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Original Article

Assessment of genotoxic effects of organophosphate and carbamate pesticides by comet assay

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ABSTRACT

Background and Aims: Pesticide poisoning is the most widespread occupational hazard for agricultural workers in the developing world, due to the extensive presence of pesticides in the environment. The aim of this study was to investigate the cytotoxicity and DNA damaging effects of organophosphosphate and carbamate pesticides.
Methods: In the present study, the cytotoxicity of chlorpyrifos methyl, azinphos ethyl, [(O-Ethyl O-(p-nitrophenyl) pherilyphosphonothioate] (EPN), aldicarb sulfone, and ethiofencarb were assessed by the trypan blue dye exclusion method. An alkaline comet assay was performed to assess the genotoxic effects of applied pesticides in human peripheral blood lymphocytes.
Results: We demonstrated the cytotoxic effect of EPN following 30 and 120 min exposure at 100 µg/mL concentration. Although chlorpyrifos-methyl and azinphos ethyl seem to be safer concerning cytotoxicity compared to other pesticides, significantly higher DNA damage levels were determined after exposure of these pesticides for 120 min at 100 µg/mL concentration by in vitro comet assay. The potential DNA-damaging effects of these pesticides were sorted from high to low, as chlorpyrifos-methyl, aldicarb sulfone, EPN, and azinphos ethyl after 30 min of exposure, and were sorted as chlorpyrifos-methyl, azinphos ethyl, aldicarb sulfone, and EPN after 120 min of exposure. Our results revealed that these pesticides tend to increase DNA damage in a dose- and time-dependent manner.
Conclusion: The genotoxic effects of these widely used pesticides may cause prominent and serious health risks for human populations; hence, the DNA-damaging potential of pesticides can lead to genotoxic risk and adverse health effects like cancer.

Keywords: Carbamate pesticides, Organophosphate pesticides, In vitro comet assay, Genotoxicity

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INTRODUCTION

Pesticides are physical, chemical, and biologically active substances that are widely used in agriculture to improve the efficiency of food production processes, lower food costs, and ensure high-quality produce. Weeds, pests, and diseases cause over 40% of global food production to be lost each year (Jamil, Shaik, Mahboob, & Krishna, 2004; Suratman, Edwards, & Babina, 2015). In addition, pesticides help to reduce the spread of infectious diseases. Despite the advantageous effects of pesticides, the residues of these chemicals in the soil, air, water, and food may pose a threat to human health and the natural environment (van der Werf, 1996; Ahmed, 2001). As a result, the balance of the ecological system may be disturbed, and they may cause acute and chronic poisoning in the immediate environment. Additionally, humans in the developing world are exposed to these chemicals in various job processes such as spraying, handling, manufacturing and packing. Besides eradicating insects or weeds, pesticides can show their toxic effects on other non-target organisms like birds, fish, beneficial insects, plants, and humans (Mohanty, Mohanty, Jena, & Dutta, 2011). The toxic effects of pesticides, including bioaccumulation, biomagnification, chronic toxicity, acute immune response, hypersensitivity reactions, and mutagenic, carcinogenic, and teratogenic responses are propounded due to exposure (Ecobichon, 2001).

Organophosphates (OPs), synthetic pyrethroids, and carbamates are the most widely used pesticides in the world today for the control of agricultural and domestic insects; as a result, these substances come into direct contact with humans. OPs in particular have been manufactured since 1943 and until today, more than one hundred OP compounds have been identified and utilized worldwide (Suratman et al., 2015). OPs and carbamate insecticides are toxic to insects and mammals by virtue of their ability to deactivate the acetylcholinesterase enzyme (AChE), which catalyzes the hydrolysis of acetylcholine (ACh), a neurotransmitter. Whereas OP insecticides inhibit the acetylcholinesterase enzyme irreversibly, carbamate insecticides inhibit it reversibly. This inhibition leads to an excess of ACh accumulating and overstimulating cholinergic neurons. If the concentration of pesticide is high, in some instances even death can occur in a matter of minutes (Munoz-Quezada et al., 2016).

