

DNA Damage after Acute and Chronic Treatment with Malathion in Rats

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Malathion is an insecticide widely used in agriculture and in public health programs that when used indiscriminately in large amounts can cause environmental pollution and risk to human health. However, it is possible that during the metabolism of malathion, reactive oxygen species can be generated, and malathion may produce oxidative stress in intoxicated rats that can be responsible for alterations in DNA molecules related in some studies. As a result, the present study aimed to investigate the DNA damage of cerebral tissue and peripheral blood in rats after acute and chronic malathion exposure. We used single cell gel electrophoresis (Comet assay) to measure early damage in hippocampus and peripheral blood and the Micronucleus test in total erythrocytes samples. Malathion was administered intraperitoneally once a day for one day (acute) or for 28 days (chronic) protocols (in both protocols, malathion was administered at 25, 50, 100, and 150 mg/kg). Our results showed that malathion (100 and 150 mg/kg) increased the DNA damage index in the peripheral blood and in the hippocampus after both chronic and acute treatment. Malathion increased the frequency of micronuclei only in chronic treatment at 150 mg/kg dose, and induced a cytotoxic dose-dependent decrease in the frequency of polychromatic erythrocytes in the peripheral blood of rats. In conclusion, since malathion increased both the peripheral blood and hippocampus DNA damage index using the Comet assay and increased the frequency of micronuclei in the total peripheral blood, it can be regarded as a potential mutagen/carcinogenic agent.

KEYWORDS: Malathion; Comet assay; Micronucleus test; DNA damage

1. INTRODUCTION

Pesticides are the only toxic chemicals deliberately spread into the environment in large amounts with the aim of controlling undesired living species. Their potential to cause adverse effects to human and wildlife populations has been the subject of intense study and has led to the development of increasingly stringent and encompassing regulations for the risk assessment of novel formulation and to control the use of existing compounds (1). Organophosphorus pesticide (OP)

compounds are widely used throughout the world as insecticides in agriculture and utilized in a variety of domestic and industrial settings (2). The number of intoxications with OPs is estimated at some 3 000 000 per year, and the number of deaths and casualties, some 200 000 per year (3).

OP compounds are recognized for their ability to induce physiological toxicity in humans and animals via the inhibition of acetylcholinesterase, leading to accumulation of the neurotransmitter acetylcholine and subsequent activation of cholinergic muscarinic and nicotinic receptors (4, 5). In chronic and subchronic exposures, added to cholinesterase inhibition, induction of oxidative stress has been reported as the main mechanism of toxicity (5–7).

Malathion [*S*-(1,2-dicarbethoxy)ethyl *O,O*-dimethyl-phosphorodithioate] is one of the most widely used organophosphorus

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pesticides for agriculture and public health programs. Malathion is known to induce cholinergic crisis through its bioactivated analog, malafoxon (8). The more common neurotoxic effect related to malathion poisoning can be memory impairment, difficulty in concentration (9–11), anxiety, confusion, irritability, and depression (12). Malathion also impairs aversive memory retention in the step-down inhibitory avoidance task (13) and induces depressive-like behavior in the forced swimming test (14).

Recently, we have reported that malathion induced oxidative stress, lipid peroxidative damage, inhibition of AChE activity, and inactivation of mitochondrial respiratory complexes in selective brain regions in rats (7, 14–16).

Studies have shown that oxidative stress can cause DNA damage (17, 18). This damage can include chemical and structural modifications to purine and pyrimidine bases and 2'-deoxyribose and the formation of single- and double-strand breaks. Strand breaks within DNA can occur either directly due to damage from reactive oxygen species exposure or indirectly due to cleavage of the DNA backbone during DNA base excision repair (19, 20).

