Effects of malathion exposure on glucose tolerance test in diabetic rats; emphasis on oxidative stress and blood concentration of malathion by gas chromatography mass spectrometry

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ABSTRACT
Malathion is one of the widely used broad-spectrum organophosphate insecticides (OPI) in Iran. Malathion affects carbohydrate metabolism, causes hyperglycemia and increases the risk of diabetes. The present study was undertaken to investigate the potential of malathion to exacerbate diabetes-induced oxidative stress and impairment in blood glucose level and glucose tolerance in a sub-acute study. Malathion concentration in blood was analyzed with gas chromatography mass spectrometry (GC-MS) after sample preparation of blood samples based on magnetic Fe₃O₄-supported graphene oxide (Fe₃O₄@ GO) nanoparticles. Type 1 diabetes was experimentally induced by intraperitoneal administration of streptozocin (65 mg kg⁻¹). Diabetic and non-diabetic rats were treated with malathion at the dose of 150 mg kg⁻¹day⁻¹ or 0.5-4.0 mg L⁻¹ in blood for 4 weeks. Fasting blood glucose was measured every week. At the end of the study, blood samples were investigated for markers of oxidative stress. Exposure to multiple doses of malathion decreased the total antioxidant capacity of plasma and the activity of catalase and superoxide dismutase enzymes in diabetic rats. Blood glucose and glucose tolerance test (GTT) and oxidative damages did not change significantly in diabetic rats exposed to malathion. However, malathion concentration in blood caused to increase GTT in malathion-treated non-diabetic rats. Taking together, our findings provide evidence that daily exposure to malathion for 4 weeks tends to exacerbate the decrease in blood antioxidant status and protein carbonylation in diabetic rats.

Keywords: Malathion, Blood samples, Diabetes, Oxidative stress, Erythrocyte, Magnetic graphene oxide, Gas chromatography mass spectrometry

1. Introduction
The agricultural application of pesticides in the world has been linked to a wide range of human health hazards through occupational, accidental, and intentional exposures [1]. It seems that among all pesticides, organophosphate insecticides (OPI) are more toxic to vertebrates with low mammalian toxicity [2, 3]. OPI inhibit acetylcholinesterase (AChE), which leads to the accumulation of acetylcholine in the cholinergic synapses and interfere with the normal function of the nervous system [4]. However, it has been shown that these pesticides have different toxicities in vivo and in vitro through AChE-independent mechanisms [5, 6].
OPI influences normal glucose homeostasis and carbohydrate metabolism and induces oxidative and nitrosative stress [7, 8]. Many techniques such as, UV-VIS, gas chromatography mass spectrometry, High Performance Liquid Chromatography (HPLC) and Liquid Chromatography [9, 10] were used for OPI and pesticides determination. The blood had difficulty matrixes and so must be treatment. Many sample preparation were used for treatment of blood samples for determination seven pesticides (malathion, methyl isofenphos, dichlorvos, chlorpyrifos, phenhoste, \( p,p' \)-DDD, \( p,p' \)-DDE) in blood samples based on a quick, easy, cheap, effective, rugged and safe (QuEChERS) sample preparation method. Occasionally, the \( \text{Fe}_3\text{O}_4 \) magnetic nanoparticles (MNPs) as the new adsorbing material was used for treatment of blood samples [11-14]. Although the foremost mechanism for hyperglycemia induced by OPI has not been recognized yet, some explanations are mentioned such as physiological stress, oxidative stress, paraoxonase enzyme inhibition, nitrosative stress, pancreatitis, cholinesterase inhibition, adrenal gland stimulation, and disturbance in liver tryptophan metabolism [15]. The human body is constantly exposed to various factors that contribute to the production of reactive oxygen species called free radicals. Imbalance between free radicals production and antioxidant systems lead to oxidative stress, which contributed to occurrence of the pathological conditions such as diabetes and development of diabetic complications [16-19]. Many toxic chemicals can generate reactive oxygen and trigger diabetes and hyperglycemia by induction of apoptosis in beta cells [20, 21]. Some evidence points to the long-term effects of OPI on glucose metabolism and increased risk of diabetes [15]. Malathion, one the of most popular OPI, has been used widely in agriculture, industry, and also for therapeutic purposes in humans (anti-louse) and animals (anti-ectoparasites) [22]. Malathion alters the pathways of carbohydrate metabolism mainly though increase in the activity of glycogen phosphorylase, phosphofructokinase, phosphoenolpyruvate carboxykinase, and hexokinase which affects glycolysis, gluconeogenesis, and glycogenolysis [15, 23]. Induction of oxidative and nitrosative stress in hepatocytes and pancreas beta cells are other contributing factors in hyperglycemia caused by Malathion [7, 23]. Activation of redox sensitive kinases and induction of oxidative stress in muscle cells after exposure to sub-toxic dose of malathion impairs insulin signaling and muscle glucose uptake and consequently causes insulin resistance state [24]. Hence, the present work has been designed to determine whether sub-acute exposure to repeated non-lethal dose of malathion can impair blood glucose control and exacerbate oxidative stress in diabetic rats. To do so, fasting blood glucose (FBG), glucose tolerance test (GTT), and biomarkers of oxidative damage were measured in non-diabetic and diabetic rats treated orally by sub-lethal dose of malathion for 4 weeks.