Human exposure to pesticides and the genotoxic effects of these chemicals has remained a global concern over the last decade. *In vitro* and *in vivo* genotoxicity studies have shown that DNA damage and oxidative stress are the underlying causes of pesticide toxicity, including OPs and carbamates (Muniz et al. 2008; Mohanty et al. 2011). Micronucleus (MN) tests, sister chromatid exchange (SCE), chromosome aberrations (CA) and the alkaline comet assay (single cell gel electrophoresis assay, SCGE) were utilized to assess these compounds' genotoxicity *in vitro*. The comet assay is a well-known genotoxicity assay for determining DNA damage at the individual cell level. It identifies DNA strand breaks, alkali-labile sites, and inadequate excision repair processes in individual cells (Singh, McCoy, Tice, & Schneider, 1988; McKelvey-Martin et al., 1993). As a result of these characteristics, it becomes possible to evaluate physiologically relevant levels of oxidative DNA damage. Up to now, insufficient information has been obtained concerning *in vitro* genotoxic influence of pesticides on human peripheral blood lymphocytes. The purpose of this study was to assess potential genotoxic effects of aldicarb sulfone, ethiofencarb from the carbamate class, and azinphos ethyl, chlorpyrifos methyl, EPN from the organophosphate class of pesticides.

MATERIAL AND METHODS

Pesticide exposure

Five milliliters of heparinized blood samples from two healthy non-smoking female volunteers, aged 30-32 years were collected in heparinized syringes. Volunteers provided informed consent. The study protocol was conducted in accordance with the Declaration of Helsinki. The samples were used immediately for the determination of viability by trypan blue dye exclusion assay and DNA damage by comet assay.

In our preliminary study, the genotoxic effects of certain carbamates (aminocarb, carbaryl, methiocarb, promecarb and propoxur) were evaluated in Maden-Darby Canine Kidney (MDCK) cell lines and human blood lymphocytes. Pesticides were applied to MDCK cell lines at several concentrations. The studied pesticide concentrations and incubation time in the current study were determined based on our preliminary study. Histopaque 1077 separating solution was used to isolate lymphocytes, which were then rinsed with PBS. Cell concentrations were adjusted to around $2x10^5$ per mL in the buffer. Isolated lymphocytes were incubated with 10, 50 and 100 µg/mL concentrations of pesticides (aldicarb sulfone, azinphos ethyl, chlorpyrifos methyl, ethiofencarb, EPN) for 30 min and 120 min at 37°C. In parallel with the pesticide standards, negative controls were established by incubating lymphocytes with the solvent DMSO at a final concentration of 1% at the same temperature and exposure duration as the pesticide standards. As a positive control, the cells were treated with 30% H_2O_2 at 100 µM and incubated for 5 min at 37°C. Blood samples from the same donor were taken at different time periods in triplicate tests. Positive and negative controls were used in each experiment.

Cell proliferation assay

The cell viability was assessed using the trypan blue dye exclusion method. The lymphocytes were washed in PBS after pesticide exposure. Subsequently, the cell suspension was mixed with the 0.04% trypan blue solution at a ratio of 1:1, and living cells were counted manually using a hemacytometer in duplicate. The mean percentage of living cells was calculated.

