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Original Research

Single Cell Gel Electrophoresis (Comet Assay) as a Tool for Environmental Biomonitoring. An Example of Pesticides

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Abstract

Methods that permit the sensitive detection of DNA damage and repair are of special significance in the field of environmental research due to the long latent period between exposure to environmental agent(s) and genetic effect(s) becoming apparent. Malathion is a commonly used organophosphorus insecticide reported to be genotoxic both *in vivo* and *in vitro,* but the reports are conflicting. In order to elucidate the genotoxic potency of a compound present in commercial preparations of malathion, the DNA damaging effect of the insecticide and its isomer isomalathion was investigated using alkaline single cell gel electrophoresis (comet assay). Freshly isolated human peripheral blood lymphocytes were incubated with 75 and 200 μM of the chemicals for 1 h. The concentrations from the range we used are relevant to that in blood following various nonlethal human exposure to pesticides. Malathion caused no significant changes in the comet length of the lymphocytes throughout the range of concentrations tested. Isomalathion introduced damage to DNA in a dose-dependent manner. Treated cells were able to recover within 60 min incubation in insecticide-free medium at 37ºC. The reported genotoxicity of malathion might, therefore, be a consequence of its thermal and/or photochemical conversion to isomalathion and the presence of isomalathion as well as its oxidation products and other unspecified impurities in commercial formulations of malathion. In this regard, the results of our study indicate that malathion used as commercial product, i. e. containing isomalathion, can be considered as a genotoxic substance *in vitro.* This means that it can produce DNA disturbances *in vivo.* Therefore, malathion can be regarded as a potential mutagen/carcinogen and requires further investigation.

Keywords: Comet assay, DNA damage, pesticides, malathion, isomalathion.

Introduction

Techniques that permit the sensitive detection of DNA damage and repair are critically important in the field of environmental research. DNA damage caused by environmental agents is often tissue- and cell-type specific so the technique dealing with individual cells seems to be an optimal one. The single cell gel electrophoresis technique (comet assay) detects migration of DNA from individual cell nuclei following alkaline treatment [1, 2]. This technique is especially sensitive in detecting DNA double- and single-strand breaks, alkali-labile damage, and excision repair sites in individual cells. It can be applied to virtually any eukaryotic cell population that can be obtained as a single-cell suspension; it only requires extremely small cell samples (from 1 to 10,000 cells) and results can be obtained in a single day. Additionally, the cost of performing the assay is reasonably economical.

In this technique a small number of cells suspended in a thin agarose gel on a microscope slide is lysed, electrophoresed and stained with a fluorescent DNA-binding dye. The basis of the method is that broken DNA molecules can migrate more readily in an electric field than intact molecules. When cells are embedded in agarose and subsequently

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lysed to remove proteins, smaller DNA molecules are able to migrate away from the residual nucleus. When DNA is subsequently stained with a fluorescent DNA-binding dye like ethidium bromide, propidium iodide or DAPI and viewed using a fluorescence microscope, the observed objects resemble comets with a head region containing undamaged DNA and a tail containing broken DNA. The amount of DNA able to migrate and, to a lesser extent, the distance of migration, are indications of the number of strand breaks present in that cell. Cells with increased DNA damage display an increased migration of chromosomal DNA from the nucleus towards the anode. In the alkaline version of the comet assay, DNA single strand breaks and alkali labile sites become apparent, and the extent of DNA migration indicates the level of DNA breakage in the cell [3]. It has been shown that the comet assay is able to detect a broad spectrum of mutagens [4].

The comet assay, because of its simplicity, sensitivity and need for only small numbers of cells, was suggested as an ideal technique for assessing the possible environmental consequences of hazardous waste pollution by the assessment of genotoxic damage in sentintel organisms [5]. The comet assay was used to examine the extent of DNA damage in coelomocytes collected from earthworms maintained in different soil samples as an indicator of soil pollution [6]. It was also used with erythrocytes of bullheads, a species of bottom feeding fish, collected from various regions of Lake Erie as an indicator of polluted sediment and in the presence of genotoxic pollutants [7].