Malathion under *in vitro* and *in vivo* conditions has been shown to induce DNA damage, chromosomal aberrations (8, 21–24), malignant transformation (25) and apoptosis (26). Except for several reports describing the effects of malathion on cognitive skills (9, 12), we can not find in the literature the effects of malathion intoxication at the DNA damage level at the hippocampus. Therefore, the present investigation was performed to determine the genotoxic effects in the hippocampus and peripheral blood DNA damage in rats after acute and chronic malathion exposure, since patients with organic insecticide poisoning present a wide spectrum of adverse health effects ranging from gastrointestinal symptoms to cardiac, immunological, or neurotoxic diseases (27–29).

2. MATERIALS AND METHODS

2.1. Animals. The subjects were adult male Wistar rats (weighting 250–350 g) obtained from our breeding colony. Animals were housed five to a cage with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.) at a temperature of 22 ± 1 °C. All experimental procedures were performed in accordance with the approval of the local Ethics Committee.

2.2. Treatment. Four doses of malathion (25, 50, 100, and 150 mg/kg body weight) dissolved in saline solution (0.9% NaCl) in a volume of 1 mL/kg were administered intraperitoneally (*i.p.*) in two treatments: (I) acute, a single injection of malathion was administered; and (II) chronic, malathion was administered once a day for 28 consecutive days. All control rats for both treatments received injections of saline solution (0.9% NaCl). The doses were chosen on the basis of other studies made by our group (7, 13–16).

The dose of 150 mg/kg of malathion corresponds to 1/9 of the LD₅₀ calculated for this pesticide. Thus, the doses of malathion employed in the present study were far below the LD₅₀ of this pesticide and caused neither overt signs of cholinergic toxicity nor mortality. In addition, Broccardo and colleagues (30) did not find any sign of cholinergic toxicity in rats intraperitoneally treated with malathion at 250 mg/kg. However, at 700 mg/kg, the oral administration of malathion induced important signs of cholinergic hyperactivity, gross behavioral abnormalities, and some cases of mortality (4).

Wistar rats were anesthetized with ketamine (80 mg/kg of body weight, *i.p.*) and xylazine (20 mg/kg body weight, *i.p.*) 24 h after the malathion administration in acute treatment and 24 h after the last malathion administration. The peripheral blood was collected by cardiac puncture, and then the animals were killed by decapitation, and the brains were removed and the hippocampus was dissected. Both peripheral blood and the hippocampus were submitted for genotoxic analyses. These tissues were chosen because

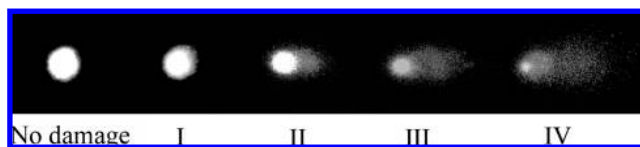


Figure 1. Comet assay. Evaluation of DNA damage using ethidium bromide (400 \times). The cells are assessed visually and received scores from 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail.

peripheral blood is the main tissue for genotoxic assays, and the brain was chosen because our previous studies showed significant oxidative stress in this tissue after malathion exposure (7, 15).

2.3. Single Cell Gel Electrophoresis: Comet Assay. A standard protocol for Comet assay preparation and analysis was adopted from Tice et al. (31). The slides were prepared by mixing 5 μ L of whole blood or hippocampus cells (washed and minced in cold PBS solution) with 90 μ L of low-melting-point agarose (0.75%). The mixture (cells/agarose) was added to a fully frosted microscope slide coated with a layer of 500 μ L of normally melting agarose (1%). After solidification, the coverslip was gently removed, and the slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, pH 10.0–10.5, with freshly added 1% Triton X-100 and 10% DMSO) for 1 day. Subsequently, the slides were incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 10 min. The DNA was electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA. After electrophoresis, the slides were neutralized with Tris buffer (0.4 M; pH 7.5). Finally, the DNA was stained with ethidium bromide. Images of 100 randomly selected cells (50 cells from each of two replicate slides) from each animal were blindly analyzed using a fluorescence microscope equipped with an excitation filter of BP546/12 nm and a 590 nm barrier filter. Cells were scored from 0 (undamaged) to 4 (maximally damaged) according to the tail intensity (size and shape), resulting in a single DNA damage score (damage index) for each sample and, consequently, for each group. Thus, a damage index (DI) of the group could range from 0 (completely undamaged = 100 cells \times 0) to 400 (maximum damage = 100 cells \times 4) (32) (Figure 1). The percentage damage frequency (DF) was calculated for each sample on the basis of the number of cells with a tail versus with no tail.

