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ORIGINAL ARTICLE

Lack of micronuclei formation in bone marrow of rats after oral exposure to thiocyclam insecticide

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Abstract In this study, a nereistoxin analogue insecticide, thiocyclam, was administered to adult male albino rats by gavage dose of 135, 270 and 540 mg/kg b.w. repeated for 5 days at 24 h intervals. Control animals received only water. Thiocyclam was tested for its potential to cause genotoxic effects in rat bone marrow cells using an *in vivo* micronucleus assay. After 24 h of the last treatment, rats from all dose levels were sacrificed. Bone marrow cells were collected and assayed for the presence of micronuclei. Thiocyclam did not cause any increase in the incidence of micronucleated polychromatic erythrocytes in rats bone marrow at any of the dose levels. The polychromatic erythrocytes/normochromatic erythrocytes (PCE:NCE) ratio was found to be in the range from 0.50 ± 0.11 to 0.55 ± 0.02. The results of this study demonstrate that the effect of thiocyclam is not significant in the rat *in vivo* micronucleus assay.

1. Introduction

Mutation occurs spontaneously or under the influence of external factors, such as a broad spectrum of chemicals, including pharmaceutical, pesticides, and petroleum products, that are present in the air we breathe, the water we drink, the food we eat and the region in which we work and live (WHO, 1985, 1992). Therefore, monitoring and evaluating the activities of the mutagenic substances in the environment are important, as they often induce genotoxicity. The purposes of genotoxicity testing are to assess the mutagenicity of chemicals, in order to protect the human gene pool and to identify potential carcinogens (Vanhauwaert et al., 2001).

Pesticides are agents used to kill or control undesired pests, such as insects, weeds, rodents, fungi, bacteria or other organisms. Until recently, pesticides were not considered a problem. On the contrary, the use of these compounds was considered to have a vital role in controlling agricultural and a sign of progress and modernization. With this attitude an increase use of pesticides among the agricultural establishment, farmers' was observed. Unfortunately, this increase has not been accompanied by a full understanding of the potential risk and possible adverse health effects to humans, domesticated

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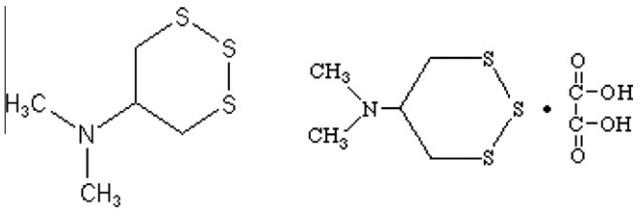

Figure 1 Structural formula of (a) thiocyclam (*N*,*N*-dimethyl-1,2,3-trithian-5-amine) and (b) thiocyclam hydrogen oxalate.

animals and the environment (Weiss et al., 2004). Since several studies have shown that exposure to pesticides may are mutagenic and induce genotoxic effects (Dulot et al., 1985; Garaj-Vrhovac and Zeljezic, 2001, 2002; Leiss and Savitz, 1995; Zeljezic and Garaj-Vrhovac, 2004), the evaluation of the genotoxicity of pesticides in use is of urgent concern.

The micronucleus assay using immature bone marrow erythrocytes has been widely used as a simple and sensitive short-term screening method *in vivo* for determining the mutagenicity of chemical substances (Heddle, 1973, 1990; Heddle et al., 1991; Schmid, 1975). Micronuclei arise from acentric fragments, or whole chromosomes, that fail to incorporate into the daughter nuclei during mitosis. Consequently, enumeration of micronuclei in the cytoplasm of the interphase daughter cell has been used to quantitate clastogenic or aneugenic chromosome DNA damage (Heddle, 1973; Schmid, 1975).

Currently, a lot of pesticides are used in Asser region (South west region of Saudi Arabia) among them is thiocyclam hydrogen oxalate (the trade name is Evisect®; Figure 1). Thiocyclam is a nereistoxin analogue insecticides, selective stomach insecticide with contact action for *lepidopterous* and *coleopterous* pests; some dipterous and *thysanopterous* pests. Evisect was reported to have many side effects, including irritating to skin and eyes, may cause sensitization by skin contact and it is harmful if swallowed (Syngenta, 2001; Ware and Whitacre, 2004). At low concentrations thiocyclam act as acetyl choline receptor agonists and as channel blockers at higher concentrations (Ware and Whitacre, 2004). Evisect has not been tested for mutagenicity, in this context; the current study was conducted to provide additional data on the effect of thiocyclam on induction of micronuclei in rat bone marrow cells.

