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GENOTOXICITY OF THE ORGANOPHOSPHORUS COMPOUND METHYLPARAOXON EVALUATED BY THE COMET ASSAY

Pesticides are widely used in agriculture, food production and household. Due to the broad spectrum of their activity, their biological action is extensively investigated. We assessed the genotoxicity of methylparaoxon – the main metabolite of commonly used organophosphorus insecticide, methylparathion. Freshly isolated human peripheral blood lymphocytes were incubated for 1 h with methylparaoxon at 37° C, and then, were incubated in methylparaoxon-free medium to examine DNA repair. Alkaline single cell gel electrophoresis (the comet assay) was used to assess DNA damage and repair. Methylparaoxon induced moderate DNA damages that were almost completely repaired after 60 min. Obtained results suggest that methylparaoxon could induce single-strand breaks in DNA both directly and by methylathion of DNA bases.

1. INTRODUCTION

Organophosphorus insecticides are commonly used pesticides due to their high toxicity to insect and little or no hazard to mammals. However, some reports suggest that they can affect also man and useful animals. Genotoxicity of those compounds is of a special significance due to long latent period between the exposure and the effect becoming apparent. It was reported that organophosphorus compounds could induce chromosomal aberrations [6], sister chromatid exchanges [7], micronuclei [18], disturbances in DNA synthesis [20].

Methylparaoxon is the main metabolite of commonly used organophosphorus insecticide, methylparathion [2]. Its acute toxicity is due to the phosphorylathion of serine residues of the active center of acetylcholinesterase that results in build up of acetylcholine and death of affected animal. Methylparaoxon could be found in commercial formulations of methylparathion. It was reported that methylparathion could induce DNA strand

breaks [8], produce micronuclei in bone marrow cells [9], exert a genotoxic effect to bacteria [5].

Single cell gel electrophoresis (the comet assay) is a fast and sensitive method of DNA damage detection in individual cells [13, 15, 17]. It requires small number of cells.

In the present work the ability of methylparaoxon to induce DNA damage in human peripheral blood lymphocytes as well as DNA repair were evaluated by the comet assay.

2. MATERIALS AND METHODS

2.1. Chemicals

Methylparaoxon (*O,O*-dimethyl *O*-4-nitrophenyl phosphate) (Fig. 1) at purity at least 95% was purchased from Institute of Organic Chemistry (Warsaw, Poland). RPMI 1640 medium without glutamine, low melting point agarose and DAPI (4',6-diamidino-2-phenylindole) were obtained from Sigma (S. Louis, MO, USA). Gradisol L was from Polfa (Kutno, Poland).

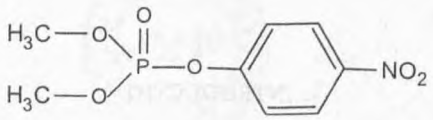

Fig. 1. Chemical structure of methylparaoxon

All other chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO, USA).

2.2. Lymphocytes isolation

Blood was obtained from young, healthy, non-smoking donors. Peripheral blood lymphocytes (PBL) were isolated by centrifugation in density gradient of Gradisol L [10]. The viability of the cells was measured by the Trypan blue exclusion [10] and it was found to be about 99%. Lymphocytes was about 92% of leukocytes in obtained cell suspension. The final concentration of the lymphocytes was adjusted to $1–3 \times 10^5$ cells/ml by adding RPMI 1640 medium to the single cell suspension.

2.3. Lymphocytes exposure to methylparaoxon

Methylparaoxon was derived from stock (50 mM) ethanolic solution and added to the suspension of lymphocytes to give final concentrations of 150 mM. The solubility of the chemical was checked spectrophotometrically. The control received, instead of the organophosphate, ethanol, at a concentration (0.29%) not affecting the processes under study [16]. To examine DNA damage the lymphocytes were incubated with the chemicals for 1 h at 37° C.

2.4. DNA repair

To examine DNA repair the lymphocytes after treatment as well as control samples were washed and incubated in fresh, methylparaoxon-free RPMI 1640 medium at 37° C. The repair incubation was stopped by placing the samples in an ice-bath and then samples were centrifuged.

