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Genotoxic effects of diazinon on human peripheral blood lymphocytes

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The aim of this study was to evaluate the genetic damage in human peripheral blood lymphocytes following 24 and 48-hour exposure to a commercial diazinon formulation Basudin 60EM® at concentrations between 0.01 and 40 μg mL^{-1}. For this purpose we used the micronucleus (MN), fluorescence *in situ* hybridization (FISH), and alkaline single cell gel electrophoresis (comet) assay. Diazinon significantly increased the frequency of micronucleated cells compared to control. Forty-eight-hour exposure increased this frequency even at lower concentrations (0.01-10 μg mL^{-1}). The FISH results revealed aneugenic effects at 10 μg mL^{-1}. The comet assay also confirmed DNA damage at concentrations between 10 and 40 μg mL^{-1}. Our findings have confirmed the genotoxic potential of diazinon and its cytotoxic effect on human lymphocytes. The increased DNA damage in our study raises concern about the current assessment of the health risk posed by this pesticide and calls for a high level of caution in agricultural and household use.

KEY WORDS: *comet assay; FISH; genotoxicity; micronucleus; organophosphate pesticides*

Organophosphorus pesticides, which are commonly used in agriculture, are known neurotoxic agents. Being cholinesterase inhibitors, they are less persistent but also more toxic to mammals than organochlorine pesticides (1). Diazinon (0,0-diethyl-0-(2-isopropyl-4-methyl-6-pyrimidinyl phosphorothionate) is a thionophosphorous organophosphate pesticide used to control a variety of insects in agriculture and domestic settings (2). Most studies have confirmed the genotoxic and cytotoxic effects of diazinon on animals. For example, it had a cytogenetic effect on bone marrow cells of mice (3) and a clastogenic and probably genotoxic effect on humans exposed to the insecticide (4).

Some *in vivo* and *in vitro* studies of diazinon reported controversial results. It increased the frequency of sister chromatid exchange and decreased replicative indices, suggesting toxic and genotoxic effects *in vitro* (5, 6). In a Chinese hamster cell line V79, it had cytotoxic effects but did not induce sister chromatid exchange (7). It also failed to increase chromosome aberrations or sister chromatid exchange in cell cultures (1, 8). In cultured human lymphocytes diazinon (0.04 μg mL^{-1}) induced a weak increase in the number of micronucleated cells (9). However, in another study of cultured human blood cells, it increased the frequency of micronuclei and inhibited cell proliferation (10). Contradictory findings obtained so far highlight the need to use different assays and different test materials for diazinon genotoxicity studies.

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Commercial formulations are often more toxic than the pure pesticide compound, as they contain surface active ingredients, dyes, stabilisers, activity enhancers, and organic solvents with unknown or poorly characterised toxicity. Yet, only a few studies have investigated the effects of commercial formulations of diazinon. One has found that commercial formulations of insecticides could be toxic and harmful to the developing embryo and *in vitro* fertilisation (11). Another has shown that diazinon is toxic to mammalian spermatogenic cells (12).

The genotoxic potential of commercial formulations of diazinon in cultured human lymphocytes has not yet been investigated. With our study we wanted to fill this gap and learn more about the mechanisms involved in the cytotoxic and genotoxic action of commercial diazinon in peripheral blood lymphocytes of healthy human volunteers. To do that, we used the micronucleus (MN), comet, and the fluorescence *in situ* hybridisation (FISH) assay.

MATERIALS AND METHODS

Test chemicals

We used a commercial formulation obtained from a local market (Basudin 60EM®, Syngenta, Basel, Switzerland) containing 630 g of diazinon per litre of the product. The test substance was prepared in sterile double-distilled water. The concentrations were selected based on our preliminary study whose aim was to determine mitotic index inhibition concentrations.

Mitomycin C (cat. no.: 50-07-7; Sigma, Steinheim, Germany) was used at a concentration of 0.1 μg mL^{-1} as positive control in the MN assay. Vinblastine sulphate (0.1 μmol L^{-1}, cat. no.: 143-67-9; MP Biomedicals, California, USA) was used as aneugenic agent in the FISH assay, and hydrogen peroxide (H_2O_2; 20 μg mL^{-}1, cat. no.: 7722-84-1; Sigma) was used as positive control in the comet assay.