Comet assay

The alkaline version of the comet assay was used in this investigation, and it was modified slightly from Singh et al. (1988) method. At the end of the incubation period, pesticide-treated and control cells were mixed with 0.7% low melting agarose (LMA) and spread on microscope slides covered with 0.7% normal melting agarose. The slides were submerged in a lysing solution (10 mM Tris, 100 mM Na_2EDTA, 2.5 M NaCl pH 10 with 10% DMSO and 1% Triton X-100) for at least 1 h at +4 °C after

the LMA had solidified. To enable for DNA unwinding and the appearance of alkali labile damage, the slides were submerged in electrophoresis buffer (0.3 M NaOH, 1mM EDTA, pH 13) for 20 min. Afterwards, the DNA was electrophoresed for 30 min at 300 mA and 15 V. The cells were neutralized with 0.4 M Tris buffer, pH 7.5 and stained with 50 µL ethidium bromide (EtBr - 20 µg/mL). The stained DNA images were examined using a fluorescent microscope with a 200x objective (Olympus BX51 microscope, Tokyo, Japan). To calculate DNA damage, 100 cells were chosen at random from each sample and visually examined for comet appearance. The degree of DNA migration in the cells was classified into five categories by eye. Five classes, ranging from class 0 (no DNA damage) to class 4 (maximum DNA damage), provided sufficient declaration (Collins, 2014).

The parameter total comet score was used to assess DNA damage (TCS). Total comet score (TCS) was then calculated according to the formula:

TCS= 0 (n)+1(n)+2(n)+3(n)+4(n), where "n" indicated the number of cells in each class.

Statistical analysis

The SPSS software (ver. 22.0. Chicago: SPSS Inc.) was used for statistical analysis. The results were statistically compared using the non-parametric Kruskal-Wallis test and the Mann Whitney *U* test as the *post hoc* analysis of differences between the groups with the least significant difference test. All results were expressed as mean±SD and a *p*-value less than 0.05 was determined to be statistically significant.

RESULTS

The cytotoxic effects of the pesticides on the cells were determined by the trypan blue dye exclusion method. According to the data obtained from three separate experiments, the cytotoxicity was increased in a concentration- and time-dependent manner after the incubation of the pesticides. The highest toxicity was observed with EPN at 50 and 100 µg/mL concentrations after 30 min incubation (p>0.05). The cell viability percentage of lymphocytes after treatment with chlorpyrifos methyl was found to be statistically higher than the other tested pesticides (*p>0.05*). After 120 min incubation of the tested compounds, the toxicity of EPN at 100 µg/mL concentration was determined as statistically significant (p>0.05). Chlorpyrifos methyl and azinphos ethyl at 50 µg/mL concentrations were found to be safer than other tested pesticides, while cell viability percentages were over 60% in lymphocytes treated with azinphos ethyl at 100 µg/mL concentration. Cell viability percentages of lymphocytes after exposure to increasing concentrations of pesticides for 30 min and 120 min are shown in Figure 2 and 3.

After visualizing the comet tail using a fluorescent microscope, the length of DNA migration in the comet tail was determined as comet tail length, which is an estimate of DNA damage for each cell. It was observed that the comet tail length extended with increasing concentrations of pesticides. Pesticides caused DNA damage at all concentrations compared to the negative control; however, responses of DNA damage varied. The highest toxicity was observed with 100 µg/mL exposure after 120 min incubation for chlorpyrifos methyl (TCS: 222±85, p<0.05). An increase in DNA damage after OPs (chlorpyrifos methyl, azinphos ethyl and EPN) exposure at 50 µg/mL concentration and carbamates (aldicarb sulfone) exposure at 100 µg/mL concentration for 30 min incubation was found statistically significant (*p<0.05*). Figures 4 and 5 show the mean TCS distribution

