The Ameliorative Effect of Royal Jelly against Malathion Genotoxicity in Bone Marrow and Liver of Rat

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Abstract: Malathion an organophosphate insecticide is used extensively in public health, agriculture and household purposes. The present investigation aimed to evaluate the genotoxicity of malathion and the possible protective effect of royal jelly against this genotoxicity. Male albino rats were orally administered royal jelly at the doses of 100 or 250 mg/kg body weight for 5 consecutive days then challenged with malathion at a dose of 100 mg/kg body weight by gavage. 24 and 48hours thereafter, animals were sacrificed and bone marrow samples were collected for micronucleus test and liver samples were used for DNA damage detection by comet assay. Malathion alone-treated mice presented significant (P<0.001) increase in the frequencies of MnPCEs compared with the controls. Malathion also lowered the PCEs/NCEs ratios than in controls (P<0.01). Furthermore, the alkaline comet assay showed significantly increased tail moment in liver cells of animals treated with malathion alone compared to control group. On the other hand, oral pre-treatment with RJ significantly ameliorated the genotoxicity induced by malathion. RJ provided significant protection against malathion-induced genotoxicity, in a dose dependent manner. Results indicate that malathion treatment induces cytotoxic and genotoxic effects in bone marrow cells, and liver cells of albino rats and RJ is a potent antioxidant against this effects.

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1. Introduction

Malathion (O,O-dimethyl-S-1,2-bis ethoxy carbonyl ethyl phosphorodithionate) is an organophosphate insecticide used in agriculture, commercial extermination, fumigation, veterinary practices, domestic, and public health purposes (**Brocardo *et al*., 2007**). Environmental contamination with malathion and its potential to cause adverse health effect in humans, animals and wildlife are a matter of concern (Eskenazi *et al*., 2007). Malathion toxicity has been associated with the inhibition of acetyl cholinesterase activity (**Sidell, 1994**), it binds with acetyl cholinesterase (**Kralj *et al*., 2007; Sparling and Fellers, 2007**), leading to the interference with the transmission of nerve impulse, and accumulation of acetylcholine at synaptic junctions. The adverse health effects due to malathion exposure including headache, dizziness, nausea, vomiting, bradycardia, and miosis (**Cox, 2003**). In addition, malathion induced oxidative stress and cytotoxicity in rat brain regions (**Fortunato *et al.*, 2006**); in human erythrocytes (**Durak *et al*., 2009**) and in human liver carcinoma (HepG2) cells (**Moore *et al*., 2010**).

Additionally, several studies indicated the genotoxic effect of malathion in both human and animals, such as the induction of DNA in the form of chromosomal aberrations (**Réus *et al*., 2008**), sister chromatid exchange (**Giri *et al*., 2002**) and micronuclei formation (**Kumar *et al*., 2010; Giri *et al*., 2011**). Moreover, malathion induced DNA damage in cerebral tissue and peripheral blood in rats (**Réus *et al*., 2008**); in human liver carcinoma cells (**Moore *et al*., 2010**); in the gill, kidney, and lymphocytes of teleost fish *Channa punctatus* (**Kumar *et al*., 2010**).

Royal jelly (RJ) is a honey bee secretion that is used in the nutrition of larvae, as well as adult queens (**Viuda-Martos, 2008**). This secretion which excreted by the mandibular and hypo-pharyngeal glands of worker honey bees, is a health tonic widely consumed with various perceived benefits (**Viuda-Martos, 2008**). It has been used since ancient times as a remedy for wound healing (**Fujii, 1990**). Recently, RJ has received particular attention because of studies that have reported that it is a highly efficient antioxidant and has free radical scavenging capacity (**Silici, 2010**). It contains many important compounds with biological activity such as free amino acids, proteins, sugars, fatty acids, minerals, and vitamins (**Nakajima *et al*., 2009**). RJ has been demonstrated to possess numerous functional properties such as antibacterial activity, anti-inflammatory activity, vasodilative and hypotensive activities, disinfectant action, antioxidant activity, antihypercholesterolemic activity, and antitumor activity (**Viuda-Martos *et al*., 2008**). RJ also stimulates bone formation (**Narita *et al*., 2006**) and has immunomodulatory activity (**Şimşek *et al.*, 2009**). In addition, several studies revealed biological evidence supports the use of RJ in the treatment of chemical-induced hepatotoxicity (**El-Nekeety, 2007; Kanbur *et al*., 2009 and Cemek *et al*., 2010**) and genotoxicity (**Cavuşoğlu *et al*., 2009**; **Türkmen *et al.*, 2009**). This study aimed to examine the genotoxicity of

