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Cytogenetic evaluation of malathion-induced toxicity in Sprague-Dawley rats

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Abstract

Malathion is a well known pesticide and is commonly used in many agricultural and non-agricultural settings. Its toxicity has been attributed primarily to the accumulation of acetylcholine (ACh) at nerve junctions, due to inhibition of acetylcholinesterase (AChE), and consequently overstimulation of the nicotinic and muscarinic receptors. However, the genotoxicity of malathion has not been adequately studied; published studies suggest a weak interaction with the genetic material. In the present study, we investigated the genotoxic potential of malathion in bone marrow cells and peripheral blood obtained from Sprague-Dawley rats; using chromosomal aberrations (CA), mitotic index (MI), and DNA damage as toxicological endpoints. Four groups of four male rats each, weighing approximately 60 ± 2 g, were injected intraperitoneally (i.p.) once a day for five days with doses of 2.5, 5, 10, and 20 mg/kg body weight (BW) of malathion dissolved in 1% DMSO. The control group was made up of four animals injected with 1% DMSO. All the animals were sacrificed 24 h after the fifth day treatment. Chromosome preparations were obtained from bone marrow cells following standard protocols. DNA damage in peripheral blood leukocytes was determined using alkaline single-cell gel electrophoresis (comet assay). Malathion exposure significantly increased the number of structural chromosomal aberrations (CA) and the percentages of DNA damage, and decreased the mitotic index (MI) in treated groups when compared with the control group. Our results demonstrate that malathion has a clastogenic/genotoxic potential as measured by the bone marrow CA and comet assay in Sprague-Dawley rats.

1. Introduction

Organophosphate insecticides (OPI) are potent toxicants that target the nervous systems of insects and other pests [1]. OPIs operate primarily through the inhibition of acetylcholinesterase, an enzyme that degrades the neurotransmitter acetylcholine, resulting in a buildup of acetylcholine in the neuronal junction [2]. They are recognized as a serious source of environmental pollution and health hazard due to their ability to exert significant adverse toxicity in non-target species including humans through the inhibition of acetylcholinesterases (AChE). This leads to the accumulation of acetylcholine and subsequent activation of cholinergic muscarinic and nicotinic receptors [3–5]. Exposure to OPI's is also a potential cause of longer-term damage to the nervous system, with reports of poor mental health, deficits in memory, concentration, tremors, seizure, and death [2, 6]. Modern agricultural practices depend on the production and use of pesticides to control pests and

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increase productivity. There are presently over 1000 chemicals that are classified as pesticides [7]. In 2004, California reported using over 3.0 million kg of active ingredient organophosphate compounds [8]. Due to the increase use of various pesticide compounds, large amounts of these chemical compounds have therefore been released into the environment, prompting concern from public health officials [7, 9].

Malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithionate) is a nonsystemic, wide-spectrum pesticide in the organophosphate chemical family, is the most widely used throughout the world. It is one of the earliest organophosphate pesticides introduced in the 1950's [10]. It is used widely for agricultural, residential and public health purposes; providing enhancement in food production, and protection from disease vectors [11, 12]. According to the Environmental Protection Agency malathion is one of the most commonly used insecticides in the United States, with an estimated use of 30 million pounds a year. Approximately 60% of this total is used for various insect eradication programs [13]. Often termed as one of the safest organophosphate insecticides, malathion is widely used in many areas because of its low persistence in the environment compared to other organophosphorus insecticides [10, 11, 13]. Once malathion enters into the environment it goes through hydrolysis, biodegradation, and photolysis [14].

Toxicity of malathion is compounded by its metabolites and also depends upon the product purity, route of exposure, amount of protein in the diet and gender. Malaoxon, the metabolites produced by the oxidation of malathion in mammals, insects and plants, is the primary source of malathion's toxicity and it is 40 times more acutely toxic than malathion (10,11 from jbb journal) [15, 16]. Numerous malathion poisoning incidents such as acute and chronic cases have occurred among pesticide workers and small children through accidental exposure [17]. According to the World Health Organization, the occurrence of pesticide-related poisonings has doubled over the past ten years due to their widespread use [18]. Symptoms of malathion toxicity can range from common early signs or mild symptoms to more serious, life-threatening adverse health effects. Some of the common signs of exposure include: numbness, tingling sensation, headache, dizziness, difficulty breathing, weakness, irritation of skin, exacerbation of asthma, abdominal cramps, along with other related symptoms [13]. The mechanisms of toxicity seen in organophosphorus compounds in general are due to their ability to inhibit cholinesterase enzyme [17]. Malathion binds with acetylcholinesterase (AChE), which is responsible for normal nerve function [8, 19]. AChE inhibition leads to the accumulation of acetylcholine which in turn results in stimulation of cholinergic, muscarinic, and nicotinic receptors leading to common signs and symptoms seen in malathion poisoning [11].