Malathion is a widely used organophosphorus insecticide because of its relatively low toxicity to mammals and high selectivity compared to other organophosphorus insecticides. The selectivity of malathion is due primarily to mammals having a high level of carboxyesterases, the enzymes that can hydrolyze malathion and its metabolites to non-toxic intermediates that can be easily eliminated from cells. Insects lack or have a low level of these esterases, so they are severely affected by malathion. It was employed in major eradication programs against insect infestations in metropolitan areas of Florida, Texas and California [8]. The aerial application of malathion over large urban areas in southern California as a part of the 1990 Mediterranean Eradication Program raised concerns over the potential of malathion to cause genetic damage. Isomalathion results from the thermal or photochemical isomerization of malathion (Fig. 1) and has been identified in certain commercial formulations [9]. It was implicated in the 1976 malathion poisoning epidemic of 2,800 Pakistani spraymen (including 5 deaths) during a malaria control program [10].

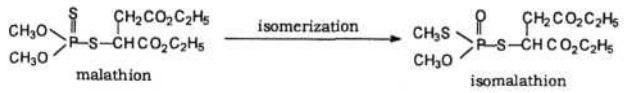

Fig. 1. Chemical structures of malathion and isomalathion.

In test animals, technical grade malathion appears to have the potential to produce chromosomal changes including chromosome aberrations and micronuclei [11-13]. The chromosomal effects produced by technical grade malathion in test animals are generally supported by results from *in vitro* studies in human lymphocytes [14-18]. Pesticide applicators exposed to technical grade malathion and other insecticides had higher levels of chromosomal damage (aberrations) or SCEs than non-exposed populations [19, 20]. These and others studies provide evidence that malathion can be genotoxic for humans. Imamura and Talcott [21] demonstrated that malathion (98% pure) and several of its impurities alkylate 4-(p-nitrobenzyl)pyrimidine, as a nucleophile for reaction of alkylation. Malathion was rated as a strongly positive alkylating agent [22]. Griffin and Hill [23] and Richardson and Imamura [24] demonstrated malathion-induced breakage of plasmid or bacteriophage DNA *in vitro;* the purity of the test material was not reported. Malathion (97-99% pure) caused specific mutations (deletions) in human T lymphocytes [25]. A strong decrease in mitotic index was shown in *in vitro* and *in vivo* studies using technical grade or other than pure malathion [9, 19, 24]. Malathion has therefore been shown to interact with DNA but the major mechanism(s) of interaction is essentially unknown.

Taking into account the above results one can consider that genotoxic effects observed for malathion may, at least in part, arise from impurities, including isomalathion, in the technical grade mixtures. It should be emphasized that humans are exposed to the technical grade. To resolve the ambiguities related to the major causative agent(s) of the genotoxicity observed in impure mixtures, it is recommended that the composition of the technical grade malathion should be determined and the components should be tested individually for genotoxicity.

In the present work the genotoxicity of pure malathion and its analogue isomalathion present in commercial formulations was investigated using the comet assay.

Material and Methods

Lymphocytes Isolation and Incubation with Pesticides

Blood was obtained from non-smoking donors. Peripheral blood lymphocytes (PBL) were isolated by centrifuge on density gradient of Gradisol L. The viability of the cells was constantly found to be over 95%. The final concentration of the lymphocytes was adjusted to 1-5 • 10^5 cells/ml by adding RPMI 1640 medium to the single cell suspension. Malathion and isomalathion were derived from stock (50 mM) ethanolic solutions and added to the suspension of the lymphocytes to give final concentrations of 25 and 200 μM. The control received ethanol instead of the organophosphate, the concentration of which (0.18%) did not affect the processes under study. To examine DNA damage the lymphocytes were incubated with the chemicals for 1 h at 37°C. Each experiment included a positive control that was hydrogen peroxide at 20 μM. H_2O_2 produced pronounced DNA damage that indicated the accessibility of the lymphocytes to tested chemicals.