Blood sampling and lymphocyte culturing

Samples of venous blood were collected from four healthy, non-smoking volunteers, two women and two men, aged between 20 and 25 years, with no history of pesticide exposure. The donors were informed about the study and signed the consent form allowing the use of their blood samples. The investigation observed the ethical standards laid down by the Declaration of Helsinki.

Blood lymphocytes were cultured at 37 °C for 70 h by adding 0.3 mL of whole blood to 4.7 mL of Ham's F-10 medium (Sigma) supplemented with 20 % foetal bovine serum (Sigma), 2 % phytohaemagglutinin (Gibco, Paisley, UK), and antibiotics (penicillin at the concentration of 100 IU mL^{-1} and streptomycin at 100 μg mL^{-1}) (IE Ulagay, Istanbul, Turkey.)

MN assay

The cytokinesis-block micronucleus (CBMN) assay is used to detect early biological effects of DNA-damaging and spindle-damaging compounds (13). Combined with hybridisation and general or chromosome-specific centromeric/telomeric probes, CBMN can identify the mechanisms most responsible for micronucleation.

To block cytokinesis, we added cytochalasin B (cat. no. 14930-96-2, Sigma) at the final concentration of 6 μg mL^{-1} 44 h after the cultures were started. Diazinon was added to the cultures in the concentrations of 0.01, 0.1, 1, 5, or 10 μg mL^{-1} on hours 22 and 46 for 48 and 24 h, respectively. The CBMN assay followed the method described by Fenech (14). We scored the number of micronuclei in 1500 binucleated cells per donor (a total of 6000 binucleated cells per concentration and treatment period). To determine lymphocyte proliferation, we analysed 500 cells for each concentration per donor (a total of 2000 cells per concentration and treatment period). The nuclear division index (NDI) was calculated as follows: NDI= (M1+2M2+3M3+4M4)/n. M1-M4 indicates the number of nuclei per cell from, and *n* is the total number of cells recorded (15).

FISH assay

We used the FISH assay with a centromere-specific α-satellite DNA probe (PanCentromeric, DiaGen, Ankara, Turkey) to distinguish the micronuclei produced by clastogens from those produced by aneugens. The FISH protocol was based on a procedure described elsewhere (16). The cells used for FISH were treated with diazinon at the concentrations of 5 and 10 μg mL^{-1} for 48 h. The slides were prepared in line with the protocol for the CBMN assay and stored at -20 °C for later analysis. After pre-treatment with RNase, HCl, and pepsin, the slides and the probe were denatured by baking on a hot block at 70 °C, then hybridised at 37 °C overnight, and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (DiaGen). For analysis we used a fluorescence microscope (Olympus BX51, Tokyo, Japan) with 360 nm, 460 nm, and 510 nm excitation filters. Micronuclei were scored as centromere-positive when the brightness of the pancentromere probe signal in the micronucleus was comparable with the spots in the nucleus. The scores are expressed as percentage. Only the cells with micronuclei showing bright fluorescent spots were classified as centromere-positive, and the rest were classified as centromere-negative micronuclei. Fifty micronuclei were scored for positive or negative centromeres for each diazinon concentration and centromere-positive micronuclei expressed as percentage.

Comet assay

Lymphocytes were isolated from 5 mL of heparinised blood using the Ficoll-Hypaque (Sigma) density gradients and washed with the RPMI 1640 medium (Sigma). The concentration of cells in the medium was about 2×10^5 mL^{-1}. Before the experiment started, we checked the lymphocytes for viability using the Trypan blue dye (Sigma). The test was continued if cell viability was more than 90 %. As in our preliminary study we found no DNA damage at concentrations below 5 μg mL^{-1}, the lymphocytes were exposed to the final diazinon concentrations of 5, 10, 20, and 40 μg mL^{-1} at 37 °C for 0.5 h. The cells were washed twice with RPMI 1640 and re-suspended in the medium.