2. Materials and methods

2.1. Chemicals and methods

Technical-grade malathion, which contains >96% malathion, was obtained from the Shimi-Keshavarz Pesticides Production Company (Tehran, Iran). The name of malathion based on IUPAC (International Union of Pure and Applied Chemistry) is diethyl(dimethoxythiophosphorylthio) succinate; S-1,2-bis(ethoxycarbonyl) ethyl-O,O-dimethyl phosphorodithioate (CAS N.: 121-75-5, Sigma, Germany) and UV spectrum of malathion in acetonitrile (CAS N.: 75-05-08, Merck, ACN) was shown in Schema 1. All other materials were purchased from the Merck and Sigma-Aldrich Chemical Company (St. Louis, MO). The \( \text{HNO}_3 \), HCl, polyoxyethylene octyl phenyl ether (MTX-100, CAS N: 9002-93-1, Sigma, Germany), acetone and toluene (CAS N: 108-88-3, Merck) were purchased from Merck, Germany. Anhydrous magnesium sulfate (CAS N: 10034-99-8), sodium chloride was purchased from Sigma (Germany). Acetonitrile (ACN) and methanol were purchased from Sigma Company (Germany). GC–MS (Agilent 7890A/5975C, USA) with HP-5MS column (30 m \( \times \) 0.25 mm i.d.,) with flow of 1 mL per minute of
He was used for qualitatively and quantitatively detecting pesticides in blood. Because blood is a complex matrix, and pesticides in blood are usually at low concentrations, the separation of malathion and elimination of interference in blood have needed a special sample treatment. Working standard solutions were prepared in DW. All these solutions were stored at 4 °C without any light. The range of this study of malathion in blood is 0.3–4.4 μg mL\(^{-1}\) by GC-MS after dilution 1 mL of blood with DW.

2.2. Synthesis of magnetic Fe\(_3\)O\(_4\)-supported graphene oxide
The magnetic Fe\(_3\)O\(_4\)-supported graphene oxide (MNGO, Fe\(_3\)O\(_4\)@NGO) were prepared by co-precipitation of FeCl\(_2\)·4H\(_2\)O and FeCl\(_3\)·6H\(_2\)O, in the presence of NGO [19]. firstly, a liquid solution of FeCl\(_2\)·4H\(_2\)O / FeCl\(_3\)·6H\(_2\)O was prepared (molar ratio= 1:2). The weight ratio of FeCl\(_3\) / NGO in the product was mFeCl\(_3\) / mGO = 20:1. To prepare the magnetic graphene oxide (Fe\(_3\)O\(_4\)@NGO), 10 mg of graphene oxide mixed with 10 mL of DW and ultrasonicated for 30 min [19]. Then, 12.5 mL solution of FeCl\(_2\)·4H\(_2\)O (125 mg) and FeCl\(_3\)·6H\(_2\)O (200 mg) in DW was added to the mixture. Finally, the pH of 11 was achieved by 30% ammonia solution and the temperature was adjusted to 70 °C (Fig.1).