Chemicals

The organophosphorus compounds malathion (S-(l,2-dicarboetoxyethyl) 0,0-dimethyl phosphorodithio-ate) and isomalathion (S-(1,2-dicarboetoxyethyl) CS-dime-

thyl phosphorodithioate) (Fig. 1) at a purity of at least 99.8% was purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). RPMI 1640 medium and low melting point agarose was from Sigma (St. Louis, MO, USA). Gradisol L was from Polfa (Kutno, Poland). DAPI (4',6-diamidino-2-phenylindole) was obtained from Serva (Heidelberg, Germany).

All other chemicals were of the highest purity available.

Single Cell Gel Electrophoresis

The comet assay was performed under alkaline conditions essentially following the procedure of Singh *et al.* [1] with a slight modification. A freshly prepared suspension of PBL in 0.7% low melting point agarose dissolved in RPMI 1640 medium was casted to fully frosted microscope slides (Labcraft, Houston, TX, USA) precoated with 1% normal melting 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. On the lysis the microscope 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 for 30 min at electric field strength 0.56 V/cm (300 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 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.

DNA Repair

To examine the DNA repair lymphocytes after treatment (as well as control samples), they were washed and incubated in fresh, insecticide-free RPMI 1640 medium for 1 h at 37°C. Repair incubation was stopped by placing the samples in an ice-bath. Subsequent steps were as already described.

Comet Analysis

The objects were observed at 320x magnification in a Zeiss Axhiophot fluorescence microscope attached to a video camera (IMAC-CCD S30, Computer System GmbH, Boblingen, Germany) connected to a personal computer-based image analysis system ISIS3 v. 2.0 (Metasystem, Althussheim, Germany). Fifty images were randomly selected from each sample and the comet length (diameter of nucleus plus migrated DNA) was measured on the screen. Two parallel tests were performed per individual for a total of 100 cells, and mean comet length was calculated.

Comet length is positively correlated with the level of DNA breakage in a cell [1]. Because the distribution of the comets was heterogeneous, histograms were applied to display information. The mean value of the comet length in a particular sample was taken as an index of DNA damage in this sample.

Data Analysis

All the values in this study were expressed as mean ± SEM from two separate experiments.

Table 1. Comet length *(μm)* of the lymphocytes exposed to organophosphorus compounds malathion and isomalathion[a]

Concentration [μM]	Treatment	
	Malathion	Isomalathion
0	33.23 ± 1.29	32.41 ± 1.04
25	33.07 ± 1.21	34.96 ± 1.29*
200	32.26 ± 1.19	56.01 ± 2.19**

[a] The number of cells in each treatment was 100; mean ± SEM, * – $p < 0.05$, ** – $p < 0.01$.

Two-way analysis of variance (a chemical at a given concentration x time) was used to compare effects evoked by a chemical at a particular concentration and the control. No statistically significant interaction was found, so one-way analysis of variance was applied. The differences between means were compared using Scheffe's multiple comparison test [26].

Results

DNA Damage

Figure 1 shows examples of the comets resulted from the exposure of lymphocytes to 200 μM isomalathion and the controls. The comets resulting from exposure to malathion did not differ from that of the controls. Relatively undamaged cells (upper panel) give comets consisting of a compact head with or without a very short tail, indicating double-stranded DNA. Comets originating from damaged cells (lower panel) have a distinct head with a tail.

The mean comet lengths for the lymphocytes exposed for 1 h to malathion and its analogue, as compared with appropriate controls, are presented in Table 1.

It can be seen from this Table that malathion at the applied concentrations did not evoke a significant effect on DNA migration.

Isomalathion evoked an increase in comet length - at concentration of 200 μM the increase was 72% as compared with the control. The increase was dose-dependent.

The most basic way of viewing the data from the comet assay is the distribution of cells according to the percentage of DNA in tail moment, which is positively correlated with comet length [27]. Fig. 3 shows such a distribution. It can be seen from this Figure, that comets resulting from lymphocytes exposed to isomalathion contain more DNA in their tails than comet resulting from control lymphocytes and lymphocytes exposed to malathion.