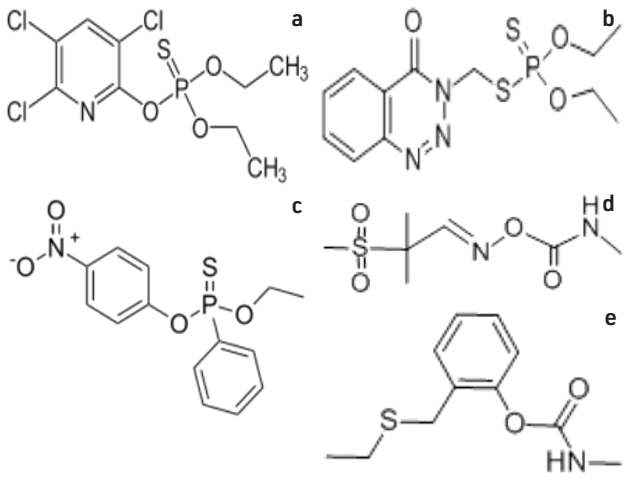

Figure 1. Chemical structures of tested pesticides (a. chlorpyrifos methyl; b. azinphos ethyl; c. EPN; d. aldicarb sulfone; e. ethiofencarb)

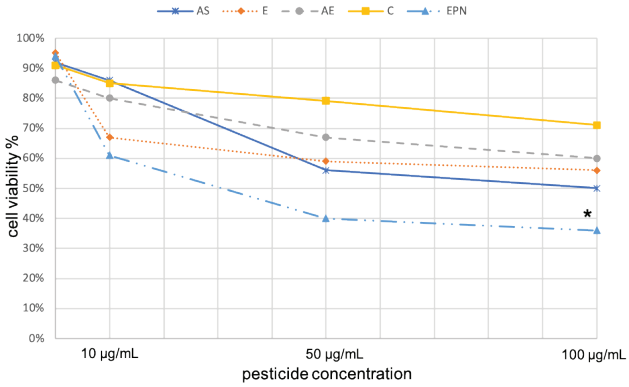

Figure 2. Cell viability percentage in lymphocytes after exposures to increasing concentrations of pesticides for 30 min (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (**p*<0.05).

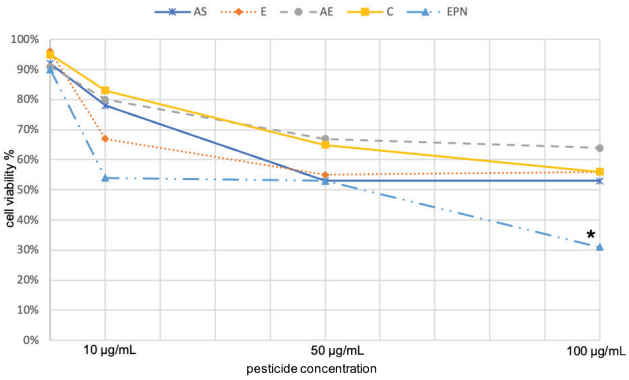

Figure 3. Cell viability percentage in lymphocytes after exposures to increasing concentrations of pesticides for 120 min. (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (**p*<0.05).

of DNA damage in lymphocytes after pesticide treatments at various concentrations for 30 and 120 min. 50 µg/mL of chlorpyrifos methyl, azinphos ethyl, and 100 µg/mL of EPN aldicarb sulfone significantly increased DNA damage after 120 min exposure. On the other hand, ethiofencarb did not induce DNA damage in a dose- and incubation time-dependent manner. The potential DNA damaging effects of these pesticides were sorted from high to low, as chlorpyrifos methyl, aldicarb sulfone, EPN, azinphos ethyl after 30 min of exposure. After 120 min of exposure, they were sorted as chlorpyrifos, azinphos ethyl, aldicarb sulfone, EPN.

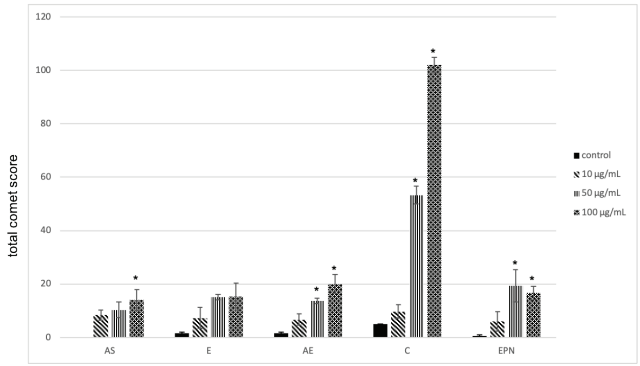

Figure 4. CDNA damage levels in lymphocytes after exposures of pesticides at different concentrations for 30 min (*p<0.05). (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