2.4. Micronucleus Test. The micronucleus (MN) assay for peripheral blood was carried out following standard protocols (33). Smears for the MN assay were made with the same blood samples used for the Comet assay. Two whole blood smears from each animal were made on precleaned microscope slides, air-dried at room temperature, and stained in 5% Giemsa (Merck) in phosphate buffer (pH 5.8) for 10 min. The slides were coded, and the cells were blindly scored by light microscope at 1000 \times magnification. For each individual, the ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) was determined in a total of 1000 erythrocytes per animal to evaluate cytotoxicity, and the frequency of micronucleated polychromatic erythrocytes in 4000 erythrocytes per rat was recorded to assess genotoxicity. For the acute treatment, we considered only the micronucleus in PCEs, and in the chronic treatment we considered the micronucleus in the total erythrocytes, both PCEs and NCEs.

2.5. Statistical Analysis. All data are presented as mean \pm SD. Data were compared using multifactorial analysis of variance (ANOVA) followed by the Tukey *post hoc* test when ANOVA was significant. The factors of variance were doses and the dependent variables were the ratios PCEs/NCEs and MNPCEs/PCEs. In all experiments, the level of significance was $p < 0.05$.

3. RESULTS

In this work, we evaluated through Comet assay and MN test DNA damage on Wistar rats cells submitted to acute and chronic treatment with different doses of malathion.

3.1. DNA Damage in Acute Treatment by Comet Assay. In the acute treatment with malathion, 100 and 150 mg/kg

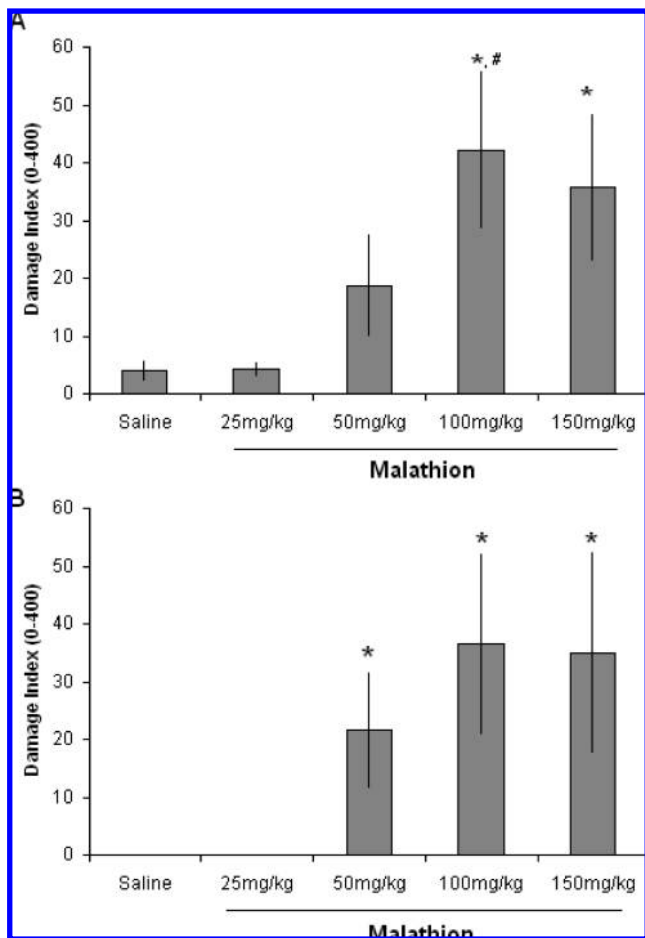


Figure 2. Mean values (\pm SD) of the damage index in blood cells (A) and in the hippocampus (B) with Comet assay. Four doses of malathion (25, 50, 100, and 150 mg/kg), dissolved in saline solution (0.9% NaCl) were administered i.p. in acute treatment. *Data significantly increased in relation to saline at $P < 0.01$ (ANOVA, Tukey). #Data significantly increased in relation to saline and malathion 25 mg/kg at $P < 0.01$ (ANOVA, Tukey). °Data significantly increased in relation to malathion 50 mg/kg at $P < 0.05$ (ANOVA, Tukey).