![Fig.1. Synthesis of magnetic Fe\(_3\)O\(_4\)-supported graphene oxide [19]](image-url)
2.3. Sample Extraction Procedure for malathion in blood

The sample preparation of blood samples in rat were prepared based on quick, easy, cheap, effective, rugged and safe (QuEChErs) method based on Fe$_3$O$_4$ magnetic nanoparticles (MNPs) functionalized with NGO. The free of DDC, DDT and malathion pesticide in blood samples were used as blank solution. 1 mL of rat blood sample was added into 10 mL of vial. Standard volumes of DDC, DDT and malathion pesticide were added to the vial, and then shaken for 1 min. The samples were extracted with 2 mL acetonitrile for 30 s. Anhydrous NaCl (0.1g) / MgSO$_4$ (0.3 g) were added to the mixture centrifuging at 4000 rpm for 5 min and then, the supernatant moved to 10 mL of vial include Fe$_3$O$_4$@NGO (0.04 g). The vial shake for 1 min, and the supernatant separated with an external magnet. Finally, the sample was dissolved in 50μL of acetonitrile and 1μL of solution was determined by GC–MS. The detection limits (LOD) and linear range (LR) of the QuEChErs method based on Fe$_3$O$_4$@NGO obtained 0.1 μg mL$^{-1}$ and 0.3–4.4 μg mL$^{-1}$ with recoveries more than 95% [9-12].

2.4. Animals

Male Wistar rats weighing 211.5 ± 10.6 grams were fed with standard diet and kept under 12:12 hour light:dark cycle, at the temperature of 20 °C and relative humidity of 25 to 30%. This study received ethical approval (Code: IR.79.KMU.REC.1395-79) from the local ethical committee of the Kerman University of Medical Sciences.

2.5. Pilot experiment

A pilot test is designed to determine an oral dose of malathion which inhibits 30% of plasma ChE activity without significant physiological consequences and mortality within 4 weeks [25]. The treated groups (Ten rats in each group) received the oral doses of 75, 100, 150, and 300 mg/kg/day of malathion dissolved in corn oil for 4 weeks, while the controls received only corn oil. Blood samples were taken at the end of each week for measurement of plasma ChE activity according to the Ellman’s colorimetric method with slight modification [26]. Briefly, 300 µl of 5,5’-Dithiobis(2-nitrobenzoic acid) (0.25 mM in 0.1M phosphate buffer, pH 7.4) was added to 10 µl of plasma and after 5 minutes 10 µl of acetylthiocholine iodide (3 mM) was added and the absorbance was measured at 412 nm for 5 minutes. The activity of ChE was calculated according to the molar extinction coefficient of 5-thio-2-nitrobenzoate ($13.6 \times 10^3$ M$^{-1}$cm$^{-1}$) and expressed as nMol min$^{-1}$mL$^{-1}$. As depicted in Table 1, malathion at the dose of 150 mg kg$^{-1}$day$^{-1}$ during 4 weeks inhibited 30% of the plasma ChE activity (663.40±72.09) compared with the control group (1029.67±84.52) with no mortality or acute toxic effects in rats.

<table>
<thead>
<tr>
<th>Week</th>
<th>Malathion (mg kg$^{-1}$ day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1st</td>
<td>99.4 ± 4.3</td>
</tr>
<tr>
<td>2nd</td>
<td>101.4 ± 9.1</td>
</tr>
<tr>
<td>3rd</td>
<td>99.5 ± 5.8</td>
</tr>
<tr>
<td>4th</td>
<td>100.6 ± 7.5</td>
</tr>
</tbody>
</table>

Data was expressed as mean ± SD; n = 10; * $P < 0.05$ and *** $P < 0.001$, significantly different from the control values (One-way ANOVA followed by multiple comparison test).
2.6. Measurement of malathion
GC–MS (Agilent 7890A/5975C, USA) with HP-5MS column (30 m × 0.25 mm i.d.) with flow of 1 mL per minute of He was used for DDC, DDT and malathion pesticide in blood rats. The splitless injector was used. By GC–MS, the main parameters such as, the inlet and interface temperature set at 250 and 280 °C, respectively. The source of MS tuned 220 °C and ionization energy was less than 65-eV. The oven temperature was first at 100 °C (1.5 min), and increased up to 200 - 280 °C (20 - 6 °C/min). The Chromatographic of DDC, DDT and malathion pesticides was shown in Figure 2 [9-13].

Fig.2. The Chromatographic of DDC, DDT and malathion pesticides by GC-MS

2.7. Induction of diabetes in rats
Type 1 diabetes was induced by intraperitoneal injection of a single dose streptozotocin (STZ) solubilized in 0.1 M trisodium citrate buffer (pH, 4.5) at the dose of 65 mg kg⁻¹, according to the method described by Furman [27]. STZ-treated rats received 10% of sucrose instead of water for 48 h. Induction of diabetes was verified by measurement of FBG four times (1, 3 and 28 days after the beginning of treatment) to ensure that the hyperglycemia (FBG≥250 mg dL⁻¹) was established. Polyuria and polydipsia were also monitored by observation of the amount of consumed water and the frequency of bedding exchange.