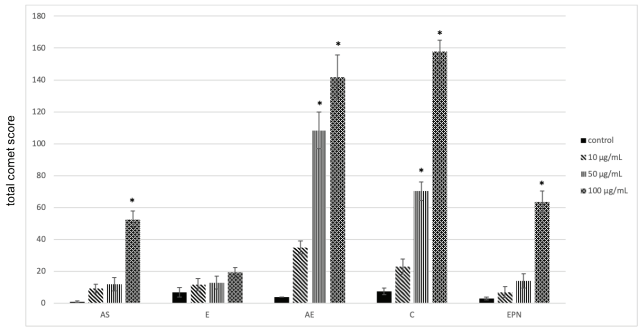

Figure 5. DNA damage levels in lymphocytes after exposures of pesticides at different concentrations for 120 min (*p<0.05). (AS: aldicarb sulfone; E: ethiofencarb; AE: azinphosethy; C: chlorpyrifos methyl) (*p<0.05).

DISCUSSION

Pesticide poisoning is the most widespread occupational hazard for agricultural workers in the developing world, due to the extensive presence of pesticides in the environment. Therefore, the mechanisms of action of these chemicals are still being investigated *in vitro* and *in vivo* (Gaikwad, Karunamoorthy, Kondhalkar, Ambikapathy, & Beerappa, 2015). Investigations which were performed using *in vivo* and *in vitro* comet assays have shown that long-term exposure to OPs is associated with increased DNA damage (Ündeğer & Başaran, 2002; Shadnia et al., 2005; Muniz et al., 2008). The trypan blue exclusion test is commonly used to assess cell viability as a part of *in vitro* genotoxicity studies conducted by comet analysis (Vigreux et al., 1998; Das, Shaik, & Jamil, 2007). In our preliminary study,

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: 0 x 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.

the genotoxic effects of certain carbamates (aminocarb, carbaryl, methiocarb, promecarb and propoxur) were evaluated in Maden-Darby Canine Kidney (MDCK) cell lines and human blood lymphocytes. Pesticides were applied to MDCK cell lines at several concentrations (3, 10, 30 and 100 µg/mL) for 48 hours at 37ºC. The DNA damage of lymphocytes treated with pesticides at 30 µg/mL concentration for 30 min or 16 h were analyzed with a comet assay. The study revealed that the highest damage was observed in cells treated with carbaryl and methiocarb, which showed the correlation with toxicity on MDCK cells. Lower DNA damage levels were determined in MDCK cells treated with pesticides in contrast to lymphocytes. No significant difference was observed in cell growth in the presence of pesticides at 10 µg/mL concentration (data not shown). Therefore, in the present study, we demonstrated the cytotoxic effect of EPN in human peripheral blood lymphocytes following 30 and 120 min exposure at 100 µg/mL concentration by the trypan blue exclusion test. Although chlorpyrifos methyl and azinphos ethyl seem to be safer regarding cytotoxicity compared to other pesticides, significantly higher DNA damage levels were determined after exposure of these pesticides for 120 min at 100 µg/mL concentration by *in vitro* comet assay. The toxicity mechanisms of these pesticides vary according to their chemical structures or their fate in the biological system.

