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·*Original Article*·

Effect of malathion on the male reproductive organs of earthworms, *Eisenia foetida*

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Abstract

Aim: To observe the cytotoxic effect of the organophosphate insecticide malathion in the reproductive tissues of the earthworms, *Eisenia foetida*. **Methods:** Worms were nourished in soil treated with malathion at single sub-lethal doses of 0, 80, 150, 300 and 600 mg·kg^{-1} soil. (LD_{50} = 880 mg·kg^{-1} soil) and evaluated on days 1, 5, 15 and 30 after exposure. The body weights were recorded and male reproductive organs evaluated. **Results:** Malathion-treated animals showed a significant reduction in body weight in a dose-dependent manner. Malathion treatment modified the disposition of spermatozoa in the basal epithelium of the spermatheca. The Br-deoxyuridine test showed a significant rise in cells in phase S on days 5 and 15. Also, a higher percentage of spermatogonia with fragmented DNA were observed by means of the TdT-mediated dUTP nick-end labeling (TUNEL) technique in the spermatheca of treated animals. **Conclusion:** Treatment with malathion decreased the body weight and the spermatic viability in spermatheca, altering the cell proliferation and modifying the DNA structure of spermatogonia. *(Asian J Androl 2005 Mar; 7: 97–101)*

Keywords: *Eisenia foetida*; malathion; reproductive organs; apoptosis

1 Introduction

Organophosphorates are frequently used in pesticides due to their low half life. Malathion (5-1, 2-di-etoxycarbonyl ethyl O, O dimethyl phosphorodithioate) is considered one of the most innocuous organophosphorates for humans. This fact has led to an indiscriminate use of this pesticide [1]. Malathion-induced morphologic alterations have been found in the mouse testes [2]. In birds, malathion induces germ cell degeneration possibly by altering cholinergic functions [3].

Routine evaluation of the effect of xenobiotics on human health has required the use of mammals as biological models [4]. Because of restriction on experimental subjects, there has been an increasing interest in alternative models. *Eisenia foetida* (Annelida, Oligochaeta) is a good candidate as a complementary model for *in situ* and laboratory studies [5]. Worms make direct contact with the ground and absorb pesticides both from the skin and the digestive system, thus the pollutants are absorbed 5 to 10 times [6].

The present work evalu ates the changes produced by the insecticide on male reproductive organs.

2 Materials and methods

2.1 Animals

Earthworms *E. foetida* (agricultural "Los Nogales", Paine, Chile) were kept in plastic boxes with soil-like substrate (size of particle: smaller than 2 mm) and dampened with water (pH 6.5 with a final humidity of 50 % at

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21–22 ºC). There were forty individuals in each treatment group.

2.2 Pesticide and treatment

A commercial solution of malathion (57 %, Laboratorios Farmagro S.A., Lima, Perú) was used. Groups of 40 worms were treated in duplicated assay (n=80). Malathion (LD_{50} = 880 mg·kg^{-1} soil) was applied at a single dose of 80, 150, 300 and 600 mg·kg^{-1} soil dissolved in 10 mL of distilled water. Control group was given only distilled water. The effects were evaluated on days 1, 5, 15 and 30.

2.3 Observations

Body weights were recorded at the beginning and the end of each treatment day. Male gonad segments were fixed in Bouin's solution for 12 hours and embedded in paraffin. Longitudinal sections (5 μm) were processed for histology and immunocytochemistry.

For the determination of cell proliferation, the BrdU staining kit was used (Zimed, San Francisco, California, USA) based on the streptavidin-biotin and diaminobencidine system. Nuclei were stained brown when there was BrdU incorporation. Cells that did not incorporate BrdU showed blue staining by hematoxyline. DNA fragmentation was detected using the TdT-mediated dUTP nick-end labeling (TUNEL) method. The marked cells (dark brown) had their DNA fragmented. A negative reaction was recognized by the nuclei turning blue, which indicates intact DNA.

The changes on male gonad segments were observed according to the method used by Sobarzo and Bustos-Obregón [7]. Four longitudinal sections for each animal were observed under light microscopy at ×400. To evaluate the percentage of cell proliferation and DNA fragmentation, 300 cells from each slide were examined.

2.4 Statistical analysis

Comparison of results was performed using the non-parametric ANOVA test (Kruskal–Wallis) and Dunn's multiple comparisons posttest. INSTAT software (Graphpad software, USA) was used to analyze the data. $P < 0.05$ was considered significant.

3 Results

3.1 Body weights

A significant reduction in the body weight of the animals treated with malathion was observed each day of treatment with 80, 150, 300 and 600 mg·kg^{-1} (Figure 1).