2. Materials and methods

2.1. Test chemical and positive control

Thiocyclam (Evisect®; CAS No.: 31895-22-4) was obtained from the local market. Mitomycin C was obtained from Sigma Chemical Company.

2.2. Animals

Young adult albino rats *Rattus norvegicus* were obtained from the animal house at King Khalid University, Department of Biological Sciences. Animals were about 2 months old and of 120–150 g weight. The animals were housed in a controlled environment of temperature (22 °C ± 3). All animals were received normal rat food and tap water *ad libitum*.

2.3. Selection of dosage

The Syngenta Safety Data Sheet (Syngenta, 2001) showed that the acute oral toxicity 540 mg/kg body weight is the LD50 in rat for Evisect giving orally. In this study, 540 mg/kg body weight was fixed as the maximum dose. Subsequent dose levels were fixed at 50% and 25% of the LD50 values amounting to 270 mg/kg and 135 mg/kg body weight, respectively. Three male mice were administered mitomycin C in normal saline (10 ml/kg) by intraperitoneal injection at 0.75 mg/kg served as positive controls.

2.4. Groups of animals under investigation and plan of thiocyclam administration

In preliminary assay, a group of five male albino rats receiving the maximum recommended dosage of thiocyclam. 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.

Animals were divided into four main groups (groups A–D) and housed in four cages; each containing three rats. The treated groups were dosed by oral gavage once daily at 12 noon for five consecutive days. The dose levels used were: Group A: This group is the control animals received only the standard diet and distilled water. Group B: Rats of this group received thiocyclam (25% of the LD50 for 5 days in distilled water by gavage). Group C: Rats of this group received thiocyclam (50% of the LD50 for 5 days in distilled water by gavage). Group D: Rats of this group received thiocyclam (LD50 for 5 days in distilled water by gavage). All animals were examined immediately after each dose, approximately 1 h after each dose, and at least daily for the duration of this assay for toxic signs and/or mortality.

2.5. Micronucleus assay

At the end of the experimental period, animals (mice from each of the treatment groups, and the positive control group) were sacrificed by cervical dislocation at the morning 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 by Agarwal and Chauhan (1993). Three bone marrow smears per animal were prepared.

2.6. Scoring

The micronucleus frequency (expressed as percent micronucleated cells) was determined by analyzing the number of micro-

nucleated PCEs from at least 2000 PCEs per preparation (Fenech, 2000). The unit of scoring was the micronucleated cell, not the micronucleus; thus, the occasional cell with more than one micronucleus was counted as one micronucleated PCE. The PCE:NCE ratio was calculated to evaluate the cytotoxic effect of thiocyclam by scoring the number of PCEs and NCEs in 2000 cells per animal (Ouanes et al., 2003).

2.7. Statistical analysis

Micronucleus data were analyzed using Student's *t*-test. Statistical analysis was performed using SPSS/10.5 Software; *p* values of <0.05 were considered to indicate statistical significance. All results were expressed as mean ± SD for three animals in each group.

3. Results

Results obtained by the micronucleus analyses are summarized in Table 1. In a preliminary assay involving a group of five male albino rats receiving the maximum recommended dosages of the thiocyclam, no mortality was observed after 48 h, suggesting this is an adequate dosage for micronucleus induction evaluation.

The micronucleated polychromatic erythrocyte (MNPCEs) in both investigated dose groups (135 and 270 mg/kg) were 8.0 ± 1.7 and 9.0 ± 1.0, respectively. When these data were compared with control group they were not statistically significant. However, the highest dose of thiocyclam (540 mg/kg) induces statistically significant MnPCEs ($p < 0.05$). One micronucleus was mainly observed at all dose levels but at high concentration two micronuclei were observed in some polychromatic cells. Mean number of MPCE per 2000 PE in positive controls receiving mitomycin C was significantly increased, suggesting that the test method was valid and the results were not an artefact.

The PCE:NCE ratio in groups treated with different doses of thiocyclam range from 0.50 ± 0.11 to 0.55 ± 0.02, while the PCE:NCE ratio in the group treated with mitomycin C (positive control) was 0.41 ± 0.02.

4. Discussion

In this *in vivo* study, the rat was chosen since rats are the most widely used animals for general toxicologic, carcinogenic, pharmacokinetic and toxico-kinetic studies. Many data are available and would be useful for the overall assessment of chemical substances (EPA, 1998). The oral route was selected because it was the one route of exposure relevant to humans that could maximize delivery of thiocyclam to the target tissue. Gavage was used instead of drinking water to allow more precise dosing (Oller and Erexson, 2007).

