration of 100 µg/mL for 30 minutes of incubation ($p<0.05$). The average TCS distribution of DNA damage in lymphocytes after pesticide treatments is shown in Figures 4 and 5. different amounts for 30 and 120 minutes. DNA damage was considerably enhanced after 120 minutes of exposure to 50 µg/mL of chlorpyrifos methyl, 100 µg/mL of EPN aldicarb sulfone, and azinphos ethyl. There was no dose-or incubation time-dependent DNA damage inducing effects of ethiofencarb. After 30 minutes of exposure, the insecticides chlorpyrifos methyl, aldicarb sulfone, EPN, and

azinphos ethyl were ranked according to their potential DNA damaging effects. Chlorpyrifos, azinphos ethyl, aldicarb sulfone, and EPN were the four groups they were divided into after 120 minutes of exposure.

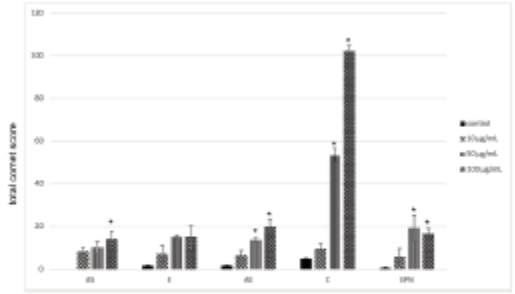

Figure 4. CDNA damage levels in lymphocytes after exposures of pesticides at different concentrations for 30 min (*p<0.05). (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

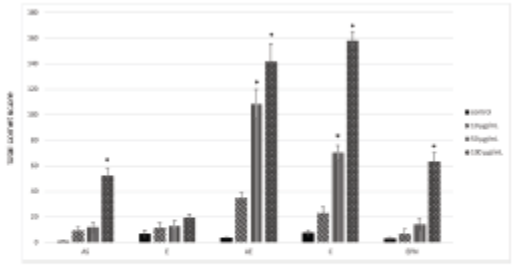

Figure 5. DNA damage levels in lymphocytes after exposures of pesticides at different concentrations for 120 min (*p<0.05). (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

DISCUSSION

Due to the pervasiveness of pesticides in the environment, pesticide poisoning is the most common occupational hazard for agricultural workers in the developing world. Gaikwad, Karunamoorthy, Kondhalkar, Ambikapathy, and Beerappa (2015) state that the compounds' action mechanisms are still under investigation in both laboratory and living organism settings. According to studies conducted utilizing in vivo and in vitro comet tests, an increase in DNA damage is linked to long-term exposure to OPs (Ündeğer & Başaran, 2002; Shadnia et al., 2005; Muniz et al., 2008). For in vitro genotoxicity investigations using comet analysis, the trypan blue exclusion test is often used to evaluate cell viability (Vigreux et al., 1998; Das, Shaik, & Jamil, 2007). According to our first research,

Table 1. Total comet scores in lymphocytes after exposures of pesticides at different concentrations for 30 min and 120 min.

Pesticides	Concentrations (µg/mL)	TCS[a] (Mean±SD)	
		30 min	120 min
Chlorpyriphos methyl	10	6.67±2.5	23.01±4.7
	50	53.31± 3.4*	70.32±5.8*
	100	101.99±2.8*	157.66±7.2*
Azinphos ethyl	10	6.64±2.1	34.98±4.1
	50	13.67±1.2*	108.32±11.5*
	100	20.01±3.6*	141.67±14.2*
EPN	10	6.01±3.6	6.99±3.5
	50	19.34±6.2*	14±4.4
	100	16.66±2.5*	63.67±6.7*
Aldicarb sulfone	10	8.33±2.3	9.32±2.6
	50	10.34±3.4	11.99±4.2
	100	13.98±4.1*	52.65±5.3*
Ethiofencarb	10	7.31±4.3	11.66±4.3
	50	15.02±1.4	12.97±4.5
	100	15.31±5.2	19.33±3.2

[a]Totalcometscore: 0x No Migration (NM) + 1 x Low Migration (LM) + 2 x Medium Migration (MM) + 3 x High Migration (HM) + 4 x Extensive Migration (EM). *P <0.05.