increased DNA damage in the total blood (**Figure 2A**), as compared to the saline group and malathion 25 and 50 mg/kg for Comet assay analysis (DF and DI, both $P \leq 0.01$). In the hippocampus, 100 and 150 mg/kg of malathion (**Figure 2B**) increased DNA damage in acute treatment in relation to the saline and malathion at 25 mg/kg (DF and DI, both $P \leq 0.01$). Additionally, a significant increase was also observed for the malathion at 50 mg/kg in relation to the saline group (DF and DI, both $P \leq 0.05$).

3.2. DNA Damage in Chronic Treatment by Comet Assay. In total blood after chronic treatment with malathion, increased in DNA damage was observed in doses of 100 and 150 mg/kg (**Figure 3A**). In the hippocampus, chronic treatment with malathion also increased DNA damage in the highest doses, 100 and 150 mg/kg (**Figure 3B**) as compared to the saline group (DF and DI, both $P \leq 0.01$). In addition, malathion at 150 mg/kg showed a statistically significant increase in comparison to 25 and 50 mg/kg doses (DF and DI, both $P < 0.05$).

3.3. Micronucleus Test. In acute treatment, there was no difference in the frequency of the micronucleus in PCEs among the studied malathion doses and the saline group (**Figure 4A**). However, the frequency of a micronucleus in the erythrocytes in the chronic treatment group increased in the 150 mg/kg of

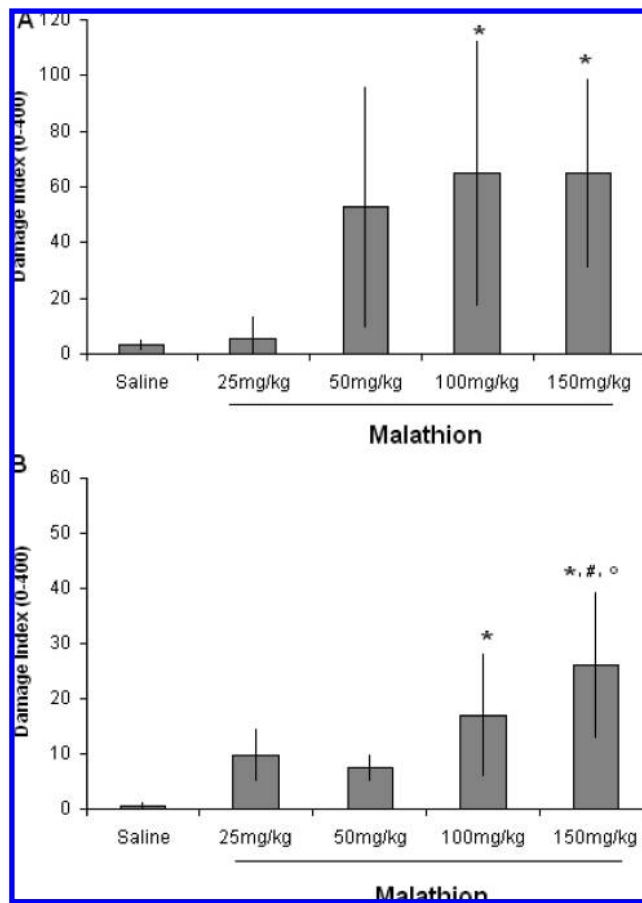


Figure 3. Mean values (\pm SD) of the damage index in blood cells (A) and in the hippocampus (B) with the Comet assay. Four doses of malathion (25, 50, 100, and 150 mg/kg), dissolved in saline solution (0.9% NaCl) were administered i.p. in chronic treatment. *Data significantly increased in relation to saline at $P < 0.01$ (ANOVA, Tukey). #Data significantly increased in relation to malathion 25 mg/kg at $P < 0.05$ (ANOVA, Tukey). °Data significantly increased in relation to malathion 50 mg/kg at $P < 0.05$ (ANOVA, Tukey).

malathion subgroup as compared to the saline group and malathion doses of 25, 50 and 100 mg/kg, $P < 0.001$ (**Figure 4B**).

