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# Cytogenetic Effects of Methidathion Pesticide on Rat Bone Marrow Cells

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**Abstract:** Methidathion is a non-systemic organophosphorus insecticide. Genotoxicity potential of methidathion was evaluated in rat bone marrow cells (*in vivo*) using different doses based on $LD_{50}$ by means of micronucleus test. MNNCE (Micronucleated Normocromatic Erythrocytes) and MNPCE (Micronucleated Polychromatic Erythrocytes), NDI (Nuclear Division Index) and NDCI (Nuclear Division Cytotoxicity Index), necrotic and apoptotic cells were recorded in rat's bone marrow samples.

**Key words:** Methidathion, micronucleus, NDI, NDCI, toxicity, clastogenic effect

## INTRODUCTION

Attempts to increase agricultural production often face difficulties like control or kill unnecessary pests like rodents, fungi, weeds or insects. So, huge quantities of chemicals are produced and released to the environment every year. Some of these chemicals affect other organisms and become possible danger to human health (Pastor *et al*., 2003). Unfortunately, pesticides were not considered as a problem causing agents, on the contrary, utilizing these compounds still sign of progress and modernization in increasing agricultural yields (Weiss *et al*., 2004). Joy *et al*. (2005) confirmed that pesticides not only affect target organisms but also have some side effects on non-target organisms. Pesticides are considered harmful pollutants according to numerous publications concerning their genotoxicity (Villarini *et al*., 1998; Grisolia, 2002; Aldemir and Ege, 2004). The steady increase in the use of pesticides in agriculture has drawn special attention in environmental pollution research and their relevance to human health (Kong and Ma, 1999; Hernandez *et al*., 2013). Biological monitoring provides a useful tool to estimate the genetic risk deriving from an integrated exposure to a complex mixture of chemicals (Bolognesi, 2003; Hernandez *et al*., 2013).

Methidathion (O, O-dimethyl-phosphorodithioate, S-ester with 4-(mercaptomethyl)-2-methoxy-Δ-1, 3, 4-thiadiazolin-5-1) was first registered in 1974 by Ciba Geigy Corporation. The Department of Pesticide Regulation (DPR) in the California Environmental Protection Agency placed methidathion on the high-priority list for risk assessment based on possible adverse effects identified in chronic toxicity, oncogenicity and chromosomal aberrations studies submitted under the Birth Defect Prevention Act (SB 950). In 1989, the California Assembly passed AB2161 which requires DPR to conduct dietary risk assessments for all pesticides with food crop uses. The purpose of this current risk assessment is to address the potential adverse health effects for the general public exposed to methidathion through the foods they eat and the water they drink (Kyriakidis *et al*., 2000).

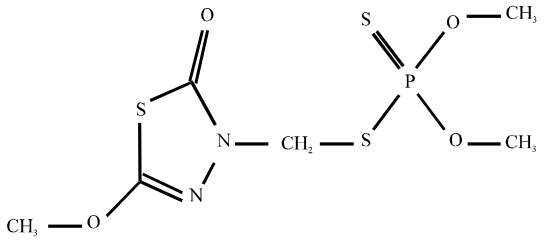

Fig. 1: Methidathion formula (Kim *et al*., 2011)

Methidathion was placed in reevaluation in 1989 along with chlorpyrifos, diazinon and ethyl parathion which were used as dormant sprays to control scale and other pests on almond trees. Field and laboratory studies indicated that Methidathion had the greatest effect on cholinesterase inhibition of these four organophosphate pesticides with the exception of ethyl parathion. The principle route of exposure to these chemicals for raptors appears to be the dermal route from perching on sprayed trees. DPR concluded that no mitigation was needed because the elimination of ethyl parathion resulted in a significant reduction in the raptors identified with lowered cholinesterase levels and therefore, continued use of the other three organophosphates did not pose a significant hazard. CDFG concurred with DPR's decision.

Methidathion may be applied directly to the soil by injection, shank or chisel. It may also be applied as a spray by ground or aerial application. The application rate for most tree crops was 4-12 lbs of product (1-4 lbs methidathion) per acre per application, except for citrus fruit and pecans which may be applied up to 20 and 16 lbs per acre, respectively. For deciduous fruit, almond and walnut trees, the product is usually applied as a dormant spray and diluted in a minimum of 20 and 50 gallons of water per acre for aerial and ground application, respectively. For citrus, olives and pecans, the product is diluted in a minimum of 1000, 400 and 200 gallons of water per acre, respectively. Generally, only one application per season is made to deciduous fruit, almond and walnut trees. Up to 2 applications per season may be permitted with citrus, pecans and olives. Application rates for artichokes, cotton and safflower ranged from 1-4 lbs of product per acre per application. No >32, 16 and 6 lbs per acre should be applied to artichokes, cotton and safflower per season, respectively. The product is diluted in a minimum of 3-50 gallons of water per acre for these crops depending on the crop and the type of application (Lino and da Silveira, 1994).