DNA Repair

Fig. 4 shows the comet lengths of the lymphocytes exposed to malathion and isomalathion immediately after exposure, as well as 15, 30 and 60 min thereafter. In all cases the comet length of the control lymphocytes was constant, indicating that preparation and subsequent processing of the lymphocytes did not introduce significant damage to their DNA.

The lymphocytes exposed to malathion behave essentially in the same way as the control - their comet length

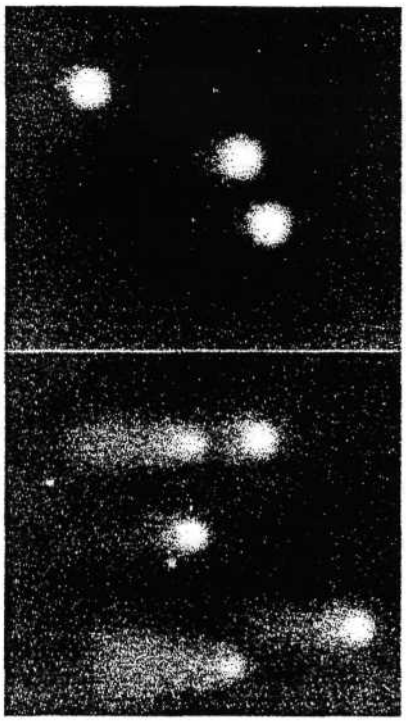

Fig. 2. Typical fluorescence microscope images (comets) of the DAPI-stained DNA of human lymphocytes exposed to 200 μM isomalathion (lower panel) as compared with the control (upper panel).

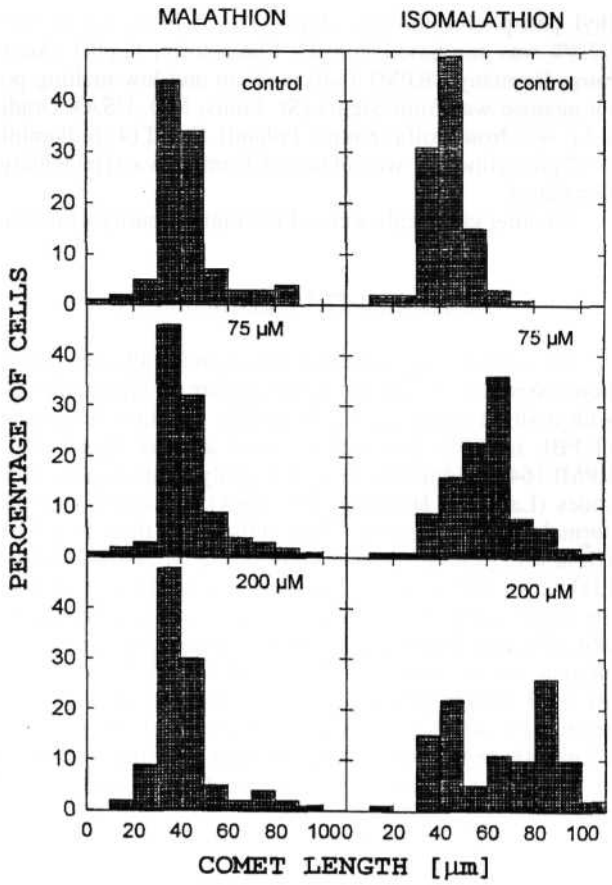

Fig. 3. Histograms of the distribution of comet length in human lymphocytes treated with malathion and isomalathion at indicated concentrations as compared with appropriate controls. The number of cells scored for each treatment was 100.

did not change during the repair incubation (Fig. 4, upper panel).

The lymphocytes exposed to isomalathion at all tested concentrations required a relatively short period of time to repair DNA damage (Fig. 4, lower panel). The comet length of the lymphocytes exposed to 200 μM isomalathion decreased from 72 to 18% during 15 min of repair incubation.

Discussion

The obtained data indicate that single cell gel electrophoresis (comet assay) is a highly sensitive technique to detect DNA damage induced by tested organophosphorus compounds, so it can be useful as a tool for environmental studies.