The comet assay followed the procedure described by Singh et al. (17) and the slides were prepared as described by Bajpayee et al. (18). They were stained with ethidium bromide (20 μg mL^{-1}) for 5 min and observed with a fluorescence microscope (Olympus BX51) using a 510 nm excitation filter. Images of 100 randomly selected comets (50 cells from each of two replicate slides) were analysed from each sample, and the tail length, tail intensity, and tail moment measured on screen using the Comet Score computer-based image analysis system (TriTek Corp., Sumerduck, VA, USA).

Statistics

The statistical analyses (IBM SPSS Statistics for Windows, version 22.0. IBM, Armonk, NY, USA) included all data from the four donors. The frequency of binucleated micronuclei in the MN assay (BNMN‰) and of the percentage of centromere-positive (C+) micronuclei in the FISH assay were compared to control using Fisher's exact test. NDI was analysed using the chi-square test. Pearson correlation was used to correlate NDI, BNMN‰, and

concentrations. The results of the comet assay were analysed with the one-way analysis of variance (ANOVA) and the *post hoc* analysis of differences between the groups with the least significant difference test. All differences equalling and below 0.05 were considered significant.

RESULTS AND DISCUSSION

Compared to controls, 24-hour exposure to diazinon significantly increased micronucleus frequency at concentrations of 1, 5, and 10 μg mL^{-1}, and 48-hour exposure significantly increased the frequency at the concentrations of 0.01, 0.1, 1, 5, and 10 μg mL^{-1} (Table 1). The insecticide also significantly decreased NDI as the concentrations increased (Pearson correlation, p=0.038) (Figure 2). Correlations were also observed between BNMN‰ and NDI following 24 (Pearson correlation, p=0.006) and 48-hour exposure (p=0.024) (Figure 1). Diazinon concentrations and BNMN‰ did not correlate.

These correlations point to the genotoxic and cytotoxic activity of the pesticide. An earlier study of human lymphocyte chromosomes in cell culture (19) reported that diazinon did not significantly increase the percentage of chromosomal aberrations but that it was clearly cytotoxic, as indicated by reduced mitotic indices. This result might be explained by the cytotoxic effect masking chromosome aberrations. Another study indicated that the percentage of metaphase cells with structural aberrations decreased in a dose-dependent manner in human lymphocyte cultures (1). All these findings are in line with ours. The cytotoxic effects of diazinon did not necessarily cause chromosome breaks but could lead to spindle disturbance.

The novelty of our study are the FISH assay findings. Diazinon exposure at the concentrations of 5 and 10 μg mL^{-1} has demonstrated that the insecticide has aneugenic effects (centromere-positive micronuclei; see Table 2). Some organophosphates with mechanisms of action similar to diazinon such as trichlorfon have been shown to induce aneuploidy *in vivo* (20). Sun et al. (21) have reported that trichlorfon is a potent spindle poison in V79 cells and that it induces aneuploidy in mouse spermatocytes. Some organophosphate pesticides also induced aneuploidy in *in vivo* studies with pesticide sprayers (22, 23). Using the comet assay, Karzmer reported carbofuran-induced DNA damage in human peripheral blood lymphocytes (24), whereas Želježić et al. (25) reported aneugenic effects, established by the FISH assay, as the frequency of micronuclei and C+ positive micronuclei increased.

