Investigating the Effect of Malathion on Cytotoxicity and Carcinogenesis in Mice

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ABSTRACT

The use of different agricultural poisons causes different effects on body tissues. In the present study, the impact of Malathion on sex hormones and its carcinogenic impact on skin contact were studied in mice. In this study, 30 adult male mice were divided into treatment and control groups. In the treatment group, Malathion with a concentration of 1 ppm was poured on the skin in the amount of 1 ml for 12 weeks. Eventually, serum levels of testosterone, luteinizing, and follicular stimulating hormones were measured, and tissue samples were taken from the desired organs after necropsy. Cytotoxicity was also measured by micronucleus test and MTT in vitro and IC50 percentage was determined. According to the results, there was no significant change in the luteinizing hormone treatment groups compared to the control group, but follicle-stimulating hormone and testosterone showed a significant decrease. Mild hepatotoxicity was observed in the histological examination in the treatment groups. The frequency of micronuclei in different concentrations of Malathion was significantly different from the control group ($p < 0.05$). From the findings of this study, it can be concluded that skin contact with Malathion can have destructive impacts on sex hormones as well as body tissues.

Key Words: *Cytotoxicity, Carcinogenesis, Malathion, Mice*

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INTRODUCTION

Contact with pesticides is one of the most important health problems. Organophosphates include many types of pesticides, the most common of which is Malathion. This class of pesticides has effects on cholinesterase enzymes, reducing insulin secretion, disrupting the normal cellular metabolism of proteins, carbohydrates, and fats, as well as having cytotoxic effects and affecting mitochondrial function, causing cellular oxidative stress and Nervous and endocrine system problems. These types of toxins are absorbed by the body through the skin, respiratory tract, and digestive system and are quickly converted into active metabolites [1-3].

Humans may be exposed to these types of toxins through occupational pollution such as agriculture, food contamination, or the environment in which they live. Some people, especially those who live in an agricultural environment, are exposed to a high dose of organophosphates, endotoxin, and allergens compared to other people. Therefore, a combination of such substances is one of the causes of various diseases and causes cancer, reproductive disorders, etc. One of the important effects of organophosphates is their effect on sex hormones. Organophosphates can reduce the secretion of some hormones by affecting the body's endocrine system [4-7].

It should be kept in mind that the effect of this category of poisons depends on the dose and duration of contact, and their continuous use in farms and homes can put the reproductive system of humans or animals at risk. Despite conducting much research regarding the possible relationship between organophosphorus pesticides and the increased risk of carcinogenesis and reproductive disorders, the studies conducted have been associated with conflicting results. Based on the conducted research, there is limited evidence regarding the carcinogenicity of Malathion in humans [8, 9], and no decisive and

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comprehensive research has been done in this regard. The purpose of this study was to investigate the toxic effects of Malathion on fibroblast cells, liver organs, and the level of sex hormones including testosterone in mice.

MATERIALS AND METHODS

This experimental study was done on 30 adult male white mice with an approximate weight of 20-30 grams and an average age of 10-12 weeks. Mice were randomly divided into two groups of 15 including control and treatment groups. In in vivo studies, the control animals were exposed during the treatment only by administration of physiological serum in any way that other substances were administered, and in the Malathion group for 12 weeks, once a day by dripping 1 ml of Malathion ppm solution on the skin in the back area of the animal was tested. All mice were kept in standard cages under conditions of 25°C and a 12-hour light-dark cycle. Also, the conditions for working with animals were based on the existing guidelines. After the last administration, sampling was done from two groups.

To collect the samples, the mice were under general anesthesia, and about 3 to 4 ml of blood was collected from their armpit area and collected inside test tubes without anticoagulant. Then the animals were necropsied. Blood samples were centrifuged at 2000 rpm for 15 minutes. After separating the blood sera, the resulting samples were divided into micro tubes (each sample inside 6 microtubes) and kept at a temperature of 70 degrees Celsius. Then the tested parameters including testosterone hormones, FSH (Follicle-Stimulating Hormone), and LH (Luteinizing Hormone) were studied. Then, using the ELISA method, samples and standards were added to the wells of the 96-well plate and after the stages of the ELISA test were read by a spectrometer at a wavelength of 450 nm. Thin tissue samples taken with a diameter of 5 micrometers from different organs were fixed in 10% buffered formalin and after preparation of tissue sections, they were subjected to histopathological examination.