Studies for genotoxicity have indicated that organophosphate compounds, such as malathion and parathion, induce DNA damage. Damage occurs in the form of chromosomal aberrations, mitotic index, and sister chromatid exchanges (SCE) in both human and animal studies [20–22]. Research performed using genotoxic evaluation in populations with malathion exposure have clearly indicated a positive genotoxic effect in human blood lymphocytes. Significantly high frequencies of chromosome aberrations have been found in a group of pesticide workers compared to an unexposed control group [23].

Although malathion and malathion-containing compounds have been the subject of important toxicology research, there exists a lack of appropriate animal models for carcinogenicity assessment, as well as a scarcity of scientific data describing the dose-response relationship with respect to their genotoxicity in *in vivo* systems (Table 1). Therefore, the present work was undertaken to study the genotoxic potential of malathion in bone marrow cells and peripheral blood leukocytes obtained from Sprague-Dawley rats. Chromosomal aberrations (CA), DNA damage, and mitotic index (MI) were used as

toxicological end-points. These endpoints were selected based on the fact that the bone-marrow assay for detecting chromosome aberrations, and DNA damage in peripheral blood leukocytes, are very sensitive, fast, inexpensive and easy to perform assays.

2. Material and Methods

2.1. Chemicals

Malathion, CAS No. 121-75-5, with purity 98.2% was purchased from Chem Service in West Chester, PA, USA. Methanol, glacial acetic acid, and superfrost microscope slides were purchased from Fischer-Scientific in Houston, TX, USA. Potassium chloride solution (0.075 M) and May-Grunwald Giemsa stain stock solution (0.4%) were obtained from Sigma-Aldrich in St. Louis, MO, USA.

2.2. Animal maintenance

Healthy adult male Sprague-Dawley rats (8–10 weeks of age, with average body weight (BW) of 60 ± 2 g) were used in this study. They were obtained from Harlan-Sprague-Dawley Breeding laboratories in Indianapolis, Indiana, USA. The animals were randomly chosen and housed in polycarbonate cages (four rats per cage) with steel wire tops and corn-cob bedding. They were maintained in a controlled atmosphere with a 12h dark/12h light cycle, a temperature of $22 \pm 2^\circ\text{C}$ and 50% humidity with free access to pelleted feed and fresh tap water. The animals were supplied with dry food pellets commercially available from PMI Feeds Inc. (St. Louis, Missouri, USA). They were allowed to acclimate for 10 days before treatment began.

2.3. Dosing

Groups of four rats each were treated with four different malathion dose levels, 2.5, 5, 10 and 20mg/kg/day BW. The present doses were chosen after preliminary studies with different dose levels of malathion to determine the rate of mortality. Malathion was diluted with 1% DMSO and intraperitoneally (i.p.) administered to animals at the doses of 2.5, 5, 10, and 20 mg/kg/day BW, one dose per 24h given for 5 days. Each rat received a total of five doses at 24h intervals. The cumulative doses of malathion given to rats were thus 12.5, 25, 50, and 100mg/kg/day BW. The control group was administered 1% DMSO in the same manner as in the treatment groups. The same dose regime was used in both chromosome aberration and mitotic index assays. Twenty four hours after the fifth day of treatment, all animals were sacrificed, and samples such as whole blood and bone-marrow were collected using the standard protocols.

2.4. Chromosome aberration assay

Experimental animals were injected (i.p.) with colchicine (4mg/kg) 1.5h prior to sacrifice. Animal sacrifice was performed using a carbon dioxide chamber. Both femora were dissected out and cleaned of any adhering muscle. Bone-marrow cells were collected from both femora by flushing KCl (0.075M, at 37°C), and incubated at 37°C for 25 min. Collected cells were centrifuged at $2000 \times g$ for 10 min, and fixed in aceto-methanol (acetic acid: methanol, 1:3, v/v). Centrifugation and fixation were repeated five times at an interval of 20min. The cells were resuspended in a small volume of fixative, dropped onto chilled slides, and allowed to dry. They were stained the following day with freshly prepared 2% Giemsa stain for 3–5 min, and washed in distilled water to remove excess stain.