2.5. Single cell gel electrophoresis

The comet assay was performed under alkaline conditions essentially following the procedure of Singh et al. [17] with slight modifications. A freshly prepared suspension of PBL in 0.75% low melting point agarose dissolved in PBS was casted to fully frosted microscope slides (Superior, Germany) precoated with 0.5% agarose. The cells were then lysed for 1 h at 4° C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X–100, 10 mM Tris, pH 10. After the lysis the slides were placed in an electrophoresis unit, allowing DNA to unwind for 40 min in the electrophoretic buffer consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at ambient temperature of 4° C (the temperature of running buffer not exceeding 10° C) for 30 min at electric field strength 1.4 V/cm (600 mA). The slides were then neutralised with neutralising buffer (0.4 M Tris, pH 7.5), rinsed with destilled water and dried on the air. After this steep, they were stained with 1 μg/ml DAPI and covered with cover slips. To prevent an additional damage all the steps described above were conducted under dimmed light or in the dark.

2.6. Comet analysis

Comets were observed at 200 × magnification in a Optiphot–2 fluorescence microscope (Nikon, Japan) attached to a Pulnix video camera (Kinetic Imaging Ldt., Liverpool, UK) connected to a personal computer-based

image analysis system Komet v. 3.0 (Kinetic Imaging Ldt). Fifty images were randomly selected for each sample and the tail moments were measured.

The tail moment is positively correlated with the level of DNA damage in a cell [14]. Because the distribution of the comets was heterogeneous, histograms were applied to display information. The mean value of tail moment in a particular sample was taken as an index of DNA lesion in the sample.

2.7. Data analysis

All the values in this study were expressed as mean $\pm$SEM. The statistically significant differences between variations were found (Snedecor-Fisher test) so the differences between means were assessed by applying Cochran-Cox test.

3. RESULTS

3.1. DNA damage

The mean tail moments for the lymphocytes exposured to methylparaoxon (without repair incubation) are presented in Table 1. Comets of lymphocytes treated with this organophosphorus compound were shown in Fig. 2. It can be seen from Table that methylparaoxon caused significant increase of cell DNA migration from the comet head into the tail ($p < 0.001$).

Figure 3 shows the results displayed in the form of histograms. Such presenting of data may provide additional information [1]. From this figure one can see that more pronounced damage of DNA in lymphocytes treated with methylparaoxon can be conected with the presence of cell population with more number of lesions of genetic material than most cells. On the other hand there was a lymphocyte population whose DNA was damaged like controls cells. In the case cells incubated with the compound under study the reason of more standard error of mean (Table 1 and Fig. 4) was a cell population with strong lesions.