South West region of Saudi Arabia is suitable for agriculture and there are many farm were spread among this region, these farms generally use methidathion to control different type of pests.

Although, there are many studies focused on methidathion structure and application, however, there are few studies were investigated methidathion effects on different body organs and systems (Altuntas *et al*., 2002a, b; Min *et al*., 2005; Ozdemir *et al*., 2009).

Consequently, the aim of this study was to provide a model fill some of the void in the evaluation of the toxicity potential of methidathion on genetic materials *in vivo* using micronucleus, NDI (Nuclear Division Index) and NDCI (Nuclear Division Cytotoxicity Index).

The micronucleus test is an *in vivo* and *in vitro* short-time screening test (Heddle, 1973; Schmid, 1975) is widely used to detect genotoxic effects (Villarini *et al*., 1998). It is one of the simple, reliable, cheap and rapid screening system for both clastogenic effects (chromosome breakage, formation of a centric fragments) and an eugenic (chromosome lagging and effects on spindle) (Heddle *et al*., 1993, 2011). Clastogenic and an eugenic agents affect the spindle apparatus which can be differentiated on basis of the relative induced micronucleus sizes or with the presence of kinetochores. Micronuclei formation can occur in any nucleated and dividing tissue of any species (Heddle, 1973).

MNi are morphologically identical to but smaller than nuclei. They also have the following characteristics: the

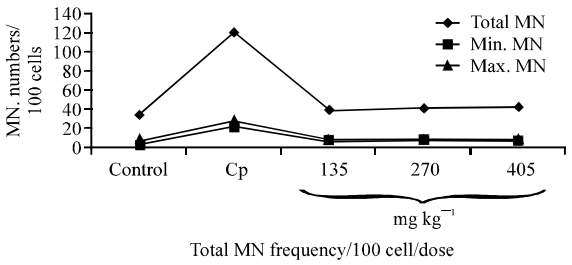


Fig. 2: Total micronucleus frequency at all groups observed in normochromatophyl and polychromatophyl bone marrow cells

diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively. MNi are non-refractile and they can therefore be readily distinguished from artefact such as staining particles. MNi are not linked or connected to the main nuclei. MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary. MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense (Fenech, 2000) (Fig. 2).

Nuclear Division Index (NDI) is useful parameters for comparing the mito-genic response of lymphocytes and cytostatic effects of agents examined in the assay. NDI is often calculated using the equation (Eastmond and Tucker, 1989; Fenech, 2000):

$$NDI = \frac{M1+2(M2)+3(M3)+4(M4)}{N}$$

Where:

M1-M4 = The number of cells with one to four nuclei

N = The total number of viable cells scored

A more accurate assessment of nuclear division status is obtained if necrotic and apoptotic cells are included in the total number of cells scored because at higher toxic doses of chemicals tested one can expect a very large proportion of cells becoming non-viable. It is therefore important to note that both binucleate ratio and the NDI are over estimated if necrotic and apoptotic cells are not included when scoring cells. A more accurate estimate of nuclear division status and cell division kinetics can be obtained using the following modified equation which takes account of viable as well as necrotic and apoptotic cells (Fenech, 2000):

$$NDCI = \frac{Ap+Nec+M1+2(M2)+3(M3)+4(M4)}{iV^*}$$

Where:

NDCI = Nuclear Division Cytotoxicity Index
Ap = Number of apoptotic cells
Nec = Number of necrotic cells
M1-M4 = Number of viable cells with 1-4 nuclei
N* = Total number of cells scored (viable and non-viable)

Necrosis and apoptosis is important for the accurate description of mechanism of action and measurement of cellular sensitivity to a chemical or radiation (Fenech, 2000).

Cells showing chromatin condensation with intact cytoplasmic and nuclear boundaries or cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane are classified as apoptotic. Cells exhibiting a pale cytoplasm with numerous vacuoles and damaged cytoplasmic membrane with a fairly intact nucleus or cells exhibiting loss of cytoplasm and damaged/irregular nuclear membrane with a partially intact nuclear structure are classified as necrotic (Fenech, 2000).