Diazinon-induced damage observed in our study might be related to its cytotoxic and apoptotic action. Table 3 shows the amount of DNA breakage (tail length), percentage of DNA (tail intensity), and DNA migrated in the tail (tail moment) determined with the comet assay. Tail length and tail moment significantly increased at the concentrations of 10 and 40 μg mL^{-1}. Tail intensities significantly increased at all concentrations compared to negative control (Figure 2), demonstrating the DNA-damaging potential of diazinon. These findings support earlier reports on diazinon-related DNA effects (26-28) and point to two main mechanisms of diazinon action on the cell level: genotoxicity, which was established by the comet assay and aneugenicity, which was

Table 1 *Micronucleus (MN) frequency and the nuclear division index (NDI) in human lymphocytes exposed to diazinon (total values of four donors)*

Exposure period	Concentration (μg mL^{-1})	BNMN‰±SE	BN%	NDI±SE
24 h	(-)Control Distilled water	4.98±0.28	24.3	1.35±0.035
	(+) Control MMC	***65.5±3.38	8.3	***1.08±0.01
	0.01	8.44±1.76	21.9	1.31±0.02
	0.1	8.85±1.15	21.4	**1.26±0.03
	1	*13.34±1.53	18.0	***1.23±0.03
	5	*13.78±0.88	12.6	***1.16±0.04
	10	*13.76±1.75	12.4	***1.15±0.04
48 h	(-)Control Distilled water	2.88±0.58	22.8	1.30±0.01
	(+) Control MMC	***196.69±14.1	2.4	***1.02±0.01
	0.01	*11.74±2.85	21.0	1.26±0.01
	0.1	*10.44±0.76	18.4	*1.23±0.01
	1	*11.50±2.09	14.7	***1.18±0.02
	5	**14.45±1.74	10.4	***1.14±0.04
	10	**15.46±0.76	9.6	***1.12±0.02

BNMN-binucleated cells with a micronucleus; BN% - percentage of binucleated cells; BNMN‰ - mean number of micronuclei per 1000 binucleated cells; SE - standard error
*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (Fisher's exact test for BNMN‰ and chi-square test for NDI)*

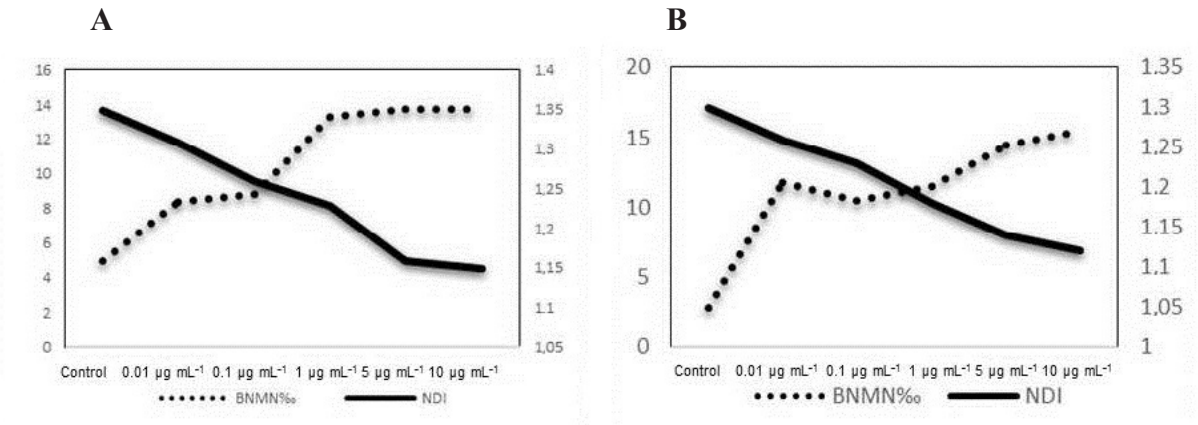

Figure 1 *Correlations between the frequency of binucleated micronucleated lymphocytes (BNMN‰) and the nuclear division index (NDI) after 24 h (A) and 48 h (B) of exposure (Pearson correlation,* p*=0.006 and* p*=0.024, respectively). The figure also shows the correlations between NDI and diazinon concentrations after 24 h (A) and 48 h (B) of exposure (Pearson correlation,* p*=0.038)*

Table 2 *The number and percentage of centromere-positive (C+) and centromere-negative (C-) micronuclei after fluorescence in situ hybridization (FISH)*