Previous investigations on OPs revealed their potential genotoxic effect; however, the results were inconsistent (Ojha & Srivastava, 2014). The present study showed that chlorpyrifos methyl led to enhanced DNA damage after 30 and 120 min treatment at the highest concentration when compared to the control. This result is in accordance with the findings of other researchers (Rahman, Mahboob, Danadevi, Banu, & Grover, 2002; Mehta, Verma, & Srivastava, 2008; Sandal & Yilmaz, 2011). These authors showed that chlorpyrifos exposure increased DNA damage in the liver and brain tissues of rats in a dose-dependent manner and their leukocytes and lymphocytes. Abuwarda et al. investigated the aneuploidy-inducing effect of chlorpyrifos in human peripheral blood lymphocyte cultures by using the fluorescence *in situ* hybridization (FISH) (Abuwarda, Alashi, & Sharif, 2021). They demonstrated that this compound has shown acceptable levels of cytotoxicity; however, frequencies of aneuploidy, chromosome loss, and chromosome gain were enhanced after exposure. The DNA damage evaluation by the comet assay of chlorpyrifos, as well as reports on bone marrow micronucleus assay of chlorpyrifos in rats, was in agreement with the DNA damage results of chlorpyrifos in lymphocytes (Okonko, Ikpeme, & Udensi, 2016). Jamil et al. (2004) studied the genotoxic effects of organophosphorus (monochrotophos, chlorpyrifos, dimethoate) and organochlorine (endosulfan) pesticides by using the comet assay (Jamil et al., 2004). They reported that two pesticides; monochrotophos, chlorpyrifos were 10 times more toxic than dimethoate.

Azinphos ethyl is highly toxic to mammals and readily absorbed by dermal exposure, inhalation of dust or spray and swallowin (Petroianu, Nurulain, Hasan, Kuca, & Lorke, 2015). There is no evidence of the genotoxicity of azinphos ethyl in human peripheral blood lymphocytes. This study presents the first report about the genotoxicity of azinphos ethyl with the comet assay. Hence, the genotoxic potential of azinphos methyl has been compared to azinphos ethyl, which is the pesticide residue found most commonly in the houses of both farm workers and growers (McCauley et al., 2001). Azinphos methyl and azinphos ethyl are in the same chemical form, both of them have phosphorodithioate linkage. Although azinphos ethyl showed a positive genotoxic effect *in vitro* micronucleus assay in Chinese hamster lung cells, the DNA damaging effects of *in vivo* micronucleus assay in mice were reported to be negative (Ni, Li, Liu, Tang, & Pang, 1993). The lack of consistency in the response between the *in vivo* and *in vitro* cytogenetic tests can be explained by the fact that the two azinphos compounds are metabolized rather quickly *in vivo* before they can induce any genotoxic effects. Kisby et al (2009) showed that cultures of human lymphocytes exposed with azinphos methyl for 24 h induced oxidative DNA damage. DNA damage is assumed to be a main underlying mechanism for the toxicity of pesticides by disrupting the function of cells (Kisby et al., 2009). The long-term exposure of OPs causes enhanced release of cytochrome c from mitochondria to cytosol and the activation of caspase-3 and results in a disrupted cellular antioxidant defense system, which causes DNA damage (Hodgson & Levi, 1996; Kaur, Radotra, Minz, & Gill, 2007; Kisby et al., 2009).

EPN [(O-Ethyl O-(p-nitrophenyl) pheriylphosphonothioate] is a nonsystemic organophosphorus insecticide and acaricide. It is highly toxic to birds and mammals through acute oral exposure (Smith, 1987). EPN was not classified as a human carcinogen, but there is no evidence of the genotoxic effects of EPN. Herein, we present the first report about the genotoxic effect of EPN in human blood lymphocytes. In our study, exposure of EPN at 100 µg/mL concentration for 120 min induces cytotoxicity in human lymphocytes, moreover led to a significant increase in DNA damage.