2.5. Mitotic Index determination

The mitotic index was used in order to determine the rate of cell division. In order to calculate the mitotic index we used the slides that were prepared for the chromosome

aberration assay. We selected random views on the slides to determine the number of dividing cells (metaphase stage). At least 1000 cells were examined in each preparation.

2.6. Scoring of slides

Bone marrow preparations for the analysis of chromosome aberrations in metaphase cells were obtained using the technique by Preston et al [24]. The slides were stained with Giemsa. Well-spread metaphases presenting 42 ± 1 chromosomes were analyzed. One hundred metaphases per animal were screened to a total of 500 metaphases for each treatment and control to obtain the total number of chromosomal aberrations. The mitotic indices were obtained by counting the number of mitotic cells in 1000 cells per animal to a total of 5000 cells per treatment and control under an Olympus microscope. The mitotic index was calculated as the ratio of the number of dividing cells to the total number of cells, multiplied by 100.

2.7. Comet Assay

Rat lymphocytes were isolated and re-suspended in PBS. Following isolation, the cells were mixed with 0.4% Trypan blue solution. After 15–20 min, cells were counted and checked for viability. The remaining cells were immediately used for single-cell gel electrophoresis [SCGE]. In a 2 ml centrifuge tube, 50 μ l (~ 10,000 lymphocytes) of the lymphocyte suspension and 500 μ l of low melting agarose was mixed and 75 μ l pipette onto a pre-warmed comet-slide. The slides were placed flat in the dark at 4° C for 10 min to allow the mixture to solidify. The slides were then placed in pre-chilled lysing solution at 4° C for 1 hr. Slides were removed from lysing solution, tapped on a paper towel to remove any excess lysis solution and immersed in alkaline solution (pH =13) for 45 min at room temperature in the dark. The slides were washed twice for 5 min with Tris-Borate (TBE) buffer. Next the slides were electrophoresed at low voltage (~0.74 V/cm) for 20 min. Slides were removed from the electrophoresis unit after the designated time, tapped to remove excess tris-borate buffer and immediately placed in 70% ethanol for 5 min and air-dried overnight at room-temperature. After overnight drying the slides were stained with SYBR-Green designed for comet assay and allowed to dry overnight. All the steps of the comet assay were conducted under yellow lamp in the dark to prevent additional DNA damage. The slides were read using an automated epifluorescence microscope and computer based DNA damage analysis software from Loats & Associates (Westminster, MD). The data was based on 100 randomly selected cells per sample, i.e., 50 cells were from each of the two replicate slides. The mean percentage of DNA damage was considered the best indicator for DNA damage.

3. Statistical analysis

Data were compared by ANOVA (one-way analysis of variance). Statistical analysis was performed using SAS for Windows 2003 package program. Using the Dunnett test, multiple comparisons were performed. All values were reported as means $\pm$ SDs. For all the experiments, the significance level was set at $p < 0.05$.

4. Results

4.1. Mitotic Index

The mitotic index was used to determine the rate of cell division. The slides prepared for the assessment of chromosome aberrations were used for calculating the mitotic index. It was found that the mitotic index significantly decreased as the malathion doses increased. Mitotic indices of 18.4 ± 0.75 , 16.1 ± 0.99 , 14.1 ± 0.96 , 8.87 ± 0.40 , and $5.87 \pm 0.67\%$ were recorded for malathion doses of 0, 2.5, 5, 10, and 20 mg/kg/day BW, respectively and are illustrated in Figure 1.

4.2. Chromosome aberrations

The results of the chromosomal aberration assay with bone-marrow cells after intraperitoneal treatment with malathion are summarized in Figure 2. The frequency of chromosomal aberrations increased with increasing doses of malathion. The mean percentages of the induced chromosome aberrations were 1.33 ± 0.58 , 5.3 ± 2.5 , 7.66 ± 2.1 , 11.3 ± 1.5 , and 17 ± 2 at malathion doses 0, 2.5, 5, 10, and 20 mg/kg/day BW, respectively.

4.3. DNA damage

Representative comet assay images of control (A) and malathion-treated leukocytes at 2.5 mg/kg/day (B), 5 mg/kg/day (C), 10 mg/kg/day (D) and 20 mg/kg/day (E), are presented in Figure 3. All the doses of malathion induced statistically significant increase in mean percentages of DNA damage (from 42 ± 0.84 to $86.5 \pm 0.57\%$ in malathion treated cells compared to only $13.5 \pm 0.2\%$ in control cells. Maximum increase in DNA damage ($86.5 \pm 0.57\%$) was observed at 20 mg/kg (BW) after 5 days post-treatment. Malathion exposure showed a clear dose-dependent increase in mean levels of DNA damage; from 2.5 mg/kg BW to 20 mg/kg BW (Figure 4).