2.8. Experimental design, animal treatment, and sample collection
Forty rats were randomly allocated to four groups of ten as follows:
Control: Healthy rats that only received corn oil orally.
DM: Diabetic rats received corn oil orally.
MT: Healthy rats received malathion (150 mg kg⁻¹ day⁻¹, oral) for 4 weeks.
DM+MT: Diabetic rats received malathion (150 mg kg⁻¹ day⁻¹, oral) for 4 weeks.

At the end of the experiment, all rats were anesthetized by ketamine (60 mg/kg) and xylazin (6 mg kg⁻¹) and after collection of blood sample through cardiac puncture sacrificed by cervical dislocation. Blood samples immediately centrifuged at 3000 g for 15 minutes for separation of plasma. To prepare hemolysate, 250 µl of distilled water was added to 50 µl of packed RBCs and mixed thoroughly. Plasma samples and hemolysate were kept at -80 °C for further experiments.

[Figure 2 showing the chromatogram of DDC, DDT, and malathion pesticides by GC-MS]
2.9. Measurement of FBG and GTT

Blood glucose was measured in the blood sample obtained by a small cut on the tip of rat’s tail immediately after overnight fasting using the commercial glucose diagnostic kit of Pars Azmoon Company (Tehran, Iran). For measurement of GTT, blood glucose was recorded every 30 min after oral administration of glucose (2% w/v). Area under the curve (AUC 0-120 min) of glucose concentration from 0 to 120 minutes after administration of glucose was calculated by the trapezoidal method [28].

2.10. Measurement of oxidative stress biomarkers by spectrophotometry

2.10.1. Measurement of total antioxidant capacity of plasma

Antioxidant capacity was measured by ferric reducing antioxidant power (FRAP) method. During the reaction in acidic pH, the colorless ferric-triprydyl triazine (Fe³⁺-TPTZ) is reduced to blue ferrous-triprydyl triazine (Fe²⁺-TPTZ) [29]. To perform this experiment, 10 µl of plasma was added to 300 µl FRAP reagent (1:1:10 mixture of FeCl₃, 10mM TPTZ, and 0.3M acetate buffer at pH 3.6). After incubation at 37 °C for 10 minutes, the absorbance was read at 593 nm. Finally, FRAP was expressed as mmol Fe²⁺/mg protein according to the standard curve of FeSO₄.

2.10.2. Measurement of glutathione (GSH) content

According to the Elman method, thiol groups react by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and produce yellow complex [30]. Briefly, 250 µL of TCA 10% were added to 500 µL of hemolysate and centrifuged at 3500 g for 35 minutes. Then, 200 µL of Tris buffer and 500 µL DTNB (10 mM in 0.1 M phosphate buffer, pH 8) were added to the supernatant and incubated in the dark at room temperature for 15 minutes. The absorbance was read at 412 nm and total thiol was expressed as nmol/mg protein according to the standard curve of GSH.

2.10.3. Measurement of superoxide dismutase (SOD) activity

SOD activity was measured based on autoxidation rate of pyrogallol at 420 nm by the Worthington method with minor modification [31]. Briefly, the absorbance of pyrogallol (2 mM pyrogallol in Tris-HCl buffer, pH 8.2) was determined kinetically alone and after the addition of 50 µl of hemolysate. The amount of SOD needed for 50% inhibition of the pyrogallol autoxidation was considered as one unit of SOD activity and expressed as U/mg protein.

2.10.4. Measurement of catalase (CAT) activity

According to Cohen method [32], 1 ml of 30 mM H₂O₂ and 50 µl of the hemolysate was added to 2 ml of phosphate buffer (50 mM, pH 7.0) and then the absorbance was measured kinetically at 240 nm. One unit of catalase activity is equal to 1 µM H₂O₂ decomposed per minute. The concentration of H₂O₂ was calculated using the following equation:

\[ \text{H}_2\text{O}_2 (\mu\text{M}) = \frac{\text{Absorbance at } 240 \text{ nm} \times 1000}{43.6 \text{ M}^{-1} \text{ cm}^{-1}} \]

Catalase activity was expressed as U/mg protein.