The concentrations of the tested organophosphorus agents in our studies ranged from 75 to 200 μM that corresponds to the range 23-66 μg/ml. It was assessed that in extreme cases agricultural workers could be occupationally exposed to doses of 10 μg/ml of malathion [25]. The estimated lethal oral dose of malathion for humans is 858 mg/kg body [25]. The concentration of malathion found in the blood of individuals autopsied after malathion overdose ranged 175-517 μg/ml [31], so the concentrations from the range we used could be achieved in the blood through various nonlethal human exposure.

The obtained results indicate that isomalathion investigated with the comet assay, using human peripheral blood lymphocytes, gives a significant increase in the length of the comets, acting therefore as genotoxic compounds. In the comet assay, the short time elapsed between treatment and measuring of the DNA damage, enables to avoid the repair of the possible lesions and makes it easier to detect whether or not the chemical is able to interact with DNA, producing different types of damage.

The presented data indicate that isomalathion, unlike its parent compound malathion, has an ability to damage DNA of the isolated human peripheral blood lymphocytes. Malathion has been proven to have genotoxic properties in many studies [12, 15, 16, 18, 25, 29] including humans *in vivo* [19, 20, 29]. However, the majority of these studies were performed using technical grade malathion or the insecticide without determined purity. An excellent study of Pluth et al. [25] demonstrated that malathion (97-99% pure) caused specific mutations (deletions) in human T lymphocytes. The impurities, that were carefully determined in this study, consisted mainly of isomalathion and malaoxon. Malathion does not appear to produce point mutations in standard gene mutation assay in bacteria, but malaoxon was positive in mammalian cell mutation tests [8, 31-33].

It must be taken into account that for pest eradication, malathion is used as a commercial or technical grade product which varies from approximately 90-95% malathion by weight and may contain impurities formed during manufacturing and storage. Among the impurities there is also isomalathion, formed from malathion by thermal and/or photochemical isomerization [34]. In this regard, the results of our study clearly indicate that malathion used as commercial formulation, i. e. containing isomalathion, can be considered as a genotoxic substance *in vitro.* This means

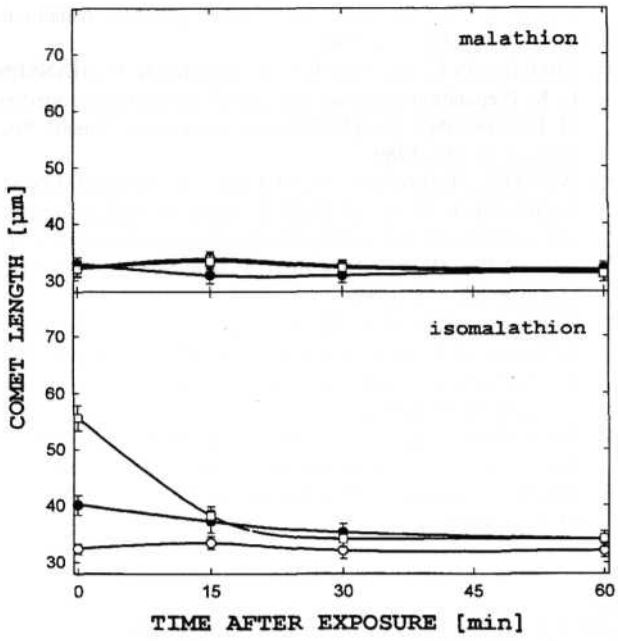

Fig. 4. The time course of the repair of DNA damage in human lymphocytes treated with malathion and isomalathion at 75 (•) and 200 µM (□) as compared with appropriate controls (O). The number of cells scored for each treatment was 100. Error bars denote SEM.

that commercially purchasable malathion can produce DNA lesions *in vivo,* potentially comprising DNA breakage at sites of oncogenes or tumor suppressor genes, and may play a role in the induction of malignancies in individuals exposed to this agent. Therefore, malathion can be regarded as potential environmental mutagen/carcinogen and needs further investigation.

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