Cytotoxicity study in vitro

The cytotoxic effect of Malathion was evaluated in vitro using MTT and micronucleus test and their inhibitory effect on normal mouse fibroblast cell lines. Also, the morphological changes of the cell line in the vicinity of Malathion were evaluated and recorded.

Cultivation of L929 cells, evaluation of cell morphology, and MIT test

First, the prepared L929 cells were cultured in 25 cc cell culture flasks in PRMI-1640 medium with 10% FCS, 100 units of penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 5.12 µM HEPES (4-(2hydroxyethyl)-1piperazineethanesulfonic acid) and cultured and passaged at 37°C and 5% CO2 until the cells reached the optimal level in terms of number and morphology (after 3-4 passages). After separating the cells from the surface of the flask by EDTA, counting and vital evaluation of the cells was done using the Trypan blue exclusion test. We cultured the number of 5×10^4 cells/ml in the wells of the six-house plates for cell culture with or without Malathion. The concentrations used were (10, 25, 50, 100 µg/ml) which were added to L929 cells [10]. Changes in the morphology and general characteristics of the cells after 48 hours were evaluated in terms of the ability to attach to the plate surface and the degree of granularity using an inverted light microscope and recorded using a digital camera.

The cytotoxic effect of the compounds was measured using the MIT colorimetric test and the quantitative assessment of cell proliferation in vitro.

Finally, according to the optical absorption values obtained by the ELISA reading device, the growth inhibition percentage related to each concentration was determined using the following formula.

$$\text{Percentage of cell growth inhibition} = \frac{Mean\ percentage\ of\ OD\ for\ each\ treatment\ group}{Mean\ percentage\ of\ OD\ for\ the\ control\ group} \times 100 \quad (1)$$

IC50 was calculated after drawing the curve using different concentrations of toxins and the percentage of living cells.

Micronucleus test

The examination method in this test was binucleate cells stopped in the cytokinesis stage based on the method proposed by Fenech. After cell culture and adding Malathion with different concentrations after 24 hours, cell harvesting was done. For this purpose, after separating the cells with 25% trypsin and centrifugation for 10 minutes at 1000 rpm, fixative (methanol 8:1 acetic acid) was added to the cell sediment. The resulting suspension was spread on a glass slide and dried at room temperature. The slides were stained with 10% Giemsa dye for 7 minutes. Cell counting was done by Olympus BH2 microscope with 1000 magnification. About 300 to 700 binucleate cells were counted on each slide. The percentage of binucleate cells with micronucleus was calculated based on the following formula. The total number of binucleated cells was also calculated.

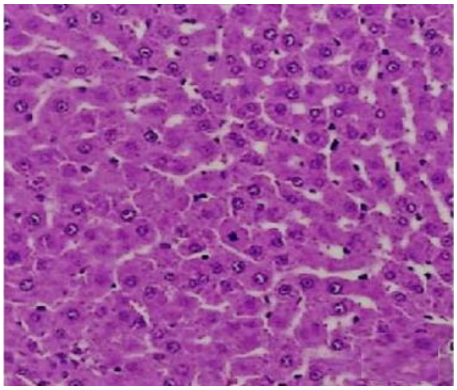
$$\text{Micronucleus percentage} = \frac{The\ number\ of\ binucleated\ cells\ with\ micronuclei}{The\ total\ number\ of\ binucleated\ cells} \times 100 \quad (2)$$

Statistical analysis method

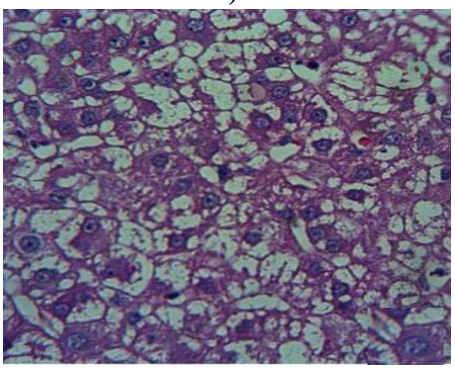
One-way analysis of variance tests was used for statistical analysis. According to the results of the Kolmogorov-Smirnov test, the data distribution was normal, parametric tests were used. Duncan's test was used to find out the location of the difference between the means in cases where the statistical difference between different groups was significant. Statistical calculations were done using SPSS23 software and a significance level of 0.05 was considered. The data in the results section are expressed as Mean ± Standard Deviation. Excel software was used to draw graphs and Prism software was used to obtain IC_{50}.