2.10.5. Measurement of lipid peroxidation

Malondialdehyde (MDA) as the end-product of lipid peroxidation was measured based on the absorbance of MDA-thiobarbituric acid (TBA) complex in acidic and high-temperature condition [33]. Briefly, 100 µl of hemolysate was deproteinized by TCA 10% and centrifuged at 3500 g for 35 minutes. One ml of sulfuric acid 0.05% and 800 µl of TBA (0.2%) were then added to the precipitant and boiled at 95 °C for 30 minutes. Then, MDA-TBA complex was extracted by 800 µl n-butanol and the absorbance was read at 532 nm. The level of MDA was expressed as nmol MDA/mg protein according to the MDA standard curve.

2.10.6. Measurement of protein carbonylation

Protein carbonylation was measured according to Levin et al. method [34]. Throughout the method, 100 µl of hemolysate was added to 500 µl of TCA 20%, kept at room temperature for 10 minutes, and
centrifuged. The supernatant was discarded and 1ml 4-dinitrophenylhydrazine (DNPH, 10 mM) was added to the pellet and incubated at 37 ºC for 50 minutes. Then, 1ml of TCA 20% was added and centrifuged. The remaining pellet was washed with 1mL of ethanol and ethyl acetate solution (1:1 ratio). Then, 1mL of guanidine hydrochloride 6 M was added and incubated at 37 °C for 30 minutes. After centrifugation, the supernatant was transferred to a 96-well plate and the absorbance was measured at 380 nm. The carbonyl content was calculated using the molar extinction coefficient of 22,000 M−1 cm−1 and expressed as nmol/mg protein.

2.11. Measurement of protein concentration
Protein concentration in the samples was measured according to the Bradford’s method [35]. Briefly, 200 µl of Bradford reagent (100 mg coomassie brilliant blue G-250 was dissolved in 50 ml 95% ethanol and then 100 ml 85% phosphoric acid and 850 ml of distilled water was added) were mixed with 50 µl of samples and bovina serum albumin (BSA) as standard in 96-well plate. After five minutes’ incubation at 37 ºC, the absorbance was measured at 595 nm and the protein concentration was expressed as mg mL−1 of samples according to the standard curve of BSA.

2.12. Statistical analysis
Data were analyzed by using commercially available SPSS software. Data was analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. Results were presented as mean ± SD (Standard Deviation) and p values less than 0.05 were regarded as statistically significant.

3. Results and discussion
3.1. Malathion blood concentration
The blood malathion firstly determined by GC-MS analysis after oral intake in rats. The blood analysis of malathion indicates that oral administration of malathion at the dose of 50-150 mg kg⁻¹ day⁻¹ caused a blood concentration of malathion in the range of 0.5–4.0 μg mL⁻¹.

3.2. Induction of diabetes in rats
As shown in Table 2, administration of STZ at the single dose of 65 mg kg⁻¹ caused significant hyperglycemia (FBG = 424.8 ± 28.9 mg dL⁻¹, p < 0.001) and loss of body weight was compared with control group (FBG = 74.3 ± 25.5 mg dL⁻¹). Polyuria and polydipsia were other findings were observed in the diabetic rats within 4 weeks.

Table 2. Fasting blood glucose (FBG), Glucose tolerance test (GGT) and weight of rats on day 1, 3, and 28 in non-diabetic rats received corn oil (Control) and malathion (MT) and in diabetic rats received corn oil (DM) and malathion (DM + MT).