3.2 Immunohistochemical studies

An evaluation of the effects of malathion on *E. foetida* spermatheca is shown in Figure 2. Slides of control individuals showed an ordered and proliferative basal epithelium. Spermatozoa were distributed occupying all the internal lumen of the structure (Figure 2A). Immunohistochemistry of the spermatheca of treated worms showed vacuolization of the basal epithelium and a very anomalous disposition of spermatozoa, which were concentrated in the center of the structure without contacting the basal epithelium (Figure 2B).

3.3 Cell proliferation in seminal vesicles

Spermatogonial morules of control worms showed a negative reaction for cell proliferation (Figure 3A). In contrast, malathion-treated worms presented positive signs of cell proliferation (Figure 3B). Figure 4 shows the percentage of positive BrdU cells in the seminal vesicles of worms treated with malathion. It could be observed that cell proliferation was altered (cf.the controls), increasing statistically at 5, 15 and 30 days with 150 mg·kg^{-1}. The higher dose of malathion increased the BrdU signal on days 1 and 5. The percentage of cell proliferation in the control group was about 15 %.

3.4 DNA fragmentation in spermatogonia

Figure 5 shows the cytotoxic damage in worms treated with 600 mg·kg^{-1} on days 5 and 15. A significant increase in cells with fragmented DNA was seen. On days 1 and 30, the percentage of DNA fragmentation did not show significant differences between the control group and the treated group. The percentage of TUNEL positive cells in germinal cells of control worms was 11 %. Figure 6 shows a positive TUNEL reaction in *E. Foetida* on day 5 with 600 mg·kg^{-1} of malathion. Morules with fragmentation of DNA had a brown nuclei and morules without fragmentation of DNA had a blue nuclei.

4 Discussion

Currently, there is much concern about the adverse effects of environmental chemical agents in biological systems [8]. Malathion is widely used in agriculture, houses and gardens and is lethal to many living systems. However, little is known about the chronic and sub-le-

thal effects it has on reproduction. The present results show a significant loss of weight in earthworms exposed to malathion (Figure 1), with a toxic dose-dependent effect, which supports the results of other authors [9, 10]. Leland *et al.* [11] detected the morphologic changes and symptomatic effects characteristic of acetylcholinesterase inhibition (including weight lost, reduced burying ability and curling) in earthworms exposed to soil polluted by organophosphates. This loss of corporal mass has a multiplicative effect in *E. foetida*, as it also alters its feeding ability, and therefore, its reproductive performance [12, 13]. In the spermatheca of animals treated with malathion (Figure 2) vacuolization of the basal epithelium and absence of integrated spermatozoa to the microvilli were seen. Bustos-Obregón and Goicochea [5] observed a similar phenomenon with parathion, another organophosphorate pesticide, which indicates that these kinds of pesticides may affect the spermatheca content, eliciting phagocytosis of spermatozoa or modifying the anchorage sites between spermatozoa and microvilli in the basal epithelium. This would explain the central disposition of spermatozoa in the