Malathion induced a significant dose-dependent decrease in the PCE/NCE ratio in all doses of malathion (25, 50, 100, and 150 mg/kg) as compared to the saline group in both treatments, acute and chronic (**Figure 5A and 5B**, $P < 0.001$).

4. DISCUSSION

In this study, we investigated the DNA damage of cerebral tissue and peripheral blood in rats after acute and chronic malathion exposure. Malathion is a widely used pesticide with high potential for human exposure. The exact mechanism of the genotoxic effects of malathion is not known. Among the potential molecular mechanisms of genotoxicity of organophosphorus compounds are induction of oxidative stress, alkylation, and immunotoxicity (5, 8, 24). However, free radicals play an important role in toxicity of organophosphorus insecticides. These compounds may induce oxidative stress, leading to generation of free radicals and alteration in the antioxidant system (6).

Although all cellular macromolecules are subject to damage by reactive oxygen species, the primary deleterious consequences of oxidative stress probably arise from damage to DNA

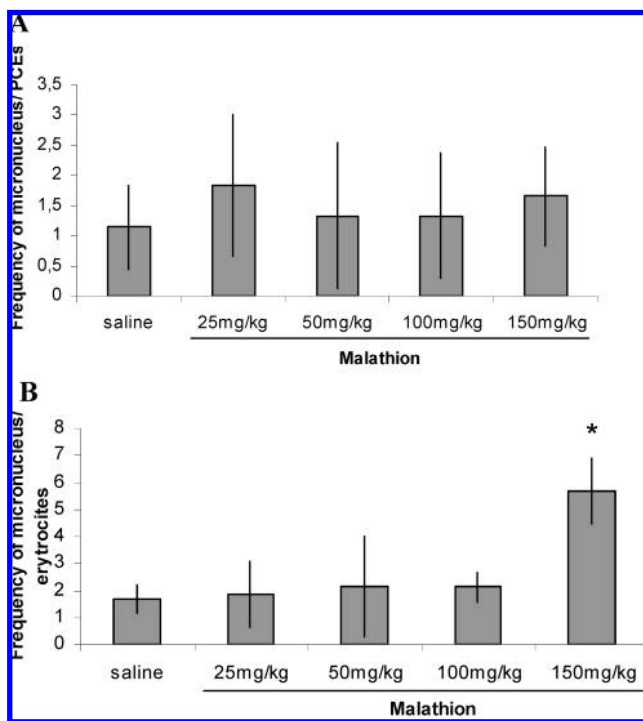


Figure 4. Mean values (\pm SD) of frequency of the micronucleus in the erythrocytes of rat. Four doses of malathion (25, 50, 100, and 150 mg/kg), dissolved in saline solution (0.9% NaCl), were administered i.p. in acute (A) or in chronic treatment (B). *Data significantly increased in relation to saline and malathion doses (25, 50, and 100 mg/kg) at $P < 0.005$ (ANOVA, Tukey).