In the light of the pervious data, the experiment is designed to assess the toxicity impact of methidathion on rat's bone marrow cells.

## MATERIALS AND METHODS

**Test chemical and positive control:** Methidathion (Fig. 1) was obtained from the local market. Cyclophosphamide obtained from Sigma-Aldrich Chemical Company.

**Animals:** Experiments were performed with female Wistar rats obtained from the animal house at King Khalid University, Department of Biological Sciences (weight range, 220-240 g) that were kept at controlled environmental conditions at room temperature (22±2°C) and under 12 h light/dark cycles. In preliminary assay, a group of five female Albino rats receiving the maximum recommended dosage of methidathion. The animals were observed for clinical signs and symptoms for reactions to treatment at 1, 4, 24 and 48 h. No clinical signs or mortality was observed. For the time-course experiment, rats were divided at random into 5 groups, 6 animals in each group. All injections were given by the intraperitoneal (i.p.) route. The control group received a single injection of 0.9% saline solution.

In order to test the effect of methidathion toxicity (cytotoxicity and genotoxicity), three treatment conditions were evaluated. Subsequent dose levels were fixed at 75, 50 and 25% of the $LD_{50}$ values amounting to 405, 270 and 135 mg $kg^{-1}$ body weight, respectively. The exposure time to Methidathion was 30 days. The positive control group was injected by single dose Cyclophosphamide. Six male rats were administered Cyclophosphamide in normal saline (10 mL $kg^{-1}$) by intraperitoneal injection at 0.75 mg $kg^{-1}$ served as positive controls and the exposure time was 24 h.

**Micronucleus assay:** At the end of the experimental period, animals (rats from each of the treatment groups and the positive control group) were sacrificed by cervical dislocation at the noon of the next day after the last injection. Both the femora were removed and cleaned with gauze by removing all the adhering muscle and tissue and subjected to micronucleus assay. The bone marrow was flushed out from both femurs using 1 mL of RPMI 1640 medium (bone marrow cells were pooled from both femurs of each animal) and centrifuged at 1000 rpm for 10 min. The cell were washed twice with Phosphate Buffered Saline (PBS) followed by centrifugation at 1000 rpm for 10 min. The supernatant was removed by aspiration and the cells were fixed in cold 3:1 methanol:acetic acid. Slides were prepared by dropping portions of the pellet on slides then air-dried for 20 min. Slides were stained with 5% solution of Giemsa in 0.01 M phosphate buffer at pH 7.4 according to the method described by Schmid (1975) with slight modifications. Two bone marrow smears per animal were prepared.

**Genotoxicity scoring:** The micronucleus frequency (expressed as percent micronucleated cells) was determined by analyzing the number of micronucleated PCEs from at least 1000 PCEs per preparation (Fenech, 2000).

**Cytotoxicity scoring:** The PCE:NCE ratio, necrotic and apoptotic cells were also calculated to evaluate the cytogenetic effect of Methidathion by scoring the number of PCEs and NCEs in 1000 cells per animal (Ouanes *et al*., 2003).

**Statistical analysis:** Collected data from all treated groups were analyzed using ANOVA. The $p<0.05$ to indicate statistical significance. All results were expressed as mean±SD for three animals in each group.

## RESULTS AND DISCUSSION

This study of the clastogenic effect of methidathion using micronucleus test revealed that there was no significant induction of micronucleus in rat bone marrow cells. Comparing results showed that control group and the experimental group have almost same value of MNE. Whereas the positive control showed more significant comparing with experimental and negative group. The microscopic investigation of micronuclei also not showed a variation in their shapes and number per cell as shown

Table 1: Clastogenic effect of methidathion comparing different control groups
Neg. and Pos. Control+treatment groups

| | | | | | Comparing to control | | |
|---|---|---|---|---|---|---|---|
| Bone marrow | Total MN | Min. MN | Max. MN | Abundant MN type | f | df | p-value |
| Control | 33 | 5 | 9 | M1 | - | - | - |
| Cp | 120 | 21 | 21 | M1 | 133.90 | 1 | <0.01 |
| 405 mg $kg^{-1}$ | 42 | 7 | 9 | M1 | 14.60 | 1 | >0.50 |
| 270 mg $kg^{-1}$ | 41 | 8 | 9 | M1 | 8.45 | 1 | >0.50 |
| 135 mg $kg^{-1}$ | 39 | 6 | 9 | M1 | 5.37 | 1 | >0.50 |