<table>
<thead>
<tr>
<th>Time</th>
<th>FBG (mg/dl)</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>62.0 ± 9.7</td>
<td>74.3 ± 25.5</td>
<td>74.0 ± 14.7</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>108.4 ± 18.6</td>
<td>424.8 ± 28.9***</td>
<td>395.4 ± 18.1***</td>
</tr>
<tr>
<td></td>
<td>MT</td>
<td>108.6 ± 19.3</td>
<td>89.1 ± 15.0</td>
<td>90.4 ± 21.0</td>
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<tr>
<td></td>
<td>DM + MT</td>
<td>133.25 ± 9.1</td>
<td>383.2 ± 19.1***</td>
<td>413.7 ± 12.1***</td>
</tr>
<tr>
<td></td>
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<td>9325 ± 409</td>
<td>NC</td>
<td>11990 ± 658.2</td>
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<tr>
<td></td>
<td>DM</td>
<td>11975 ± 521</td>
<td>NC</td>
<td>47574 ± 3758***</td>
</tr>
<tr>
<td></td>
<td>MT</td>
<td>10010.5 ± 688</td>
<td>NC</td>
<td>17886 ± 1438 *</td>
</tr>
<tr>
<td></td>
<td>DM + MT</td>
<td>10834 ± 674</td>
<td>NC</td>
<td>50577 ± 1256***</td>
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<table>
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<tr>
<th>Time</th>
<th>GTT (AUC 0-120 min)</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 28</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>211.5 ± 10.6</td>
<td>213.3 ± 9.2</td>
<td>263.7 ± 12.33</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>189.4 ± 8.5</td>
<td>182.4 ± 12.3</td>
<td>158.4 ± 8.2***</td>
</tr>
<tr>
<td></td>
<td>MT</td>
<td>198.4 ± 8.3</td>
<td>198.8 ± 7.7</td>
<td>214.6 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>DM + MT</td>
<td>212.6 ± 2.6</td>
<td>214.9 ± 3.1</td>
<td>162.7 ± 7.9***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time</th>
<th>Weight (gr)</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>211.5 ± 10.6</td>
<td>213.3 ± 9.2</td>
<td>263.7 ± 12.33</td>
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<tr>
<td></td>
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<td>189.4 ± 8.5</td>
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<tr>
<td></td>
<td>MT</td>
<td>198.4 ± 8.3</td>
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<tr>
<td></td>
<td>DM + MT</td>
<td>212.6 ± 2.6</td>
<td>214.9 ± 3.1</td>
<td>162.7 ± 7.9***</td>
</tr>
</tbody>
</table>

Data was expressed as mean ± SD; n = 10; *p < 0.05, **p < 0.01, and ***p < 0.001, significantly different from the control values (One-way ANOVA followed by multiple comparison test). NC: not calculated.
3.3. Effects on blood glucose
As shown in Table 2, malathion at the dose of 150 mg kg\(^{-1}\) did not result in a significant increase in FBG in diabetic rats compared with non-treated diabetic rats (\(p = 0.77\)) and in non-diabetic rats compared with control group (\(p = 0.72\)). On the other hand, malathion caused significant (\(p = 0.04\)) increase in AUC 0-120 of glucose concentration curve in non-diabetic rats. Most previous studies showed that hyperglycemia in both short-term and long-term exposure to OPI happened due to disruption in glycolysis, glycogenolysis, and gluconeogenesis pathways [7] and impairment in insulin signaling and insulin-stimulated glucose uptake in muscle cells [24]. Also, a meta-analysis conducted by Ramirez-Vargas et al. (2018), revealed that blood glucose concentrations were 3.3-fold higher in malathion-exposed rats than in the control group [23]. In contrast, it should be noted that some studies show gradual increase in blood glucose and even hypoglycemia after malathion exposure [36-38]. It has been also reported that blood glucose in malathion-treated rats increased (2.2-fold) after 2 h but gradually decreased within 4 h [39]. It can be concluded that duration of exposure, dose, experimental protocols, time of blood sampling, and the mode of administration are variables which affects the toxicity of malathion. As the toxicity of malathion on carbohydrates, fats, and protein metabolism pathways is approved previously, significant effects on FBG and GTT might be obtained with increase in the number of examined animal and duration of exposure to malathion.

3.4. Effects on antioxidants level
The total antioxidant capacity of plasma in diabetic and non-diabetic rats exposed to malathion decreased significantly (\(p < 0.001\)) comparing to control group. Moreover, a considerable difference (\(p = 0.009\)) was detected in malathion-treated diabetic rats compared to diabetic rats received corn oil (Fig. 3a). GSH level in RBCs decreased significantly (\(p < 0.001\)) in all groups compared to control group. However, there was no considerable difference (\(p = 0.13\)) between diabetic and non-diabetic rats received malathion (Fig. 3b). The activity of SOD in RBCs decreased significantly in diabetic groups (\(p < 0.05\)), and in diabetic and non-diabetic group received malathion (\(p < 0.001\)) compared to control group. Malathion decreased significantly (\(p = 0.0008\)) the activity of SOD in diabetic rats compared to diabetic group received corn oil (Fig. 3c). The activity of CAT in erythrocyte decreased significantly (\(p < 0.001\)) in all groups in comparison with control group. A significant (\(p = 0.007\)) decrease was also observed in diabetic rats received malathion compared to diabetic groups (Fig. 3d). The results of this study indicated that both diabetes and sub-acute exposure to the sub-lethal dose of malathion reduced the activity of CAT and SOD enzymes and total antioxidant capacity of plasma and GSH level. Interestingly, malathion in diabetic rats intensified the reduction of total antioxidant capacity and the activity of antioxidant enzymes. These results are in agreement with previous studies, which have indicated that diabetic condition and exposure to OPI reduce the total antioxidant capacity of plasma [7, 40, 41]. Reduction in total thiol content which induces oxidative and nitrosative damages were also reported in OPI exposure [42-45].