Aminocarb, carbaryl, methiocarb, promecarb, and propoxur were tested for their genotoxic effects on human blood lymphocytes and Maden-Darby Canine Kidney (MDCK) cell lines. The MDCK cell lines were treated with pesticides for 48 hours at 37ºC at doses of 3, 10, 30, and 100 µg/mL. A comet assay was used to examine the DNA damage in lymphocytes that were exposed to pesticides at a dosage of 30 µg/mL for either 30 minutes or 16 hours. Results demonstrated a connection between carbaryl and methiocarb toxicity and the most damage inflicted on MDCK cells. Compared to lymphocytes, MDCK cells treated with pesticides had lower levels of DNA damage.

At a dosage of 10 µg/mL, there was no discernible change in cell development when pesticides were present (data not shown). Thus, using the trypan blue exclusion test, we proved that EPN was cytotoxic to human peripheral blood cells after 30 and 120 minutes of exposure at a dosage of 100 µg/mL. Despite the fact that chlorpyrifos methyl and azinphos ethyl seem to be less harmful to cells than other pesticides, an in vitro comet test showed that these two pesticides caused much more DNA damage after 120 minutes of exposure at a dose of 100 µg/mL. These pesticides' harmful processes differ based on their chemical compositions or where they end up in the body.

Although OPs have been the subject of some genotoxic effects research, the findings have been contradictory (Ojha & Srivastava, 2014). Compared to the control group, those treated with the highest concentrations of chlorpyrifos methyl for 30 and 120 minutes had more DNA damage. This outcome is consistent with what other studies have found (Rahman et al., 2002; Mehta et al., 2008; Sandal & Yilmaz, 2011).

The researchers demonstrated that rats exposed to chlorpyrifos had dose-dependent increases in DNA damage in their brain and liver tissues, as well as in their lymphocytes and leukocytes. Using fluorescence in situ hybridization (FISH), Abuwarda et al. (2021) examined the aneuploidy-inducing effects of chlorpyrifos on human peripheral blood cell cultures. While they did find that this chemical exhibited tolerable levels of cytotoxicity, they also found that exposure increased the frequencies of aneuploidy, chromosomal loss, and chromosome gain. Okonko, Ikpeme, and Udensi (2016) found that chlorpyrifos caused DNA damage in lymphocytes, which was consistent with findings from the comet test and rat studies on the bone marrow micronucleus assay. Using the comet test, Jamil et al. (2004)

investigated the genotoxic effects of organochlorine (endosulfan) and organophosphorus (monochrotophos, chlorpyrifos, dimethoate) pesticides. They found that dimethoate was only 10 times as hazardous as two pesticides, monochrotophos and chlorpyrifos.

Exposure to the skin, breathing in dust or spray, or swallowing azinphos ethyl all lead to rapid absorption of the very poisonous chemical by animals (Petroianu, Nurulain, Hasan, Kuca, & Lorke, 2015). In human peripheral blood lymphocytes, azinphos ethyl does not seem to be genotoxic. The first report on the genotoxicity of azinphos ethyl using the comet test is presented in this paper. According to McCauley et al. (2001), azinphos methyl has the same genotoxic potential as azinphos ethyl, the pesticide residue most typically detected in the homes of farmers and agricultural laborers. Both azinphos methyl and azinphos ethyl share a phosphorodithioate bond, making them chemically identical. Despite azinphos ethyl's favorable results in the in vitro micronucleus test for Chinese hamster lung cells, the in vivo micronucleus assay for mice did not reveal any DNA damaging effects (Ni, Li, Liu, Tang, & Pang, 1993).

The two azinphos chemicals are metabolized rather rapidly in vivo before they can generate any genotoxic effects, which explains why the in vitro cytogenetic tests and the in vivo tests do not consistently provide the same answer. Human cell cultures treated to azinphos methyl for 24 hours caused oxidative DNA damage, as shown by Kisby et al. (2009). According to Kisby et al. (2009), one of the primary ways in which pesticides affect cellular function is by DNA damage. Excessive cytochrome c release from mitochondria to the cytosol, caspase-3 activation, and a compromised cellular antioxidant defense system leading to DNA damage are all consequences of prolonged exposure to OPs (Hodgson & Levi, 1996; Kaur, Radotra, Minz, & Gill, 2007; Kisby et al., 2009).