Cp: Cyclophosphamide as positive control; M1: One micronuclei per cell; Neg.: Negative; Pos.: Positive

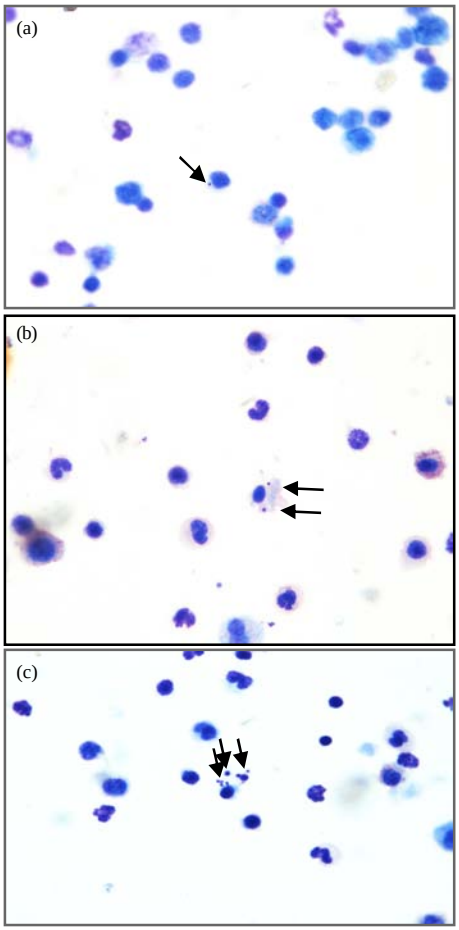


Fig. 3: a) M1 (one micronuclei per cell); b) M2 (two micronucleus per cell); c) M3 (three micronucleus per cell) and M4 (four micronucleus per cell) were observed in different groups, cells were stained by Giemsa and May-Grunwald stain and photographed by Nikon microscope 40X

in Table 1. The Micronucleus type (M1) was found in all groups while types (M2), (M3) and (M4) were not present in all groups including negative control were it was very slightly in positive control (Table 1 and Fig. 3a-c).

NDI is often calculated according to the method of Eastmond and Tucker (1989). Five hundred viable cells are scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and calculate the NDI using the equation (Table 1 and Fig. 3a-c):

$$NDI = \frac{M1 + 2(M2) + 3(M3) + 4(M4)}{N}$$

Where:
M1-M4 = The number of cells with one to four nuclei
N = The total number of cells scored

The microscopic investigation of micronuclei also showed a variation in their shapes and number per cell as shown (Table 2 and Fig. 3c). The micronucleus type (M1) was found in all groups while type (M2), (M3) and (M4) micronucleus were found very rare in all treated groups, apoptotic and necrotic cells were increased depending on the dose (Table 2 and Fig. 3a-c).

**Cytotoxicity of methidathion:** To asses cytotoxicity of methidathion, necrotic and apoptotic cells were scored. Consequently NDI and NDCI were calculated and expressed in Table 2 and Fig. 4.

The microscopic investigation of necrotic and apoptotic cells also showed very rare variation in all treated groups, apoptotic and necrotic cells were increased depending on the dose (Table 2 and Fig. 5b).

Micronucleus test is considered as a good bioindicator (Fenech, 2010). Bioindicators offer several types of unique information not available from other methods and may be summarized as: early warning of environmental damage; the integrated effect of a variety of environmental stresses on the health of an organism and the population, the community and the ecosystem; relationships between the individual responses of organisms exposed to pollution and the effects at the population level; early warning of potential harm to human health based on the responses of wildlife to pollution and the effectiveness of the remedial efforts in decontaminating waterways (Villela *et al*., 2006). It is very necessary to keep these data reachable to make evaluation for the experiment.