3.5. Effects on lipid peroxidation and protein carbonylation
As shown in Table 3, lipid peroxidation and protein carbonylation in diabetic rats as well as in diabetic and non-diabetic rats exposed to malathion significantly (\(p < 0.001\)) increased compared to control group. However, despite increase in the lipid peroxidation, no significant differences were observed between lipid peroxidation level in diabetic rats and diabetic rats exposed to malathion. Protein carbonylation was significantly (\(p = 0.042\)) increased in diabetic rats exposed to malathion compared to diabetic rats received corn oil. Generation of free radicals disables antioxidant systems and consequently exerts further destructive effects on cellular macromolecules [7, 15, 40]. Increase in protein carbonylation and lipid peroxidation revealed in the current study was in agreement with the findings of other studies [7, 46]. OPI increases lipid peroxidation and protein carbonylation in acute and sub-acute exposure
Table 3. Protein carbonylation and lipid peroxidation in non-diabetic rats received corn oil (Control) and malathion (MT) and in diabetic rats received corn oil (DM) and malathion (DM + MT) after 4 weeks.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DM</th>
<th>MT</th>
<th>DM + MT</th>
</tr>
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<tbody>
<tr>
<td>Lipid peroxidation (nmol/mg protein)</td>
<td>0.64 ± 0.13</td>
<td>0.86 ± 0.09***</td>
<td>0.92 ± 0.10***</td>
<td>0.99 ± 0.15***</td>
</tr>
<tr>
<td>Carbonyl protein formation (nmol/mg protein)</td>
<td>0.92 ± 0.15</td>
<td>1.12 ± 0.09***</td>
<td>1.20 ± 0.2**</td>
<td>1.45 ± 0.23***</td>
</tr>
</tbody>
</table>

Data was expressed as mean ± SD; n = 10; * p < 0.05, ** p < 0.01, and *** p < 0.001, significantly different from the control values (One-way ANOVA followed by Tukey’s multiple comparison test).

Fig. 3. Effects of malathion on antioxidants in the studied groups. a) Total antioxidant capacity of plasma, b) RBCs glutathione content, c) RBCs superoxide dismutase activity, d) RBCs catalase activity in healthy rats received corn oil (Control) and malathion (MT) and in diabetic rats received corn oil (DM) and malathion (DM + MT) after 4 weeks. Data was expressed as mean ± SD; n = 10; * p < 0.05, ** p < 0.01, and *** p < 0.001, significantly different between groups (One-way ANOVA followed by Tukey’s multiple comparison test).

[47-49]. Elevation in lipid peroxidation in diabetic rats exposed to monocrotophos, an OPI, was also reported by Vismaya and Rajini [44]. Increase in the MDA level indicates the susceptibility of cell membrane lipids against oxidative stress induced by malathion in diabetic rats.

4. Conclusions
Taking together, the results of this study imply that malathion aggravated decline in the enzymatic antioxidant defense system. Impairment in antioxidants capacity in long-term exposure to OPI causes further oxidative damages as a proposed
mechanism for OPI-induced hyperglycemia. According to high prevalence of diabetes, it is recommended to conduct further in vivo, in vitro, and clinical studies to investigate effects of OPI on insulin release and blood glucose tolerance in diabetic subjects exposed to OPI. It is also recommended to evaluate effects of malathion on tissues contributing to or affected by hyperglycemia. Further consideration is also required to restrain the utilization of OPI, which are widely used in developing countries.

5. Acknowledgement
This manuscript was supported by Research Deputy of Kerman University of Medical Sciences.

6. References


[41] D. Jira, Toxicity hazard of organophosphate insecticide malathion identified by in vitro