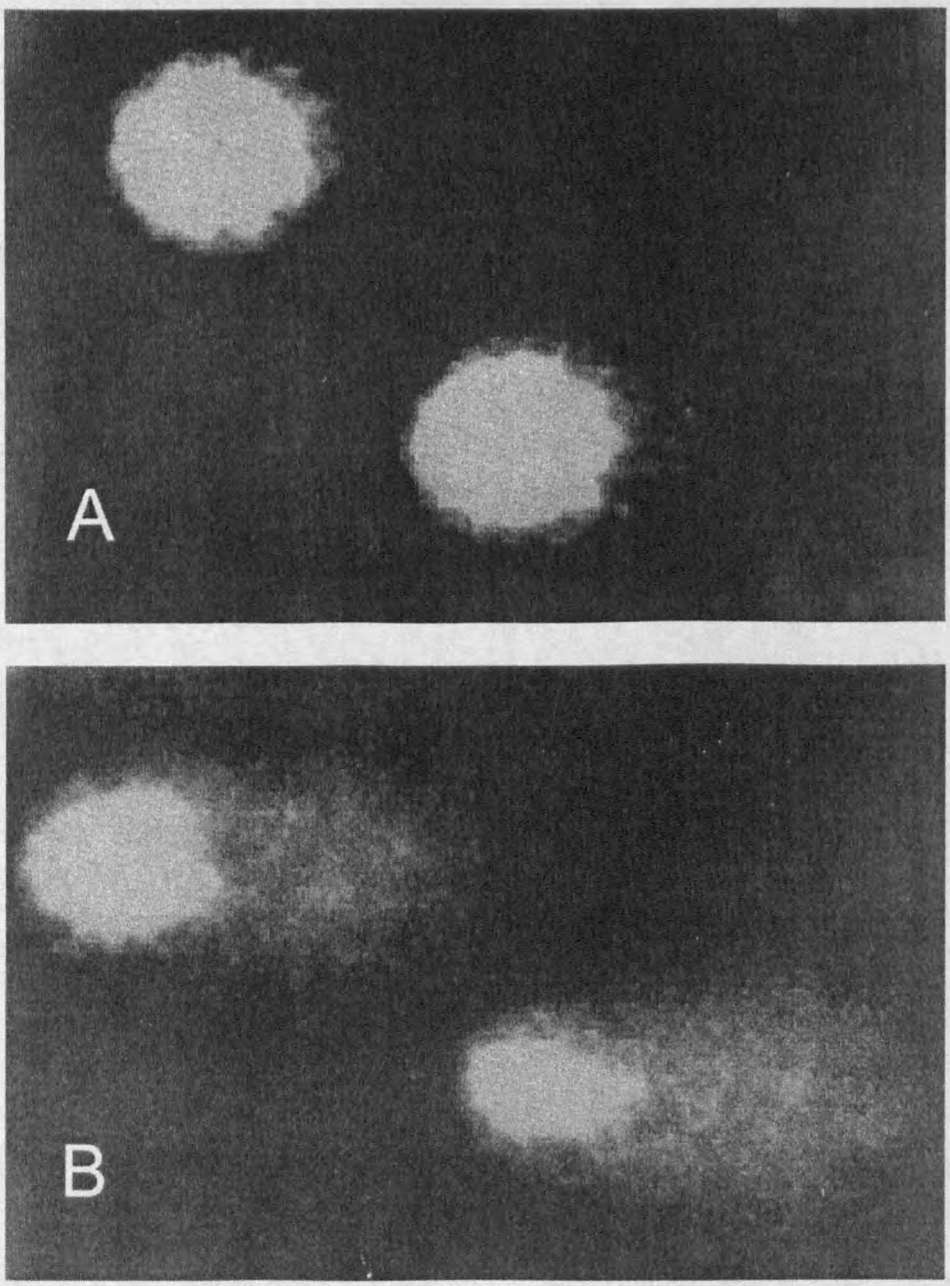

Fig. 2. Typical flourescence microscope images of the DAPI-stained DNA of human lymphocytes exposured to methylparation at the concentration of 50 μM (B) as compared with the control (A)