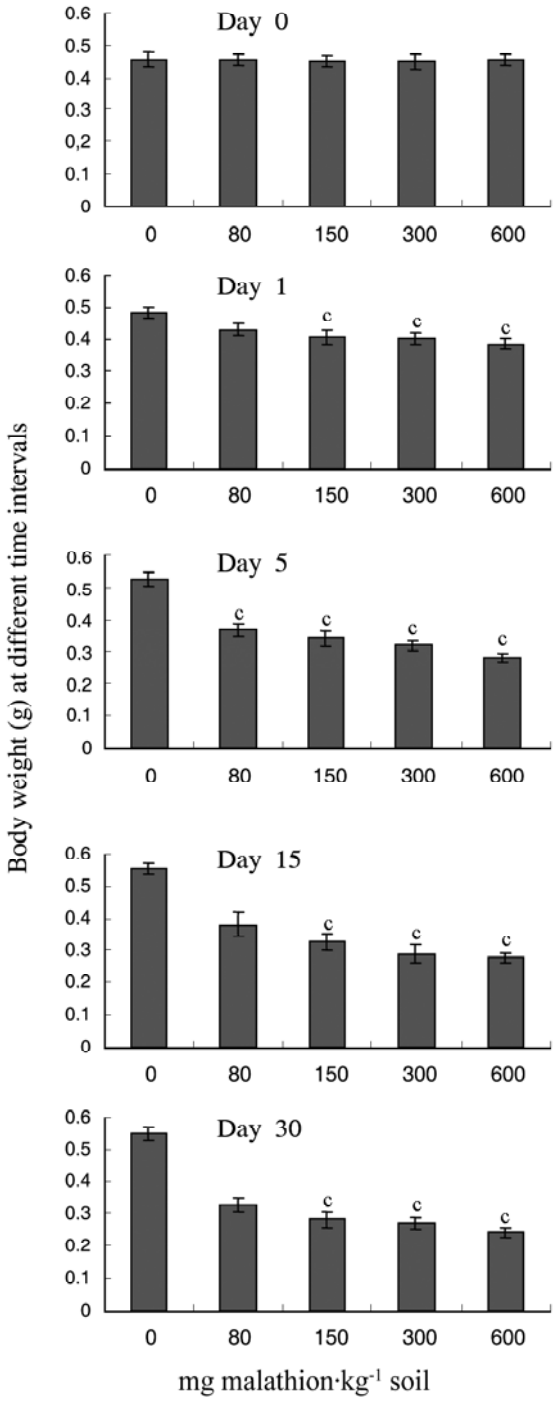

Figure 1. Changes in body weights (g) of *E. foetida* exposed to 0, 80, 150, 300 and 600 mg·kg^{-1} of malathion. Mean ± SD, $^{c}P < 0.01$, compared with control group (0 mg·kg^{-1} of malathion).

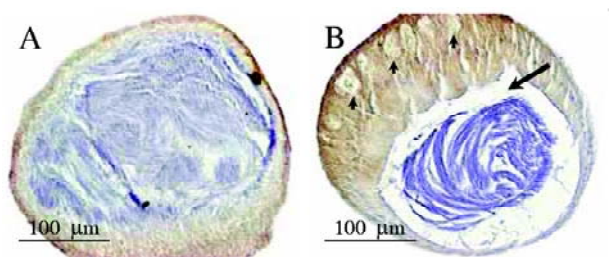

Figure 2. BrdU uptake by spermatheca of *E. oetida*. (A): control showing ordered proliferative basal epithelium. Spermatozoa are included within the microvilli. (B): spermatheca of treated worms with 150 mg·kg^{-1} malathion, day 15. Spermatozoa are located at the center without contact with the basal epithelium (black arrow). The arrowheads show vacuolization.

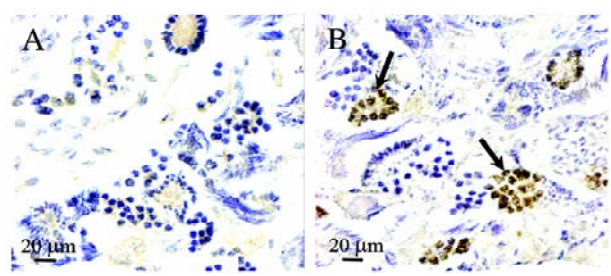

Figure 3. (A): negative BrdU incorporation in spermatogonial morulae of a control worm. Blue nuclei express hematoxyline staining. (B): spermatogonia of worms treated with 150 mg·kg^{-1} malathion, day 5. Black arrows show morules with nuclei of brown colors indicating cell proliferation (positive reaction).