Carbamates, which are potent cholinesterase inhibitors as organophosphates, constitute another important class of pesticides, which have also been revealed to be mutagenic in various test systems (Proença et al., 2004; Mohanty et al., 2011). Das et al (2007) assessed the damage caused by pesticides (organophosphate, organochlorine and carbamate) and their combinations on humans by cytotoxicity and genotoxicity assays (Das et al, 2007). High doses of certain pesticides (0.5-4.0 µM) produced considerable DNA damage, as evidenced by apparent tail lengths, according to the authors. Carbamate pesticides caused significant DNA damage in lymphocytes. However, besides direct strand breakage, DNA damage may be caused by inhibition of some other metabolic pathways and cell death. Aldicarb is a carbamate used in agriculture as an insecticide and nematicide. It is metabolized to aldicarb sulfoxide and aldicarb sulfone. Aldicarb sulfoxide is a more potent inhibitor of acetylcholinesterase than aldicarb. The genotoxic potential of aldicarb determined by genotoxicity tests (comet assay, SCE and micronucleus assay) has been previously reported (Cid & Matos, 1984; Sun et al., 2010). Sun et al (2010) investigated the genotoxicity of aldicarb and methomyl at different concentrations by micronucleus test, Ames test and comet assay. According to the results of the comet assay, high concentrations of aldicarb were observed to cause DNA damage at different levels in human peripheral blood lymphocytes (Sun et al., 2010). However, studies on genotoxicity caused by aldicarb sulfone are very limited. In our study, DNA damage was observed after treatment with 100 µg/mL of aldicarb sulfone. Venkat et al (1995) evaluated the mutagenic potential of 47 pesticides, including aldicarb sulfone using a modified SOS microplate assay (Venkat et al., 1995). Aldicarb sulfone was found as one of the ten most active pesticides. Canna-Michaelidou&Nicolaou (1996) studied the genotoxic effect of aldicarb sulfone with the Mutatox™ test, both directly and after exogenous activation with the S9 hepatic enzyme (Canna-Michaelidou & Nicolaou, 1996). The genotoxic effect of aldicarb sulfone was categorized as 'suspect genotoxic' both directly and after S9-activation in mutatox. The findings obtained from these studies have demonstrated that aldicarb sulfone significantly damages DNA.

Ethiofencarb, which is used frequently as a systemic insecticide, acts through contact and oral route. It is almost completely absorbed in mammals and excreted rapidly as metabolites, mainly in the urine (Al-Samarraie et al., 2009). There is no evidence of the genotoxic effects of ethiofencarb *in vivo* and *in vitro* mammalian test systems. This is the first data to evaluate that ethiofencarb is not cytotoxic and does not cause DNA damage in a comet assay at studied concentrations and incubation times. Further studies are required to better understand the genotoxic effects of ethiofencarb.

CONCLUSION

OPs and carbamate pesticides cause significant DNA damage in lymphocytes by inhibition of some other metabolic pathways and cell death. Genotoxic damage is considered a relevant biomarker for carcinogenic risk. These widely used agricultural pesticides should be handled cautiously since low-level, long-term exposure to pesticides can lead to genotoxic risk and adverse health effects like cancer. Further investigations on genotoxic thresholds and susceptibility to pesticide-related pathologies in human populations are required.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- E.F.İ.D., T.Y.; Data Acquisition- A E.F.İ.D., T.Y., S.K.; Data Analysis/Interpretation- E.F.İ.D., T.Y., S.K, S.Y.; Drafting Manuscript- E.F.İ.D., T.Y., S.K, S.Y.; Critical Revision of Manuscript- T.Y., S.K, S.Y.; Final Approval and Accountability- E.F.İ.D., T.Y., S.K, S.Y.

Conflict of Interest: The authors have no conflict of interest to declare.

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Ethics Committee Approval: The blood samples were collected from authors of the study (S.K. and E.F.İ.D.). The study was carried out between February 2008 and May 2009 prior to the publication of the Regulation on Clinical Trials on August 19, 2011 (https://www.resmigazete.gov.tr/eskiler/2011/08/20110819-9.htm). As a result, even though being conducted in accordance with the Helsinki Declaration, the study protocol was unable to obtain approval from an Ethics Committee.

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