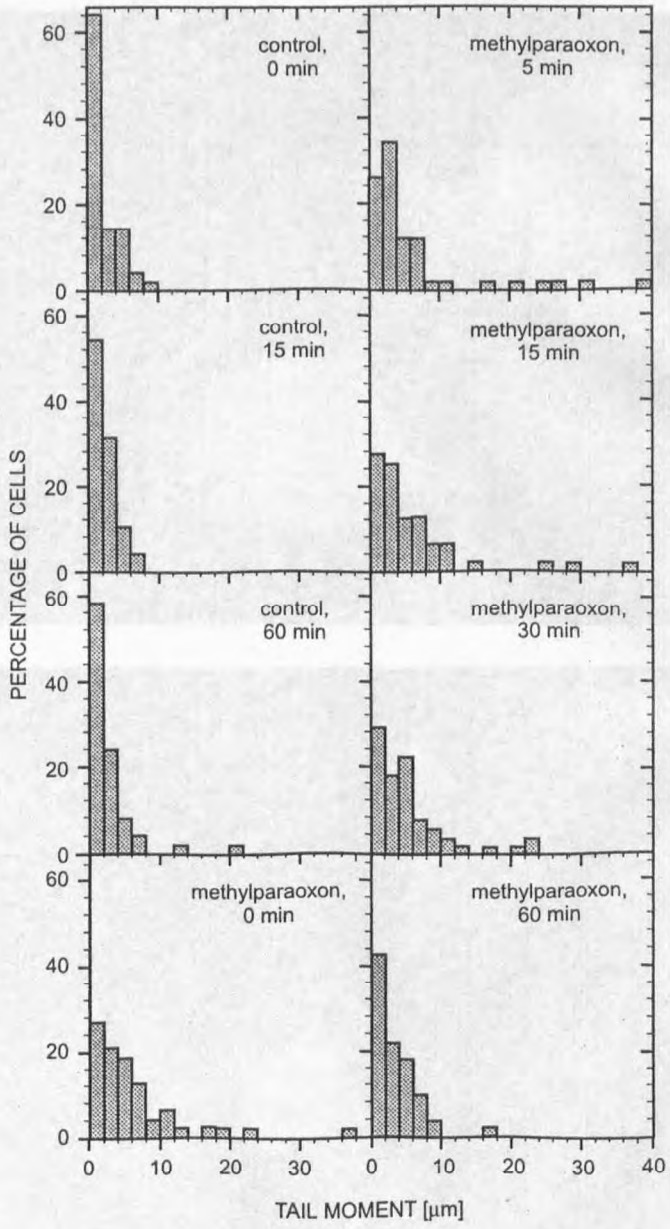

Fig. 3. Histograms of the distribution of tail moment in human lymphocytes treated with methylparaoxon at 50 μM for 1 h. The repair incubation time is indicated in each panel. The number of cells scored for each treatment was 50

Table 1

Tail moment of the lymphocytes after exposure to methylparathion. Results were expressed as mean ± SEM, $n = 50$

Concentration of methylparaoxon [μM]	Tail moment [μm]
0	2.23 ± 0.29
50	6.10 ± 0.97*

* $p < 0.001$

3.2. DNA repair

Figure 3 shows the tail moments for the lymphocytes after incubation with methylparaoxon and the repair incubation which were performed in variable period of time (0, 5, 15, 30 and 60 min). In the case of treated lymphocytes, set of highly damaged cells decreased during repair incubation. In Fig. 4, one it can observe the same process as decreasing mean and SEM of tail moment. At the period of incubation of 60 min repair of methylparaoxon-induced damages was almost complete.

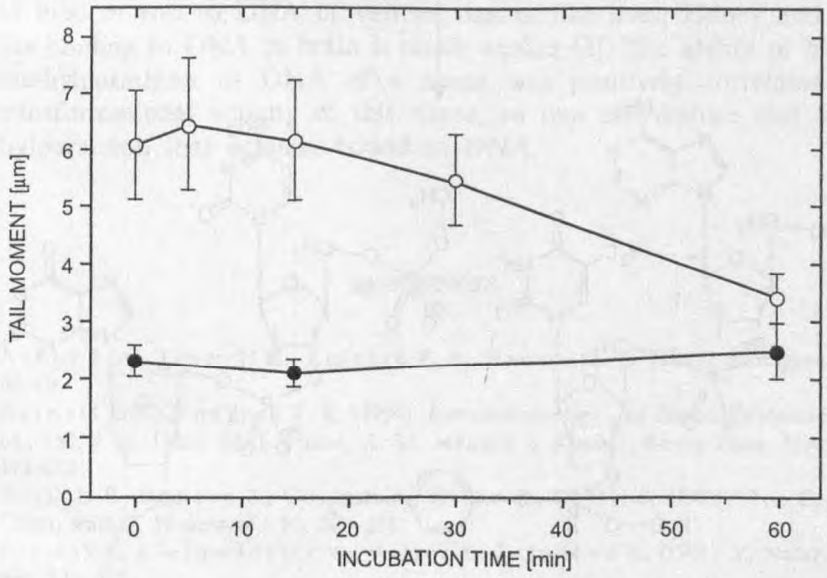

Fig. 4. The time course of the repair of DNA damage in human lymphocytes treated with methylparaoxon at 50 μM (open circles) as compared with controls (closed circles). The number of cells scored for each treatment was 10. Error bars denote SD

