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Original Article

Evidence for tissue-specific toxicity of malathion by biochemical biomarkers and histopathological index in two weeks-treated Wistar rats

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ABSTRACT

Introduction: Malathion (MAL), a kind of organophosphate pesticide (OPs), is one of the oldest phosphoric pesticides used for both domestic and commercial agricultural purposes. However, it possesses adverse effects and organ-specific toxicity for the heart, kidney, and other vertebrate organs. The exact effects of the short-term toxicity of MAL on lipid peroxidation, antioxidant activity, and pro-inflammatory cytokines have not been sufficiently elucidated yet.

Methods: We evaluated lipid peroxidation (MDA level), antioxidant activity [superoxide dismutase (SOD) and catalase (CAT)], tumor necrosis factor alpha (TNF-α), and Interleukin-1 beta (IL-1β) in different tissues of MAL-treated Wistar rats, at doses of 50, 100, and 200 mg/kg.

Results: After 14 days of exposure, CAT and SOD activities and MDA level increased in most tissues. Based on the histopathological results, the liver, kidney, and heart were the most affected, while the testes and lungs showed no damage. Also, increased TNF-α was measured as an inflammatory cytokine compared to untreated rats. IL-1β levels showed a dual response to the toxic effects of MAL, such as an increase in testis, kidney, and lung tissues and reduced in liver, heart, and blood tissues.

Conclusion: The present findings reinforce the concept that MAL can cause tissue-specific damage while enhancing the activity of antioxidant enzymes and reducing cytokine levels.

Keywords:
Organophosphates
Cholinesterase Inhibitors
Agricultural chemicals
Oxidative stress
Environmental exposure

Introduction

Pesticides are widely used around the world to kill or control unwanted pests and biological organisms in the agricultural industry. In this regard, the use of pesticides is approximately 2 million tons annually, causing approximately 200000 global deaths, which is expected

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to increase in the future (Eddleston et al., 2008; Heshmati et al., 2020). The use of pesticides is inevitable due to the widespread presence of pests in agricultural products. On the other hand, the accumulation of these toxins in agricultural products and their entry into the human body endangers the health of consumers and ensues acute or chronic side effects and human toxicity (Flehi-Slim et al., 2015; Rahimi et al., 2022). Organophosphorus pesticides (OPs) are one of the most potent pesticides. About 2 to 5% of OPs reach the target organism and the rest are transmitted to non-target organisms such as animals, plants, and the environment (Gupta et al., 2019). OPs have replaced organochlorine pesticides owing to their faster degradation in the environment and lower persistence (Lasram et al., 2009).

Malathion (MAL) [O,O-dimethyl-S-(1,2-dicarcethoxyethyl) phosphorodithioate], a kind of OP, is one of the oldest phosphoric pesticides which is a wide spectrum insecticide used by the public to kill different insects and control disease vectors (Jalili et al., 2019; Moestafalou et al., 2012). MAL is a lipophilic compound that can easily interact with cell membranes and destroy the phospholipid bilayer structure (Possamai et al., 2007). It can further cause DNA damage, lipid peroxidation (LPO), enzyme inactivation, and oxidative stress (Possamai et al., 2007). In developing countries, however, MAL also causes poisoning-induced deaths.

Previous studies have examined different times and doses of MAL; in our study, however, the short-term toxicity of MAL for histopathological disorders, lipid peroxidation, antioxidant activity, and pro-inflammatory cytokines in the rat model are investigated for the first time.

Material and Methods

Reagents

MAL was purchased from Semiran Agricultural Pesticides Company (Tehran, Iran). Corn oil was supplied by Sigma Aldrich Co. Other chemical compounds were obtained from reliable companies with analytical grades.

Experimental design

Thirty-two male Sprague-Dawley rats (weighing 250-300 g, aged 2-3 months) were purchased from the animal house of Shiraz University of Medical Sciences (Shiraz, Iran). The rats were kept at room temperature ($25 \pm 2^{\circ}C$) under controlled laboratory and natural conditions (lighting: 12h /12h, humidity: 50–55%) with *ad libitum* access to standard rodent diet and water. They *were* divided into four groups (n=8) after a *14*-day *acclimatization* period. The treatment groups were the control group which received corn oil, and MAL50, MAL100, and MAL200 groups which received 50, 100, and 200 mg/kg BW MAL (dissolved in corn oil), respectively, by gastric gavage (0.5 ml per day) for 15 days (Coban et al., 2015). Malathion dosages were equivalent to 1/40, 1/20, and 1/10 LD_{50}, respectively. In this study, the standard guidelines for the use and care of laboratory animals were followed and approved by the Animal Care and Use Committee at Fasa University of medical sciences (Iran).

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Samples preparation

At the end of the 15th day, all rats were anesthetized using ether, and blood samples of each group were collected directly from the heart. Serum was then prepared by centrifugation (3000 × g for 15 min) and frozen at -20°C until the time of analysis. Lung, testis, kidney, liver, and heart samples were removed and washed immediately with ice-cold 0.9% NaCl. To measure the antioxidant and cytokines levels, the tissue samples (left side) and apex of the heart were immediately transferred to -80 °C until biochemical analysis. The tissue samples (right side) and base of the heart were immersed in neutral buffered formalin for 24 h.

Histopathological study

Fixed tissues were processed routinely and stained with hematoxylin and eosin (H&E) according to standard instructions. The slides were evaluated using a light microscope (Olympus BX51; Olympus, Tokyo, Japan) attached to the camera (Olympus E-330, Olympus Optical Co., Ltd., Japan).