The carcinogen potential of methidathion, a dimethoxyorganic phosphorus pesticide and cholinesterase inhibitor was evaluated by the Health

Table 2: Clastogenic effect of methidathion comparing different control groups

| Control+treatment groups | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | Comparing to control | | |
| Bone marrow | Ac | Nc | NDI | NDCI | f | df | p-value |
| Control | 1 | 0 | 0.0420 | 0.008 | - | - | - |
| Cp | 1 | 0 | 0.0780 | 0.000 | 352.30 | 1 | >0.50 |
| 405 mg $kg^{-1}$ | 58 | 28 | 0.0270 | 0.047 | 121.00 | 1 | <0.50 |
| 270 mg $kg^{-1}$ | 22 | 21 | 0.0290 | 0.109 | 0.08 | 1 | <0.50 |
| 135 mg $kg^{-1}$ | 10 | 7 | 0.0305 | 0.260 | 0.06 | 1 | <0.05 |

Ac: Apoptotic; Nc: Necrotic; NDI: Nuclear Division Index; NDCI: Nuclear Division Cytotoxicity Index

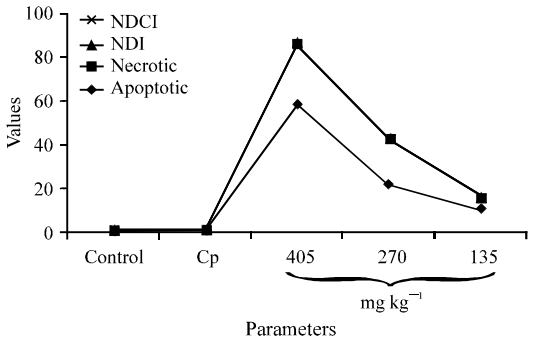


Fig. 4: Expression NDI, NDCI, necrotic and apoptotic cells comparing to negative and positive control

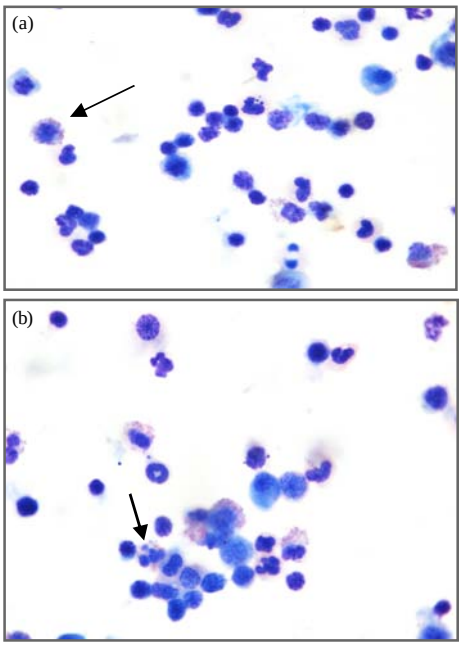


Fig. 5: a) Presents apoptotic cells; b) presents necrotic were observed in different groups, cells were stained by Giemsa and May-Grunwald stain and photographed by Nikon microscope 40X

Effects Division of the Office of Pesticide Programs using a consensus peer review process and the EPA's guidelines for risk assessment. Methidathion was categorized as a group C (possible human) carcinogen based upon evidence of an increased incidence of benign and malignant hepatocellular tumors (Quest *et al*., 1990). Although, the same study confirmed that Methidathion was not genotoxic in a variety of *in vitro* or *in vivo* tests designed to detect DNA damage, chromosome aberrations, gene mutations and sister chromatid exchange (Quest *et al*., 1990). However, it is unclear from these data if either of these effects are mediated through cytological disruption, inhibition or some other mechanism. Therefore, the mechanism of action of methidathion it seems that it is still a mystery or unclear.

Methidathion molecule can pass throw cell membrane easily. This result has appeared clear through the significant value of apoptotic cells and necrotic cells, of the second part of current study. Takayasu suggested that methidathion can distribute with different concentrations inside the body as follows; 66.2 in heart blood, 8.33 in peripheral blood, 8.80 in urine, 2000 in the brain (frontal lobe), 4800 in the left lung, 810 in the liver, 150 in the left kidney and 64,000 in the stomach contents (Takayasu *et al*., 2012) which demonstrate clearly that methidathion penetrate with high levels. But the entry of this molecule not cause any chromosomal defects either methidathion can't penetrate nuclear membrane or the chemical structure is not interacting with DNA polymer or the molecule may interact with special detoxification enzymes. Hence, more specific research to identify and explore the mechanism of action of methidathion is needed.

## CONCLUSION

Results showed that there was no significant increase in the frequency of micronucleated in bone marrow cells. However, it showed significant increase in necrotic and apoptotic cells following methidathion administration in a dose-dependent manner comparing to positive and negative control groups. In light of these results, methidathion can be considered unsafe to use as insecticide specially for human water or food resources control.

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