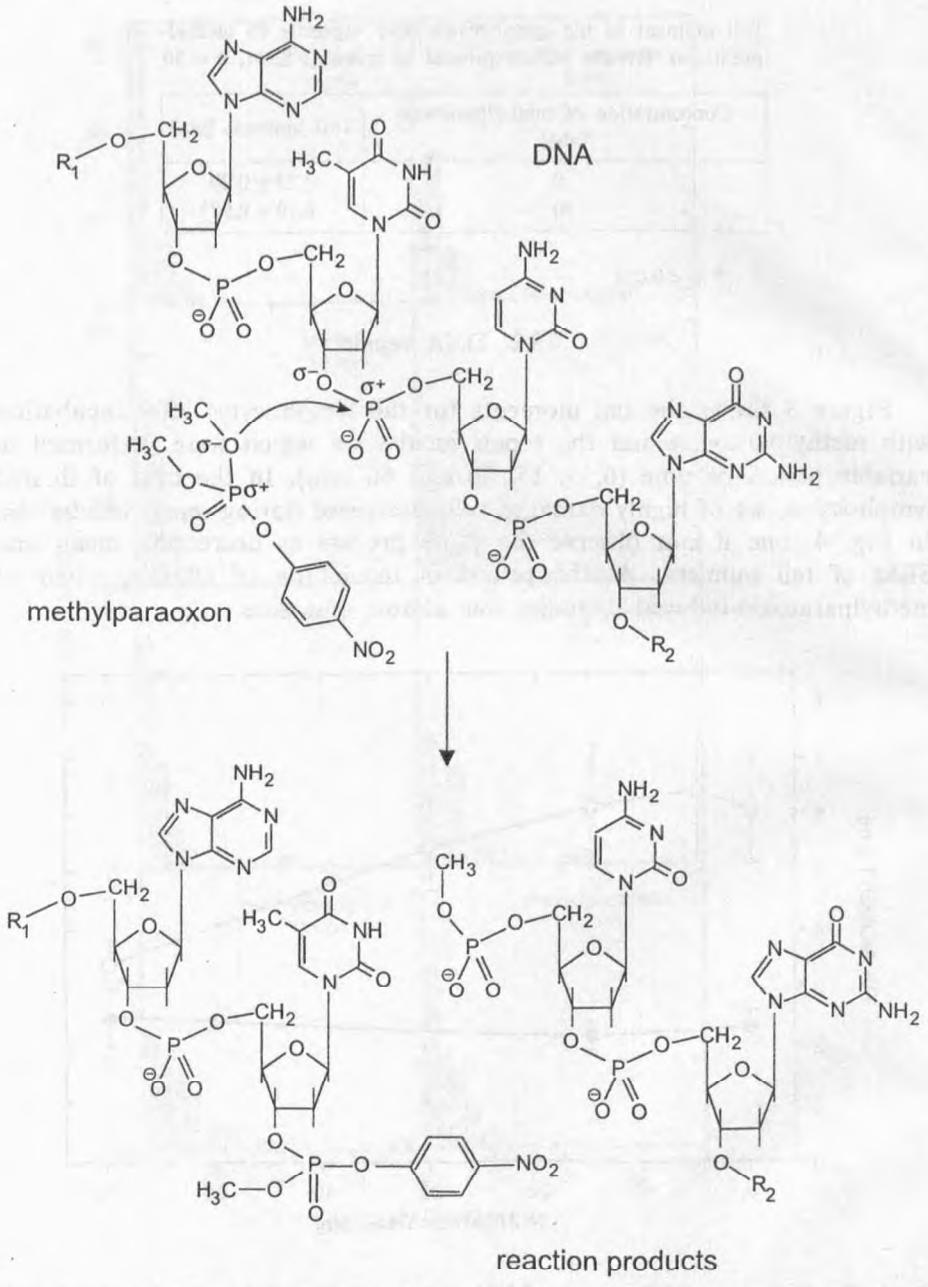

Fig. 5. Hipotetical model of the interaction of methylparaoxon with DNA

4. DISCUSSION

Obtained results suggest that methylparaoxon can induce strand breaks in DNA of human peripheral blood lymphocytes. Strand breaks could be induced either directly, by phosphodiester bond nicking. On the other hand, methylparaoxon, containg methyl groups, has a potential to methylate DNA bases. The process of methylathion may be followed by apurination or/and apirimidination as consequences of the instability of *N*-glycosidic bond and the excision of mismatched bases. These processes can underlie the indirect mechanism of strand nicking by methylparaoxon. It was reported that methylparathion could methylate DNA bases *in vitro*, suggesting that the major methylation product was N^7-methylguanine [19]. It is likely, that such ability may be attributed also to methylparaoxon.

The direct nicking of methylparaoxon can be a consequence of the presence of the electrophilic nitro group, that can decrease the electron density of aromatic ring in its *orto* and *para* configuration. The latter situation could let to a induction additional positive charge on the phosphorus atom. This could facilitate the interaction of methylparaoxon with DNA and nicking phosphodiester chain.

Methylparathion can change the thermodynamic properties of DNA [4]. Using an isotope labelling technique it was shown that methylparathion could bind *in vivo* to DNA of various tissues, like liver, kidney and lung, but its binding to DNA in brain is much weaker [3]. The ability of binding of methylparathion to DNA of a tissue was positively correlated with biotransformational activity of this tissue, so one can deduce that it was methylparaoxon that actually bound to DNA.

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