malathion and the possible protective effect of royal jelly against this genotoxicity in bone marrow cells and liver of albino rats.

2. Material and Methods

A- Animals and treatments:

The present investigation carried out on 45 male albino rats aged 3-4 months and weighted 130-150 g. Rats were purchased from Helwan Farms of the Egyptian Organization for Vaccines and Biological Preparations, Cairo. Animals were acclimatized for two weeks and the commercial food and tap water were supplemented *ad libitum* during acclimatization period. Animals were divided into 9 groups 5 animals each. Rats in the first group received nothing and served as control group. Second and third groups received orally RJ (100 or 250mg/kg b.w) alone for 5 consecutive days, and dissected 24hrs after the last injection. The fourth and fifth groups received orally 100 mg/kg b.w of malathion (**Réus *et al.*, 2008**), animals were dissected 24 and 48 hours post treatment. While, the remaining groups received 100 or 250 mg/kg b.w RJ /day (**Cavuşoğlu *et al.*, 2009**) for 5 consecutive days, followed by malathion (100mg/kg b.w) 2 hrs after the final RJ treatment. The incidence of bone marrow micronuclei and DNA damage in liver were deliberated 24 and 48hours after the last treatment.

B- Chemicals

Malathion: Commercial "malathion" 57% under the trade name Malathin® 57% EC was used (Agrochem, Egypt). Malathion was diluted with distilled water to the required concentration before treatment.

Royal jelly: Pure Royal jelly capsules were used in this study (Pharco Pharmaceuticals Co., Egypt). Each capsule (1000 mg) was dissolved in warm distilled water to get a concentration of 100 or 250mg mL^{-1}

Fetal calf serum, normal melting agarose, low melting agarose, triton X-100, sodium N-lauryl sarcosine, ethylenediamine tetraacetic acid, trishydroxymethyl aminomethane, ethidium bromide, Giemsa, and other common chemicals were purchased from Sigma-Aldrich.

C- Micronucleus test

The frequency of micronucleated polychromatic erythrocytes was evaluated based on a technique developed by **Schmid (1976)**. Rats were sacrificed by cervical dislocation. Femoral bone was dissected out and cleaned of any adhering muscle. The femoral bone marrow was flushed out using 1 ml of fetal calf serum and centrifuged at 1000 rpm for 10 min. The supernatant was discarded and few drops of fetal calf serum were added. Smears were prepared for each animal, fixed in methanol and stained with Giemsa. Smears were screened at a magnification of 1000X, using a light microscope. The polychromatic erythrocytes with one or more micronuclei were counted in 2000 polychromatic erythrocytes per animal. In addition, 500 erythrocytes were counted to determine PCE/NCE ratio.