Currently, the rat micronucleus assay is one of the methods used to evaluate the genetic toxicity of chemicals *in vivo*. The rodents (mouse and rat) bone marrow micronucleus test is one of several available *in vivo* mammalian test system for the detection of chromosomal aberrations (Morita et al., 1997). In the last three decades, toxicologists have often used the mouse and bone marrow micronucleus test since it has advantages in speed, simplicity, and cost effectiveness in comparison to other *in vivo* systems for testing chromosomal aberrations (e.g. the cytogenetic test).

In the micronucleus formation, when a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualization of micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of induced chromosome damage (Mavournin et al., 1990; OECD 474, 1997).

A single sex study was considered adequate as extensive studies of clastogens (chromosome breakage) by micronucleus assay have led to the following conclusions: (1) any mouse and rat strain is acceptable; (2) one sex, either male or female can be used; (3) treatment by either intraperitoneal injection or oral administration is acceptable; (4) examination 24–48 h after a single administration of at least one dose will be acceptable to evaluate the mutagenicity of chemicals (Sutou, 1996).

Though the micronuclei assay is very useful as a short-term screening method. It is difficult to evaluate the safety of chemical substances based on only one short-term test system. It is

Table 1 Incidence of micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow of albino rats.

Group (control)	Dose (mg/kg b.w.)	No. of animals	MNPCEs/PCE scored (individual data)	Mean ± SD	Mean ± SD (group data)	Frequency (%) (individual data)	Frequency (%) (group data)	PCE/NCE
A	–	3	7/6000 (2,3,2) 4/6000 (1,2,1) 7/6000 (3,2,2)	2.3 ± 0.6 1.3 ± 0.6 2.7 ± 0.6	6.0 ± 1.7	0.12 0.07 0.12	0.10 ± 0.03	0.55 ± 0.02
B	135	3	9/6000 (3,1,3) 6/6000 (2,1,3) 9/6000 (3,4,2)	1.6 ± 1.2 2.0 ± 1.0 3.0 ± 1.0	8.0 ± 1.7	0.15 0.10 0.15	0.13 ± 0.03	0.50 ± 0.11
C	270	3	9/6000 (3,2,4) 8/6000 (3,3,2) 10/6000 (4,3,3)	3.0 ± 1.0 2.6 ± 0.6 3.3 ± 0.6	9.0 ± 1.0	0.15 0.13 0.16	0.15 ± 0.02	0.51 ± 0.05
D	540	3	13/6000 (6,3,4) 9/6000 (2,4,3) 15/6000 (4,5,6)	4.3 ± 1.5 3.0 ± 1.0 5.0 ± 1.0	12.3 ± 3.1*	0.21 0.15 0.25	0.20 ± 0.05*	0.54 ± 0.10

* $p < 0.05$.

recommended in the guidelines for genotoxicity studies of chemical substances that the *in vitro* bacterial reverse mutation assay (Ames test), *in vitro* mammalian cells chromosome abnormal test and *in vivo* rodent micronucleus assay should be used, as in the series. The *in vivo* micronucleus assay in bone marrow cells is used to identify chromosome aberration, thus the target cells must be effectively exposed to the chemical stuff tested (Sato and Tomitab, 2001). However, the bone marrow micronucleus assay may not be suitable for clastogenic compounds, which are reactive to other cellular molecules and have barrier for reaching bone marrow cells (Sato and Tomitab, 2001). The compounds administered orally, intraperitoneally or intravenously may be inactivated before it reaches the target bone marrow cells. Thus, a micronucleus assay using other organs or tissue.

The number of MNPCEs in the positive control receiving mitomycin C showed significant increase compared to the negative control. This finding demonstrate the validity of the experiment and the sensitivity of the animal strain to the clastogenic agents as observe by Krishna and Hyashi (2000).

The results of this study clearly demonstrate that thiocyclam does not induce micronuclei formation in bone marrow cells after different gavage doses to albino rats. Thus, thiocyclam does not act as a genotoxic material based on the available *in vitro* and *in vivo* data. Our results are in agreement with the WHO and the Health Effects Division Office of Pesticide Programs US Environmental Protection Agency that considered thiocyclam as moderately hazardous pesticide and not carcinogenic to human (EPA, 2006; WHO, 2005).

The PCE:NCE ratio is used to evaluate the cytotoxicity and heavy DNA damages leading to cell death or apoptosis (Ouanes et al., 2003). The analysis of the PCE:NCE ratio at different doses of thiocyclam showed that animal groups treated had a ratio close to the values reported for mice in literature that is between 0.5 and 1.0 (Adler, 1984), these results showed that the thiocyclam is not genotoxic.

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