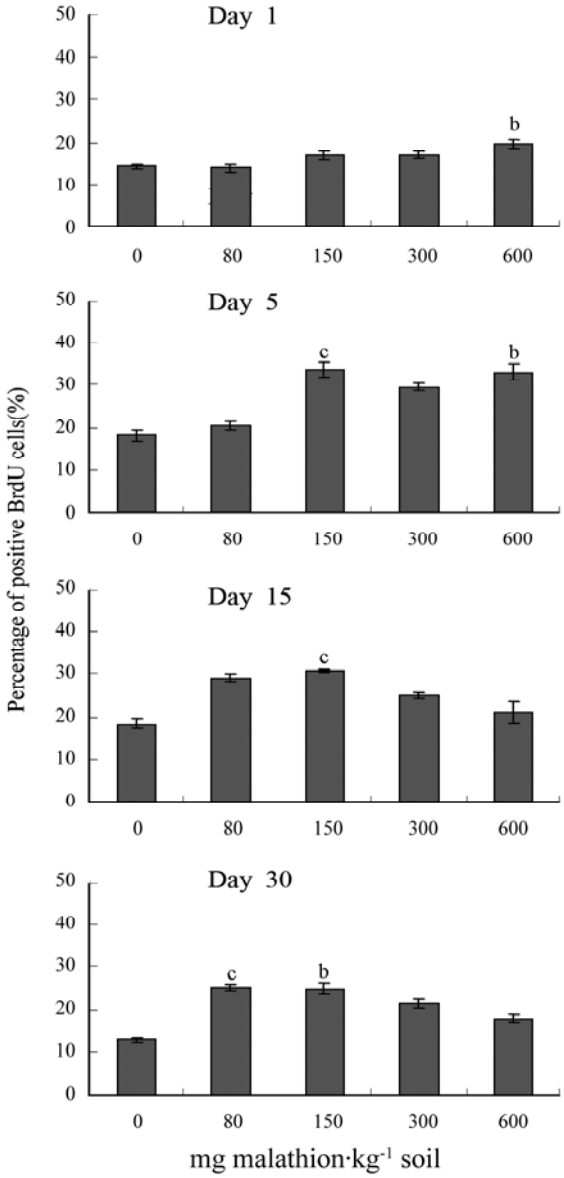

Figure 4. Results of BrdU incorporation at concentrations of 0, 80, 150, 300 and 600 mg·kg^{-1} malathion on days 1, 5, 15 and 30. Mean ± SD, $^{b}P < 0.05$, $^{c}P < 0.01$, compared with control group (0 mg·kg^{-1} of malathion).

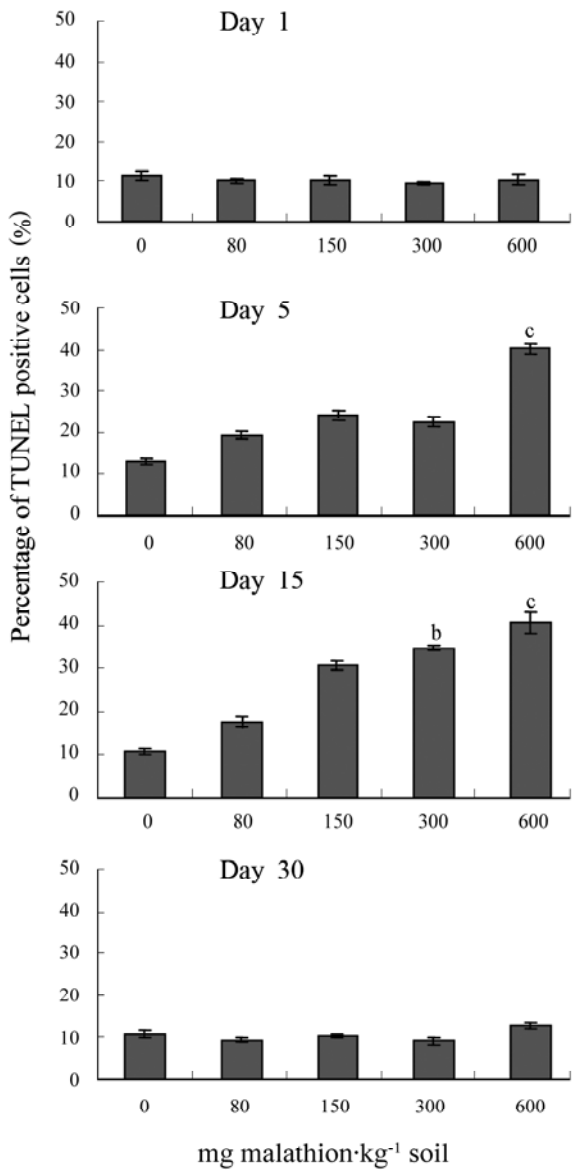

Figure 5. Percentage of TUNEL positive cells (%) in seminal vesicles of *E. foetida* with doses of 0, 80, 150, 300 and 600 mg·kg^{-1} malathion on days 1, 5, 15 and 30. Mean ± SD, $^{b}P < 0.05$, $^{c}P < 0.01$.

spermatheca. These findings are worthy of further investigations. BrdU incorporation and TUNEL studies showed that malathion affected the cell proliferation and DNA fragmentation. These results were clearly observed on days 5 and 15. Blasiak *et al.* reported that malathion has an acute cytotoxic effect leading to cellular death in human lymphocytes, without causing damage to the DNA; but its active metabolites, malaoxon and isomalathion act on DNA, breaking its chains [14]. Bustos-Obregón and González-Hormazabal observed that malathion interfered with mouse testicular function, altering the ADN structure of spermatogonia and maturing spermatids [15]. Thirty days after treatment, cell proliferation was observed, but no significant differences in DNA fragmentation were seen. Studies in mice have reported that malathion was a potent cell cycle inhibitor that induces