5. Discussion

In the present study, we evaluated the genotoxic potential of malathion in exposed Sprague-Dawley rats, using chromosome aberration (CA), mitotic index (MI), and DNA damage as toxicological endpoints. The results indicated a significant increase in cytogenetic damage in the bone marrow cells associated with exposure to malathion via intraperitoneal injection. The percentages of structural CAs in the bone marrow of Sprague-Dawley rats exposed to malathion were significantly different compared to the control group. We also noted a significant decrease in mean MI values in malathion-treated groups compared to the control group. Malathion also induced a significant increase in mean percentages of DNA damage, as measured by the comet assay.

Our data supports the earlier findings that have shown an elevated incidence of CA following exposure to malathion. The induction of CAs in rat bone marrow cells following was found to be dose-dependent. Cytogenetic effects of malathion have been evaluated in different test systems using the structural chromosome aberration assay. Balaji et al [21] reported a significant dose-related increase in chromosomal aberrations, and sister-chromatid exchanges in human peripheral blood following exposure to malathion at nominal concentrations of 0.02, 0.2, 2 and 20 $\mu\text{g/mL}$.

Genotoxicity studies of human blood lymphocytes have reported that positive results with malathion exposure; showing significant higher frequencies of chromosome aberrations in exposed workers compared to unexposed workers [23]. Similarly, studies on cytogenetic assays in individuals with occupational exposure to malathion have also indicated increased levels of chromosome aberrations [25]. In a longitudinal risk assessment of occupational workers exposed to a mixture of pesticides, Zeljezic et al [9] reported an increase in chromosome aberrations including acentric fragments of chromosomes that do not have a centromer. Furthermore, chromosomal alterations including micronuclei have been linked to malathion exposure [26, 27]. Other studies have reported that malathion-induced genotoxic damage include chromosomal aberrations, mitotic index, and sister chromatid exchanges (SCE) in both human and animal studies [20–22].

Kumar et al., [28] likewise studied the effect of malathion in root tip cells, and found an increase in chromosome aberrations with increased dose. Studying the cytogenetic effect of malathion in stored wheat residues in exposed Swiss mice, Amer et al [29] observed chromosome aberrations in bone marrow cells. The results of this study demonstrated that

malathion caused a dose-dependent increase in cytogenetic effects of exposed mice. Comparable findings in other studies have shown that malathion doses of 2.5, 5 and 10mg/kg caused a dose-dependent increase in chromosome aberration frequency [30]. Similar results were recorded using techniques for cytogenetic evaluation in Sprague-Dawley rats exposed to arsenic trioxide [31].

Another parameter used in the present study to determine the cytogenetic potential of malathion was the mitotic index (MI) rate. The mitotic index is defined as the proportion of cells undergoing mitosis (cell division) compared to the total number of cells. Hence, the mitotic index is very critical in determining the rate of cell division. We noted a significant decrease in the mean MI values in malathion treated groups compared to the control group in this study. This decrease might be due to a slower progression of cells from S (DNA synthesis) phase to M (mitosis) phase of the cell-cycle. Comparable research has indicated that human peripheral leukocytes treated with various concentrations of malathion led to a significant decrease in mitotic index in all study concentrations [21]. Another study of exposed cotton field workers exhibited cell cycle delay and a decrease in mitotic index as opposed to the control group [32]. Evidence of a moderate cell cycle delay was also recorded in mice exposed to higher doses of malathion [30]. Further cytogenetic studies of the effects of malathion on somatic and germ cells in mice revealed a measurable decrease in mitotic indices of bone marrow cells [27] and a dose dependent decrease in mitotic index in exposed root tip cells [28].

Furthermore, the induction of DNA damage in this study was measured using mean percentage of DNA damage as a toxicological endpoint. Single cell gel electrophoresis (Comet Assay) is a common method used to measure DNA damage. Results in the present study indicated a dose dependent increase in mean comet tail length in regards to malathion exposure. Malathion has been shown to increase the DNA damage index in the peripheral blood and hippocampus following both chronic and acute treatments [33]. Similar trends have been observed in human liver carcinoma cells [34], as well as in lymphocytes, gill cells, liver and brain cells due to organophosphate exposure [35, 36].