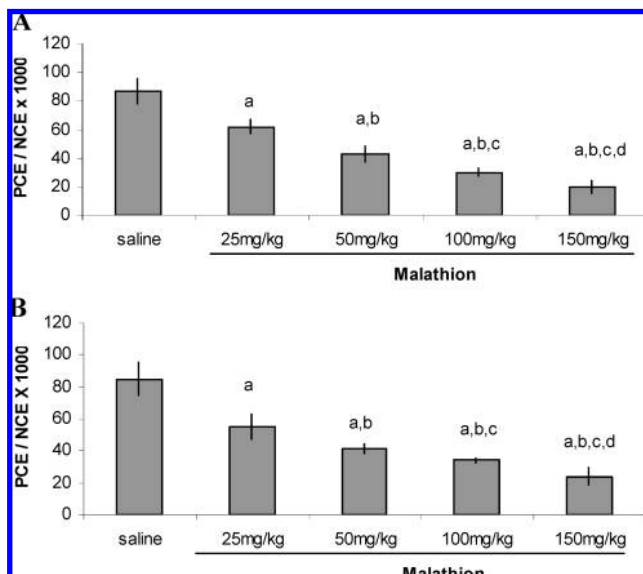


Figure 5. Mean values (\pm SD) of PCE/NCE \times 1000 observed in whole blood of rats. Four doses of malathion (25, 50, 100, and 150 mg/kg), dissolved in saline solution (0.9% NaCl), were administered i.p. in acute (A) or in chronic treatment (B). ^aData significantly increased in relation to saline at $P < 0.001$; ^bdata significantly decreased in relation to 25 mg/kg at $P < 0.001$; ^cdata significantly decreased in relation to 50 mg/kg at $P < 0.001$; ^ddata significantly decreased in relation to 100 mg/kg $P < 0.001$ (ANOVA, Tukey). PCE = polychromatic erythrocytes; NCE = normochromatic erythrocytes.

(17). Reactive oxygen species damage the DNA, and this damage leads to a loss of function. Oxidative stress has been associated with chromatin cross-linking (34), DNA base oxida-

tion (35), chromosomal aberrations, (20) and an increased risk of cancer (36). The damage can be repaired by base excision repair to maintain DNA integrity. However, when DNA damage is beyond the ability of the DNA repair system, the DNA damage is not completely repaired, and the damage accumulates (37).

Evidence also has shown that mitochondria are the primary subcellular target of the organophosphorous compounds, which inhibits enzymatic activity and ATP generation, causing structural alterations of the matrix and mitochondrial swelling and also generating free radicals (38, 39). It was shown by Delgado and colleagues (7) that there is an increase in the mitochondrial superoxide production in the hippocampus of rats subacutely exposed to malathion as well as an increase in the lipid peroxidation in the hippocampus. Deficiencies in the functioning of the mitochondrial respiratory chain can lead to a fast fall in ATP production, which is strongly related to cellular death (40).

In the results presented in this study, we have demonstrated that malathion increased peripheral and hippocampus DNA damage and did not alter the micronucleus frequency but presented signs of cytotoxicity in total erythrocytes of rats.

Micronuclei are acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division and appear in the cytoplasm of interphase cells as small additional nuclei (41). Our results showed that malathion after acute exposure was not able to increase cytogenetic damage in the MN test, but after chronic exposure, the highest dose presented a significant increase in the frequency of micronuclei. However, after both treatments, malathion showed cytotoxic results, reducing the number of polychromatic erythrocytes.

In accordance with our results, other studies had demonstrated cytotoxicity dose-dependent on malathion in culture cells and had showed that this toxicity was mediated through its effect on apoptosis (26, 42). The results presented by Titenko-Holland et al. (43) using the micronucleus assay in vitro demonstrated that concentrations higher than 100 μ g/mL of malathion were cytotoxic in vitro. Nevertheless, the significance of this finding to the in vivo situation is questionable. There is one report that the micronucleus frequency was increased in mouse bone marrow after in vivo exposure (44) but an abstract of a study from China reported that malathion induced micronuclei in vitro but not in vivo in mouse bone marrow cells (45). These results corroborate our findings that micronuclei were induced by malathion only at relatively high doses (150 mg/kg) approaching cytotoxic levels.

The mechanism of micronucleus formation caused by malathion was assessed in the work of Titenko-Holland et al. (43) using an antikinetochore antibody staining. They showed that there was a significant increase in the kinetochore-negative micronuclei in isolated lymphocytes after malathion treatment, suggesting that high concentrations of malathion can cause chromosome breakage. This is consistent with the previous data that malathion and its metabolite malathion acid are alkylating agents of nucleic acids (46).