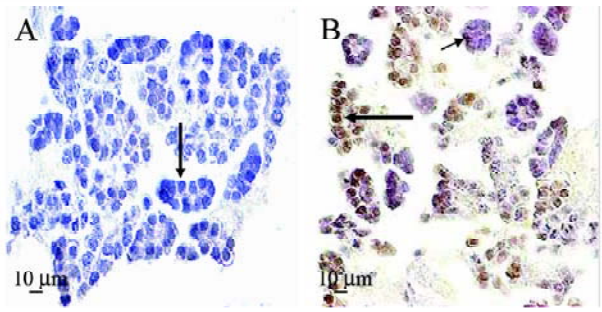

Figure 6. Immunohistochemical study using the TUNEL method. (A): morules of a control worm with a negative reaction where the nuclei are observed with blue colors (black arrow). (B): worm treated with malathion and evaluated on day 5. Morulae with nuclei of brown colors indicating fragmentation of DNA (black arrow). Nuclei of blue colors indicating DNA not fragmented (arrowhead).

DNA damage and capable of interfering with DNA replication; this suggests that malathion is a genotoxic agent and may be regarded as a potential germ cell mutagen [16].

These effects tend to disappear, possibly because of the detoxifying action of *E. foetida* through peritoneal yellow cells, also known as chloragogenous cells, that are found throughout the intestine. These cells play a detoxifying function, similar to hepatocytes in vertebrates and they would allow total biodegradation of malathion and its metabolites [17].

In conclusion, malathion is a cytotoxic agent that interferes with the reproductive function of *E. foetida*, decreasing significantly the body weight dose-dependently. This pesticide also impedes the appropriate anchorage of the sperms on the microvilli of the basal epithelium of spermatheca, possibly altering their viability. In addition malathion alter the cell proliferation and affect the DNA structure of spermatogonia.

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References

1 Thompson TS, Treble RG, Magliocco A, Roetteger JR, Eichhorst JC. Case study: fatal poisoning by malathion. Forensic Sci Int 1998; 95: 89–98.
2 Contreras H, Bustos-Obregón E. Morphological alteration in mouse testis by a single dose of malathion. J Exp Zool 1999; 284: 355–9.
3 Maitra S, Sarkar R. Influence of methyl parathion on gametogenic and acetylcholinesterase activity in the testis of whitethroated munia (Lonchura malabarica). Arch Environ Contam Toxicol 1996; 30: 384–9.
4 Luster MI, Munson AE, Thomas PT, Holsapple MP, Fenters JD, White KL Jr, *et al*. Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. Fundam Appl Toxicol 1988; 10: 2–19.
5 Bustos-Obregon E, Goicochea RI. Pesticide soil contamination mainly affects earthwor m male reproductive parameters. Asian J Androl 2002; 4: 195–9.
6 Sorour J, Larink O. Toxic effects of benomyl on the ultrastructure during spermatogenesis of the earthworm *Eisenia fetida*. Ecotoxicol Environ Saf 2001; 50: 180–8.
7 Sobarzo C, Bustos-Obregon E. Sperm quality in mice acutely treated with parathion. Asian J Androl 2000; 2: 147–50.
8 Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, *et al*. Male reproductive health and environmental xenoestrogens. Environ Health Perspect 1996; 104: 741–803.
9 Senapati B, Biswal J, Pani SC, Sahu SK. Ecotoxicological effects of malathion on earthworms. Soil Biol Biochem 1992; 24: 1719–22.
10 Martikainen E. Toxicity of dimethoate to some animal species in different soil types. Ecotoxicol Environ Saf 1996; 33: 128–36.
11 Leland J, Mullins D, Berry DF. Evaluating environmental hazards of land applying composted diazinon using earthworm bioassays. J Environ Sci Health B 2001; 36: 821–34.
12 Spurgeon DJ, Weeks JM, Van Gestel CA. A summary of eleven years progress in earthworm ecotoxicology. Pedobiologia 2003; 47: 588–606.
13 Bauer C, Rombke A. Factors influencing the toxicity of two pesticides on three Lumbricid species in laboratory test. Soil Biol Biochem 1997; 29: 705–8.
14 Blasiak J, Jaloszynski P, Trzeciaks A, Szyfter K. *In vitro* studies on the genotoxicity of the organophosphorous insecticide malathion and its two analogues. Mutat Res 1999; 445: 275–83.
15 Bustos-Obregon E, Gonzalez-Hormazabal P. Effect of a single dose of malathion on spermatogenesis in mice. Asian J Androl 2003; 5: 105–7.
16 Giri S, Prasad SB, Giri A, Sharma GD. Genotoxic effects of malathion: an organophosphorus insecticide, using three mammalian bioassays *in vivo*. Mutat Res 2002; 514: 223–31.
17 Dhainaut A, Scaps P. Immune defense and biological responses induced by toxics in Annelida. Can J Zool 2001; 79: 233–53.