D- Comet assay

Comet assay allows the detection of DNA alterations of diverse kinds, such as double-strand breaks, single-strand breaks, alkali-labile sites and cross-links. The alkaline single-cell gel electrophoresis (comet assay) was applied to detect the DNA damage induced after 24hrs of treatment with malathion and/ or RJ. The SCGE was performed by the method of **Sasaki *et al.* (1997)**. The liver was minced and suspended in 4 mL of chilled homogenizing solution (0.075 M NaCl and 0.024 M Na_2EDTA, pH 7.5), then homogenized gently at 500 to 800 rpm on ice. To obtain nuclei, the homogenate was centrifuged at 700 rpm for 10 min, and the pellet was gently resuspended in 4.0 ml of chilled homogenizing buffer. After that, 75 µl of a mixture containing equal volumes of sample (nuclei preparation) and low melting agarose (2% in phosphate buffer saline) was quickly layered on 1% normal melting point agarose (NMA) (prepared in distilled water) precoated and overnight dried slides and covered with a coverslip. Then the slides were placed on a chilled plate to allow complete polymerization of agarose. Finally 75 µl of 0.5% NMA in PBS was quickly layered in the same manner after removing the coverslip and allowed to solidify on chilled plate. Slides were then immersed in the lysis buffer (containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sarcosine, pH 10.0 adjusted with 10 N NaOH and with 5% DMSO and 1% Triton X-100 was added just before use) for 1 hr at 48°C in the dark. After lysis, the slides were transferred on a horizontal electrophoresis platform and immersed in electrophoresis buffer (300 mM sodium hydroxide and 1 m M EDTA, pH > 13.0) for 20 min for unwinding of DNA. Electrophoresis was performed for 15 min at constant voltage (1 V/cm and 300 mA). After electrophoresis, the slides were washed thrice with neutralizing buffer (0.4 M Tris-HCl, pH 7.4) for 5 min each. Slides were dehydrated in absolute methanol for 10 min and left at room temperature to dry. The whole procedure was performed in dim light or dark to minimize artefactual DNA damage. Just before visualization, each slide was stained with 50µl of ethidium bromide (20µl/ml), rinsed with water, and covered with a coverslip. The slides were examined at 200x magnification using Olympus fluorescent microscope. All slides were coded and examined blindly. A total of 100 randomly selected cells from two replicate slides (50 cells per slide) were examined per sample. The analysis of comet cells was done using TriTek Comet Score Freeware v1.5.

The DNA damage in the treated cells was quantified as tail moment (the DNA product in tail X

the migration distance in the tail) was compared with untreated cells. **(Olive *et al*., 1990).**

E- Statistical analysis

Student's *t*-test (**Fowler *et al.,* 1998**) was used for comparing the level of significance in the results between:

a) The malathion treated group and the untreated control.

b) The malathion plus royal jelly treated groups and malathion treated group.

Significance was indicated by *P* values <0.05

3. Results and Discussion

The micronucleus test is a simple method to assess *in vivo* clastogenicity of chemicals. It is known that the appearance of MN is related to the loss of chromosome fragments due to chromosome breaks (**Heddle *et al*., 1991**). In the present study, results showed that, malathion induced highly significant (*P*<0.001) increase in MnPCEs, the most frequent types of micronuclei detected were round and dot-like (Fig. 2a). This increase was observed by other researchers using the same test in human and experimental animals (**Garaj-Vrhovac and Zeljezic, 2002; Réus *et al*., 2008**; **Giri *et al*., 2011**). Also, malathion induced significant decrease in PCEs/NCEs ratios which indicates its toxicity (Table 1). This result concurs with **Réus *et al.* (2008**).

In addition, the frequencies of micronuclei 48hours post-treatment with malathion were higher than those obtained 24 hours post-treatment (Table 1). This result could be attributed to the metabolization of malathion, and the formation of malaoxon, which is the mian metabolize of malathion. Malaoxon was reported as 40 times more toxic than malathion itself **(Wankhade *et al*., 2009)**. In **1999, Błasiak *et al*.** reported that the genotoxicity of malathion might be due to its first and main metabolite malaoxon. Additionally, **Blasiak and Stankowska (2001)** stated that, malaoxon could damage DNA more potently than the parent compound.

Moreover, Comet assay revealed that, malathion induced statistically significant (*P*<0.01) increase in the mean value of the tail moment from (1.33±0.42) in control group to (13.03±3.09) 24 hours Post-treatement with malathion. (Table 2 and Fig. 2b)**.** This result agrees with **Réus *et al.* (2008)**.

Reactive oxygen species (ROS) are generated under normal cellular conditions and are immediately detoxified by major scavenger enzymes (glutathione based enzymes). However, excessive ROS production causes antioxidant imbalance and leads to lipid peroxidation and antioxidant depletion. **Akhgari *et al.* (2003)** reported that, malathion induced oxidative stress leading to generation of free radicals and alterations in antioxidant and scavengers of oxygen free radicals. As well, **Blasiak and Stankowska (2001)** suggested that the formation of ROS causing DNA strand breaks may originate from hydrogen peroxide yield by malaoxon.