RESULTS AND DISCUSSION

In the histopathological study, to investigate the effects of compounds in liver tissue, hematoxylin-eosin (H & E) staining was used to identify histological changes compared to normal tissue **(Figure 1)**. The studied parameters were tumor and cytotoxicity and necrosis in the central areas, Hepatitis, Cholestasis, and Steatosis, which was observed in the tested group of mild hepatotoxicity.



a)



b)

Figure 1. The effect of malathion on liver tissue stained with hematoxylin-eosin with 4x magnification; a) liver tissue in the control group with normal morphology, b) accumulation of fat in the cytoplasm of liver cells and Pyknosis of the nucleus of liver cells.

By affecting different concentrations of Malathion (10, 25, 50, 100 µg/ml) on mouse fibroblast L929 cells, inhibition of cell growth was significant. Thus, the highest inhibition was 27.19 and 30.24 at concentrations of 50 and 100 µg/ml, respectively **(Table 1)**.

Table 1. The effect of different concentrations of Malathion on inhibiting the growth of L939 mouse fibroblast cells after 24 hours of treatment.

Concentration (µg/ml)	Average optical absorbance ± standard deviation	Average inhibition percentage
10	0.09 ± 0.01	5.5
25*	0.04 ± 0.07	20.37
50*	0.03 ± 0.002	27.19
100*	0.032 ± 0.021	30.24
Control	0.1 ± 0.01	

*Significant difference compared to the control group (P<0.05)

The average optical absorbance in concentrations of 25, 50, and 100 micrograms/ml is significantly different from the average optical absorbance of the control group, and thus it is significant (P<0.05). The IC50 of the drug for this cell line was 22.90 µg/ml after 24 hours. According to **Table 2**, the frequency of micronuclei in different concentrations of Malathion is not significant.

Table 2. Percentage abundance of micronuclei in Malathion.

Concentration (µg/ml)	Frequency of micronucleus (%)
Control	3.04
10	3.62
25	3.85
50	3.65
100	4.04

The findings of the current study showed that the amount of testosterone and FSH hormones in the test group had a significant decrease compared to the control group **(Table 3)**. This issue can indicate that Malathion has been able to disrupt the body's hormonal system and eventually cause it to decrease. This trend was also observed in the change of LH hormone, but it was not significant.

Table 3. The effect of Malathion on the concentration of LH and FSH and testosterone in mice.

Hormone (concentration)	Control group	Treatment group
FSH (IU/L)*	280%	103%
LH (IU/L)	320%	100%
Testosterone (ng/dL)*	30.25	5.50

*Significant difference compared to the control group (P<0.05)

The findings of the current study showed that different amounts of Malathion inhibit the growth of fibroblasts in vitro. Skin contact with different amounts of Malathion decreased blood levels of FSH and testosterone in mice. Microscopic observations confirmed mild hepatotoxicity. Exposure to Malathion significantly increases breast cancer incidence in rats. Long-term dietary exposure to Malathion has been related to an increased prevalence of liver, nasal, and oral tumors in rats [11-13].

Little is known about the liver effects of Malathion in agricultural workers, however, recent scientific data have shown that Malathion and other pesticides cause kidney and kidney tissue irritations in laboratory animals [14]. Malathion has also been reported to cause genetic damage in a variety of laboratory studies, including in mice-fed malathion-laced seeds. Experimental studies have shown that the commercial insecticide Malathion causes breast cancer in laboratory animals. In addition, the use of Malathion by farmers has been related to an increase in the prevalence of non-Hodgkin's lymphoma [15].

Skin absorption of Malathion is rapid. However, the amount of absorption is highly dependent on the dose and the area of exposure [16]. In a toxicokinetic study, it was observed that male rats administered 28 mg/kg Malathion orally and also dermally exposed to 41 mg/kg Malathion absorbed more than 90% of Malathion. They are excreted in the urine within 24 hours. Residual Malathion was detected in feces, kidneys, blood, liver, and intestines. In another study, inhalation of Malathion for two weeks in rats was associated with the distribution of Malathion in the liver and kidney and tissue toxicity in these organs [17]. Based on comprehensive scientific studies, the mutagenic effect of Malathion has been shown in bacteria, fruit flies, mice, hamsters, and fish. Malathion is recognized by the NIOSH (National Institute for Occupational Safety and Health) as a mutagen. An in vitro Geno toxicity study has shown that oral Malathion causes genetic damage in mice [18].