Consistent with our findings, a group of researchers used the comet assay as an assessment tool to measure DNA damage in production workers exposed to pesticides in India. Their findings yielded an increase in mean comet tail length compared to a control group [37]. Since all of the studies show a positive increase in DNA damage, malathion's active metabolite, malaoxon, induced a significant increase in the tail moment of lymphocytes in a dose dependent manner [38]. Also, Muniz and his collaborators reported an elevated level of oxidative stress and DNA damage in human lymphocytes of agricultural workers exposed to organophosphate pesticide compared to the control group [39].

Recently, it has been demonstrated that malathion was responsible for the onset of oxidative stress which led to the alterations in DNA molecules of exposed rats. This particular study further concluded that malathion should be viewed as a potential mutagen/carcinogenic compound [33]. Malathion also has been recorded as a weak hepatocarcinogen agent [40]. Additional studies have reported that commercially purchasable malathion can cause DNA lesions *in vivo*, such as DNA breakage at sites of oncogenes or tumor suppressor genes, and induce malignancies in exposed individuals [41].

Our results indicate that malathion administration to Sprague-Dawley rats yields an increase in cytogenetic damage, which was evidenced by the production of structural chromosome aberrations, modulation of cell cycle, and increase in mean percent of DNA damage (%), as shown by the results of chromosome aberration assay, mitotic index, and comet assay,

respectively. Taken together, our findings indicate that malathion administration to Sprague-Dawley rats increases the cytogenetic damage, and confirms its high genotoxic potential.

The possible use of biomarkers representing intermediate steps in the pathway from exposure to disease to estimate the risk of cancer in human populations has gained increasing attention. The approach offers practical advantages, such as the smaller size and lower costs of studies and the potential for early detection of risk in exposed populations. CAs and MNs are among the biomarkers most commonly considered for such purposes [42, 43]. Although the presence of cytogenetic markers themselves does not necessarily lead to adverse health outcomes, high levels of these markers apparently indicate that cells have been exposed to mutagens/carcinogens.

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- We study the genotoxic effects of malathion on Sprague-Dawley rats.
- We examine its effects on mitotic index and chromosome structure in bone-marrow cells, and on DNA integrity in leukocytes.
- Malathion exposure significantly decreases the mitotic index.
- on the other hand, it significantly increases the frequencies of structural chromosomal aberrations (SCA) and the percentages of DNA damage, in a dose-dependent manner.
- Malathion has a strong clastogenic/genotoxic potential as measured by the SCA and Comet assays.

Figure 1. Effect of malathion on mitotic index. Malathion was administered in different doses of 0, 2.5, 5, 10, and 20mg/kg BW for 5 days through intraperitoneal injection. Values are expressed as means $\pm$ SDs. *Significantly different from the control group, at $p < 0.05$.

Figure 2.

Effect of malathion on structural chromosomal aberrations. Malathion was administered in different doses of 0, 2.5, 5, 10, and 20mg/kg BW for 5 days through intraperitoneal injection. Values are expressed as means $\pm$ SDs. *Significantly different from the control group, at $p < 0.05$.

Figure 3.
Representative comet assay images of control (C) and malathion-treated leukocytes at 2.5 mg/kg, 5 mg/kg, 10 mg/kg, and 20 mg/kg.

Figure 4.

Genotoxicity of malathion on blood leucocytes of Sprague-Dawley rats. Malathion was administered in different doses of 0, 2.5, 5, 10, and 20mg/kg BW for 5 days through intraperitoneal injection. Leucocytes were collected and Comet assay was performed as described in Materials and Methods. Values are expressed as means $\pm$ SDs. *Significantly different from the control group, at $p < 0.05$.

Table 1

Published studies in which malathion exposure has shown positive cytogenetic effects

Test system	Administration of malathion	Results	Reference
Swiss Albino Mice	i.p. route	Dose dependent increase in frequency of CA	Giri et al. [30]
Swiss mouse	i.p. route	Induced aberrations as conc. increased	Amer et al. [22]
Human whole blood	OE	Inc. number of chromatid and chromosome breaks	Garaj-Vrhovac et al. [25]
Mouse (spleen)	i.p.	CA	Amer et al. [29]
Human peripheral leukocytes	Treated with various amounts of malathion	Dose dependent increase in CA	Balaji et al. [21]
Human peripheral lymphocytes	OE	Increase in CA	Rupa et al. [20]

i.p.:intraperitoneal injections; CA: chromosome aberration; OE: occupational exposure