In addition, **Réus *et al.* (2008**) declared that, it is possible during the metabolism of malathion, ROS can be generated, and malathion may produce oxidative stress in intoxicated rats that can be responsible for alterations in DNA molecules. Moreover, **Fortunato *et al*., 2006** revealed the generation of oxygen free radicals and hydroxyl radicals due to the biochemical mechanism of malathion toxicity. This implicated in the initiation of membrane damaging by lipid peroxidation **(Conklin, 2000)**.

Furthermore, we used commercial malathion which might containing malaoxon and isomalathion which might be responsible for the high frequencies of MnPCEs and tail moment. The above data indicates that malathion acts as mutagenic agent. The present results are in agreement with the findings of **Amer *et al*., 2002 and Réus *et al*., 2008**. Additionally, **Amer *et al.* (2002)** reported that, malathion used as commercial product, can be considered as a genotoxic substance *in vitro* and also produce DNA disturbances *in vivo*, such as DNA breakage at sites of oncogenes or tumor suppressor genes, thus playing a role in induction of malignancies in individuals exposed to this agent.

In the present investigation pre-treatment for five days with (100 or 250mg/kg b.w) RJ which is a potent antioxidant **(Silici**, 2010**)**, induced significant decrease in the frequencies of MnPCEs from (1.6±0.64) to (1.45±1.04) and (1.35±0.95), and from (1.7±0.64) to (1.52±0.74) and (1.45±0.64) 24and 48hrs post-treatment respectively (Table 1 and Figure 1). The PCEs/NCEs ratios also increased in the RJ pre-treatment groups but didn't reach any significant level. Moreover, RJ pre-treatment induced decrease in the tail moment when compared with malathion alone (Table 2). This decrease was only significant with the high dose of RJ (250mg/kg b.w). This decrease could be due to the ability of RJ for scavenging the free radicals. In addition, **Cavuşoğlu *et al.* (2009)** revealed that RJ caused a significant recovery in antioxidant status of GSH and a significant inhibition of MDA production. Also, the protective effect of RJ which appeared in this investigation may be attributed to its constituents, such as free amino acids, proteins, sugars, fatty acids, minerals, and vitamins (**Nakajima *et al*., 2009)**. In **1987 Carlson**, stated that, the toxicity of malathion depends on the amount of protein in the diet and gender. As protein intake decreased, malathion was increasingly toxic to the rats, which indicates that RJ may have a beneficial effect in reducing malathion toxicity. In conclusion, the present results indicated that RJ ameliorated DNA damage and genotoxicity induced by malathion in rat cells.

Table 1: Incidence of MnPCEs in bone marrow cells of rats post-treatment with malathion and/or royal jelly.

Treatment	Duration	Percent of PCEs with Mn (mean% ± S.E.)	PCE/NCE (Mean±S.E.)
Control	24	0.16±0.60	1.39±0.12
Royal jelly			
100 mg/kg bw	24	0.15±0.5[a]	1.37±0.12[a]
250 mg/kg bw	24	0.14±0.6[a]	1.31±0.11[a]
Malathion			
100 mg/kg bw	24	1.6±0.64[c]	0.790±0.06[b]
100 mg/kg bw	48	1.7±0.64[c]	0.784±0.04[b]
Malathion + Royal jelly			
100 mg/kg bw	24	1.45±1.04[d]	0.800±0.03[d]
100 mg/kg bw	48	1.52±0.74[e]	0.823±0.06[d]
250 mg/kg bw	24	1.35±0.95[f]	0.856±0.02[d]
250 mg/kg bw	48	1.45±0.64[g]	0.876±0.03[d]

[a]$P>0.05$ compared with control [b]$P<0.001$ compared with control [c]$P<0.0001$ compared with control
[d]$P>0.05$compared with malathion (corresponding duration) (24hrs) [e]$P<0.01$ compared with malathion (48hrs)
[f]$P<0.01$ compared with malathion (24hrs) [g]$P<0.001$ compared with malathion (48hrs) .