Chemicals and concentrations	MN (C-)	MN (C+)	% MN (C+)
Negative control	12.6±2.3	25.6±2.3	69.3±0.6
0.1 µmol L^{-1} vinblastin sulphate	9±1.4	41±1.4	**82±2.8
5 µg mL^{-1} diazinon	14.3±2.6	31.3±5.7	68.7±1.6
10 µg mL^{-1} diazinon	7.6±3.6	29±3.5	*81±5.5

*p≤*0.05; **p≤0.01*
data are given as mean±SE

Table 3 *Tail length, intensity, and moment comparison between the treated groups and negative control*

Sample	Tail length	Tail intensity	Tail moment
Negative control	0.997	1.000	1.000
Positive control (20 µg mL^{-1} H_2O_2)	***0.001	**0.002	**0.002
5 µg mL^{-1} diazinon	-0.357	-0.234	-0.592
10 µg mL^{-1} diazinon	***0.000	***0.001	**0.004
20 µg mL^{-1} diazinon	-0.993	**0.003	-0.329
40 µg mL^{-1} diazinon	***0.000	***0.000	***0.000

*p≤*0.05; **p≤0.01; ***p≤0.001*

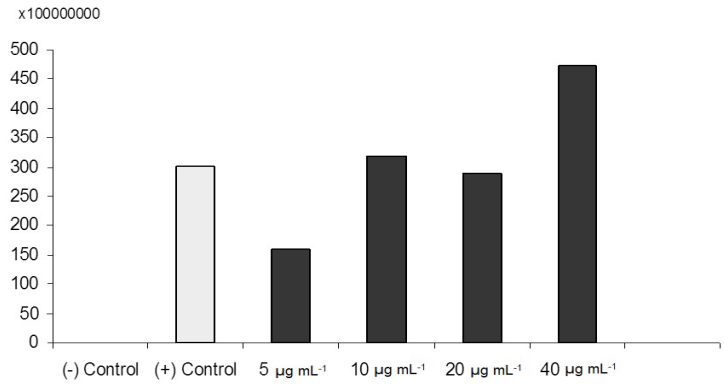

Figure 2 *Tail intensities by groups. All treated groups significantly differed from negative control*

established by the MN and FISH assays. Both mechanisms contribute to the diazinon's cytotoxic activity, possibly by triggering apoptosis. Further research should investigate the exact mechanism of diazinon action using other test methods.

The increased DNA damage in peripheral lymphocytes in our study raises concern about the current assessment of the health risk posed by this pesticide and calls for a high level of caution in agricultural and household use.

Conflict of interest statement

We declare that no conflict of interest exists in relation to this study or this article.

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Genotoksično djelovanje diazinona na limfocite ljudske periferne krvi

Cilj je ovog istraživanja bio ocijeniti genetička oštećenja u limfocitima ljudske periferne krvi nakon 24-satne odnosno 48-satne izloženosti komercijalnom diazinonu (Basudin 60EM®) u rasponu koncentracija od 0,01 do 40 μg mL^{-1}. U tu smo svrhu rabili mikronukleus (MN)-test, fluorescencijsku *in situ* hibridizaciju (FISH) i metodu elektroforeze pojedinačnih stanica u agaroznom gelu (tzv. komet-test). Diazinon je značajno povećao učestalost stanica s mikronukleusima u odnosu na kontrolu. Taj je učinak bio još izraženiji nakon 48-satne izloženosti, gdje je značajno povećanje zamijećeno već pri koncentracijama diazinona od 0,01 do 10 μg mL^{-1}. FISH je pokazao aneugeno djelovanje diazinona u koncentranciji od 10 μg mL^{-1}. Naši rezultati potvrdili su ranija saznanja o genotoksičnom i citotoksičnom djelovanju diazonona na ljudske limfocite. Oštećenje DNA koje smo zamijetili u našem istraživanju dovodi u pitanje trenutačne procjene zdravstvenih rizika povezanih s tim pesticidom te poziva na izrazit oprez pri njegovoj primjeni u poljoprivredi i kućanstvima.

KLJUČNE RIJEČI: *FISH; genotoksičnost; komet-test; mikronukleus; organofosforni pesticidi*