Alkylating agents are known to cause DNA damage (47). Since malathion practices its genotoxic effect by alkylating the DNA (46) or causing chromosome breakage (43) and that many organophosphorus compounds are reported to have the ability to bind to DNA (46) and cause mutations, our findings using the Comet assay after acute and chronic exposure can be explained. We have found using the Comet assay that this organophosphorous insecticide was capable of increasing the DNA damage both in peripheral blood and in hippocampus cells at the highest doses.

The Comet assay, or alkaline single cell gel electrophoresis, is a rapid, simple, and sensitive technique for measuring and analyzing DNA breakage in individual cells (48). Under alkaline (pH > 13) conditions, the assay can detect single- and double-stranded breaks, alkali labile sites, and also possibly both DNA–protein and DNA–DNA cross-links in virtually any eukaryotic cell population that can be obtained as a single-cell suspension (49). In addition to DNA damage, DNA strand breaks formed during excision repair may also cause DNA migration measurable in the Comet assay. Thus, DNA repair that reduces DNA damage by eliminating DNA lesions may, on the other hand, increase DNA migration due to incision-related strand breaks. The processes of DNA repair can influence the results obtained with the Comet assay in a complex way (50).

Our results are consistent with existing literature that has shown genotoxic effects of malathion. Blasiak and colleagues (8) verified in their studies in vitro that malaoxon and isomalathion (metabolite and isomer of malathion, respectively), but not malathion, introduced damage to DNA of human peripheral blood lymphocytes in a dose-dependent manner. Additionally, other studies have shown that blood samples of workers occupationally exposed to a complex mixture of pesticides, including malathion, have increased parameters evaluated through the Comet assay (21, 22). Moreover, a strong genotoxic effect on mucosal epithelial cells taken from human tonsil tissue was demonstrated (22). Balaji and Sasikala (51) showed a cytogenetic effect of malathion in in vitro culture of human peripheral blood, with a dose-dependent increase in the frequency of chromosomal aberration as well as sister-chromatid exchanges.

The present data indicate that malathion have an ability to damage DNA of peripheral blood lymphocytes and hippocampus cells in rats after both acute and chronic treatment, but also present an inability to increase cytogenetic damage after acute exposure and present a significant increase only at the highest dose of malathion in the chronic treatment detected by micronucleus test. Since both methods measure different end points, it seems that the damage caused by malathion detected by the Comet assay may be induced by strand breaks first, probably originating after action of the repair system, and this damage does not remain after a cell division, explaining why positive results do not appear during the micronucleus assay except at the highest dose, at which point the repair system was probably not capable of repairing the damage.

In summary, the results of this study indicate that malathion has induced DNA damage detected the Comet assay in the hippocampus and total blood of rats at higher doses (100 and 150 mg/kg) and increased the frequency of micronuclei of total erythrocytes only after chronic exposure at the highest dose (150 mg/kg). Additionally, we have demonstrated a dose-dependent cytotoxicity in peripheral blood after both treatments, acute and chronic. This means that commercially available malathion may produce DNA lesions in vivo and may play a role in the induction of malignancies in individuals exposed to this agent. These findings agree with the current literature and introduce results about damage in DNA in the hippocampus, suggesting a cause of neurotoxic damage for this pesticide. Our results in the hippocampus could be related to the findings of Abdel-Rahman and colleagues that showed subacute dermal exposure to malathion induced a significant reduction in the density of surviving neurons in the dentate gyrus, CA1 and CA2 subfields of the hippocampus, midbrain, brainstem, and cerebellum in rats (52).

Additional research is needed to illustrate the neurochemical substrates involved in malathion-induced DNA damage in rats

and explore how long DNA damage continues in acute and chronic malathion poisoning treatment in the brain.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of July 11, 2008, contained two incomplete author names. These have been corrected with the posting of August 1, 2008.

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