Table 2: Incidences of tail moment in liver cells of rats 24 hours after malathion treatment.

Treatment / Tail moment	Control	Royal jelly (mg/kg bw)		Malathion (mg/kg bw)	Malathion + royal jelly (mg/kg bw)	
	-	100	250	100	100RJ	250 RJ
mean	**1.33**	**1.58**	**1.51**	**13.03**	**8.03**	**7.7**
± S.D.	**0.42**	**0.81**	**0.73**	**3.09**	**12.09**	**0.94**
t-test (*P*-values)	**-**	**0.66**	**0.73**	**0.0029**	**0.53**	**0.047**

Three animals from each experimental group were used for this test.

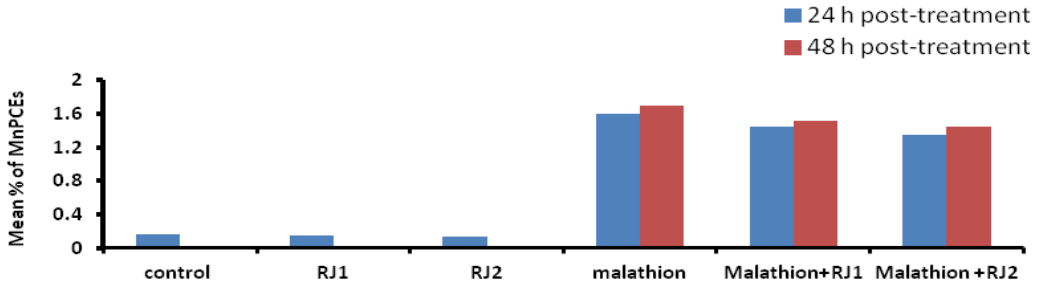
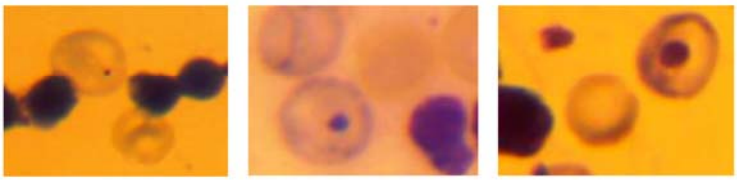


Fig. (1):Histogram represents incidence of MnPCEs in bone marrow cells of rats post-treatment with malathion and/or royal jelly.

RJ1= 100mg/kg bw royal jelly
RJ2= 250mg/kg bw royal jelly



dot-like round shape large size

Fig. (2a): Different types of micronuclei detected after malathion treatment.

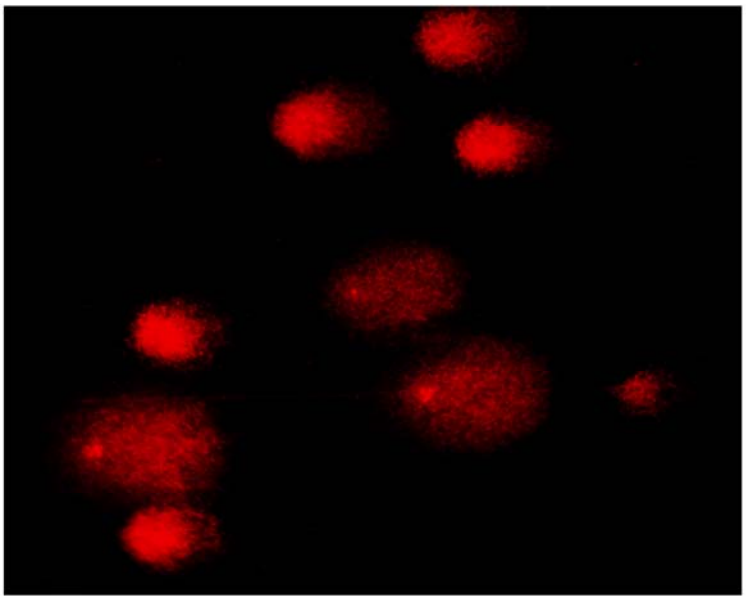


Fig. (2b): DNA damage induced after malathion treatment.

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