In a study in which rats were orally exposed to Malathion for a long time, the results showed an increase in the prevalence of oral and nasal tumors in rats and liver tumors in mice [19]. In an 80-week study in rats, Malathion was administered orally at doses of 0, 359, and 622 mg/kg per day, but no significant finding of Malathion carcinogenesis was [20]. In another study, researchers administered dietary doses of 0, 166, and 332 mg/kg per day to rats for 103 weeks, but no data on the carcinogenicity of Malathion were observed in rats [21]. In a two-year study, researchers administered oral doses of 2, 359, 739, and 868 mg/kg of Malathion per day to female mice. They observed a statistically significant increase in liver adenomas and carcinomas at the highest dose tested [22].

In a bioassay study in rats, Malathion was administered at different doses. They reported that there was evidence of carcinogenicity at doses of 1476 and 2978 mg/kg/day in males and 1707 and 3448 mg/kg/day in females, based on the incidence of liver adenomas and hepatocellular carcinoma [23]. Malathion has been classified as a potential carcinogen by the EPA in 1999. A quantitative dose-response evaluation of the possible carcinogenic potential of Malathion has not been performed. Also, the carcinogenic potential of Malathion has been investigated by FIFRA (Federation of Insecticides, Fungicides, and Rodents), and this compound is classified as a potential carcinogen.

In a study conducted in 2003, they looked at the skin effects of Malathion on the internal organs of the rat. Mice were treated with Malathion 4 hours a day for 28 days. The used doses of 8 and 16 mg did not affect the internal organs of the body [24]. In another study, the effects of non-cholinergic doses of Malathion on the apoptosis of L929 mouse fibroblasts were investigated. Using flow cytometry and caspase activation, they showed that Malathion increases apoptosis in L929 cells in a dose- and time-dependent manner [25].

In a study in 2012, to investigate the cytogenetic toxicity effect of Malathion in rats, it was observed that exposure to Malathion significantly increased the number of chromosomal structural abnormalities and the percentage of DNA damage compared to the control group, and Malathion has the potential Genotoxic-Clastogenic toxicity [26]. Studies conducted on genetic toxicity in people exposed to Malathion show an increase in the chromosomal disorder of these people compared to others. In another similar study to investigate the cytogenetic effects of workers exposed to Malathion, it was found that the chromosomal disorder increased in a dose-dependent manner [27].

Cytotoxicity risk assessment of workers exposed to a mixture of organophosphorus pesticides shows an increase in chromosomal abnormalities including acentric fragments (chromosomes without centromeres) and micronucleus by Malathion [28].

In our study, despite the dose-dependent increase in the number of micronuclei by Malathion, this increase was not significant. This finding is from another study that was conducted on the genotoxic effects of Malathion on HTC rat cells. Malathion was incubated with concentrations of 9, 0.009, and 0.0009 mg in 5 cc of culture medium, and there was no significant change in the number of micronuclei in any of the concentrations [29].

The results of our study showed that the amount of testosterone and FSH hormones in the test group had a significant decrease compared to the control group **(Table 3)**. This issue can indicate that Malathion has been able to disrupt the body's hormonal system and eventually cause it to decrease. This trend was also observed in the change of LH hormone, but it was not significant. In a study to

investigate the protective role of green tea extract on the function of ovarian tissue in rats treated with the insecticide malathion, it was shown that malathion has adverse effects on the secretion of sex hormones in female rats and the process of oogenesis, and the tea extract Green reduces the negative effects of malathion [30].

CONCLUSION

In the present study, the effect of Malathion on sex hormones and its carcinogenic effects in skin contact were studied in mice. According to the results, there was no significant change in the luteinizing hormone treatment groups compared to the control group, but follicle-stimulating hormone and testosterone showed a significant decrease. Mild hepatotoxicity was observed in the histological examination in the treatment groups. The frequency of micronuclei in different concentrations of Malathion was significantly different from the control group. From the findings of this study, it can be concluded that skin contact with Malathion can have destructive effects on sex hormones as well as body tissues.

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