A nonsystemic organophosphorus pesticide and acaricide, EPN stands for (O-Ethyl O-(p-nitrophenyl) pheriylphosphonothioate]. When exposed to the mouths of birds and animals in a short amount of time, it causes severe toxicity (Smith, 1987). Although EPN has not been shown to cause cancer in humans, there is also no proof that it has any genotoxic effects.

The genotoxic impact of EPN on human blood lymphocytes has never been reported before, and here it is. Our research shows that human lymphocytes are cytotoxic when exposed to EPN at a concentration of 100 μg/mL for 120 minutes, and that there is a significant increase in DNA damage as a result. Another major family of pesticides, carbamates are strong cholinesterase inhibitors in the form of organophosphates. Multiple test methods have shown that carbamates are mutagenic (Proença et al., 2004; Mohanty et al., 2011). Using cytotoxicity and genotoxicity experiments, Das et al. (2007) evaluated the effects of pesticides (organophosphate, organochlorine, and carbamate) and mixtures thereof on people. Evident tail lengths proved that high concentrations of certain pesticides (0.5-4.0 μM) caused significant DNA damage, as stated by the authors. The DNA of lymphocytes was severely damaged by carbamate insecticides. Inhibition of other metabolic processes and cell death are two other potential sources of DNA damage, in addition to direct strand breaking.

An agricultural pesticide and nematicide, aldicarb is a carbamate. Through its metabolism, aldicarb sulfoxide and aldicarb sulfone are produced. The acetylcholinesterase inhibitor aldicarb sulfoxide outperforms the original aldicarb. Past reports (Cid & Matos, 1984; Sun et al., 2010) have detailed aldicarb's genotoxicity potential as indicated by genotoxicity assays (comet assay, SCE, and micronucleus assay). Sun et al. (2010) used the micronucleus test, the Ames test, and the comet assay to examine the genotoxicity of aldicarb and methomyl at varying doses. Results from the comet test showed that human peripheral blood cells were DNA damaged at various degrees by high doses of aldicarb (Sun et al., 2010). Nevertheless, there is a dearth of research on aldicarb sulfone's potential genotoxicity.

Following treatment with 100 μg/mL of aldicarb sulfone, our investigation found evidence of DNA damage. Using a modified SOS microplate test, Venkat et al. (1995) assessed the mutagenic potential of 47 pesticides, including aldicarb sulfone (Venkat et al., 1995). One of the top ten most effective pesticides was identified as aldicarb sulfone.

Using the MutatoxTM test, Canna-Michaelidou and Nicolaou (1996) investigated aldicarb sulfone's genotoxic effects both in a control group and in groups that had been exogenously activated with the S9 hepatic enzyme. Whether evaluated before or after S9-activation, aldicarb sulfone was determined to have a "suspect genotoxic" impact on DNA. Results from these investigations show that aldicarb sulfone causes substantial DNA damage. One common systemic insecticide, ethiofencarb, works via both the oral and contact routes. According to Al-Samarraie et al. (2009), it is almost entirely absorbed by mammals and then quickly eliminated as metabolites, mostly via urine. Ethiofencarb

has not been shown to have any genotoxic effects in mammalian test systems, either in vivo or in vitro. For the first time, at the doses and incubation durations tested, this comet assay confirms that ethiofencarb is not cytotoxic and does not harm DNA. The genotoxic consequences of ethiofencarb need to be further studied.

CONCLUSION

By inhibiting other metabolic pathways and causing cell death, OPs and carbamate insecticides produce substantial DNA damage in lymphocytes. Genotoxic damage is an important indicator of potential cancer risk. Due of the genotoxic risk and other negative health consequences, such as cancer, that may result from low-level, long-term exposure to pesticides, it is important to treat these pesticides with caution. There has to be more research on human populations' genotoxic thresholds and vulnerability to pesticide-related diseases.

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