Biochemical analysis

All tissue samples were homogenized using radioimmunoprecipitation assay (RIPA) buffer in a mini grinder homogenizer and subjected to biochemical analysis. Supernatants were obtained after centrifugation (3000 rpm, 10min at $4^{\circ}C$) and analyzed for malondialdehyde (MDA), Catalase (CAT), Superoxide dismutase

(SOD), Interleukin 1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α). The time-tested colorimetric assay (Bradford protein assay) was used to measure the concentration of total protein in all samples (Bradford, 1976). CAT activity was measured as previously described by Aebi, 1984, based on the reduction in hydrogen peroxide at 570 nm (Aebi 1984). SOD activity was assayed according to *Marklund* method (Marklund and Marklund, 1974) by assessing the pyrogallol illumination and auto-oxidation at 440 nm for 3 min. CAT and SOD activities were expressed as U/mg protein. The thiobarbituric acid (TBA) test was used to evaluate the MDA content as described by *Ohkawa* (Ohkawa et al., 1979). The absorbance of supernatants was measured at 535 nm and MDA concentrations were determined (as nm/mg protein) based on the standard curve. The concentration of proteins was estimated by the Bradford method (Kruger, 2009) .

The contents of IL-1ß and TNF-*α* were measured by commercial kits (Karmania Pars Gene Company Kerman, Iran) according to the manufacturer's instructions. The ELISA plates were coated with antibodies specific for IL-1ß and TNF-*α*. These cytokines (pg/mg protein) were assessed as follows: In summary, the supernatant obtained from the homogenized samples was added to the wells of ELISA plates and incubated at room temperature (RT) on a shaker at 200 rpm. After washing, an anti-cytokine antibody (Anti-Avidin-HRP) was added and incubated at RT on a shaker at 200 rpm. After washing, substrate (Tetramethyl benzidine + Hydrogen Peroxide) was added to each well and dark incubated at RT. In the last step, stop solution (H_2SO_4 1 N) was added to the wells and OD was assayed at 450 nm.

Statistical analysis

Statistical analysis of data (inflammatory mediators and oxidative stress parameters) was performed using one-way ANOVA and SPSS version 25.0. Significance differences (P<0.05) between the means were followed by Tukey's multiple comparison tests. Data were expressed as mean ± SE.

Results

Antioxidant enzyme activity

CAT and SOD activities were used as indicators of oxidative stress (Tables 1 and 2). The results indicate that CAT activity in MAL-treated rats significantly increased in all tissues (Liver = 2.04±0.05 U/mg protein, Kidney = 2.35±0.15 U/mg protein, Lung = 1.96±0.03 U/mg protein, Heart = 2.52±.12 U/mg protein, Testis =1.76±0.03 U/mg protein, Blood = 96.91±6.38 U/mg protein, P<0.05). SOD activity in MAL-treated rats (Table 2) significantly increased in all tissues except in lung and testis (Liver = 181.25±10.42 U/mg protein, Kidney

TABLE 1: Effects of oral administration of Malathion (50, 100, and 200 mg/ kg, BW) on Catalase activity (U/mg pro) in the rat tissues

	Tissues					
	Liver	**kidney**	**Lung**	**Heart**	**Testis**	**Blood**
Control	0.83±0.03^{a}	1.14±0.00^{a}	1.15±0.00^{a}	1.38±0.03^{a}	1.21±0.04^{a}	77.82±4.56^{a}
MAL50	1.51±0.06^{b}	1.63±0.1^{a}	1.62±0.09^{b}	1.5±0.03^{a}	1.57±0.03^{b}	78.29±10.01^{a}
MAL100	1.55±0.05^{b}	1.58±0.11^{a}	1.83±0.07^{bc}	2.29±.14^{b}	1.78±0.1^{b}	96.91±6.38^{b}
MAL200	2.04±0.05^{c}	2.35±0.15^{b}	1.96±0.03^{c}	2.52±.12^{b}	1.76±0.03^{b}	77.54±0.83^{a}

Data were expressed as mean ± SE (n = 8), Different superscript letters (a, b, and c) indicate a significant difference (P < 0.05) among groups (column).

TABLE 2: Effects of oral administration of Malathion (50, 100 and 200 mg/ kg, b.w) on superoxide dismutases activity (U/mg pro) in the rat tissues

	Tissues					
	Liver	**kidney**	**Lung**	**Heart**	**Testis**	**Blood**
Control	106.22±2.38^{a}	129.04±9.77^{a}	171.39±5.43^{b}	123.79±2.21^{a}	137.06±2.17	41.82±0.31^{a}
MAL50	141.03±3.02^{b}	97.9±6.90^{a}	138.27±3.56^{a}	126.06±1.88^{a}	141.06±0.79	74.16±1.42^{b}
MAL100	175.98±6.28^{c}	107.87±4.36^{a}	177.22±4.64^{b}	159.69±4.15^{b}	135.55±1.17	76.32±0.58^{b}
MAL200	181.25±10.42^{c}	175.33±10.49^{b}	163.633±3.63^{b}	199.57±10.93^{c}	142.21±1.18	74.42±0.18^{b}

Data were expressed as mean ± SE (n = 8), Different superscript letters (a, b, and c) indicate a significant difference (P < 0.05) among groups (column).

TABLE 3: Effects of oral administration of Malathion (50, 100 and 200 mg/ kg, b.w) on malondialdehyde activity (nmol/mg pro) in the rat tissues

	Tissues					
	Liver	**kidney**	**Lung**	**Heart**	**Testis**	**Blood**
Control	4.03±0.44^{a}	3.43±0.3^{a}	4.26±0.1^{a}	3.49±0.21^{a}	6.79±0.43	1.61±0.48
MAL50	7.54±0.11^{b}	4.39±0.15^{ab}	7.67±0.73^{b}	5.5±0.33^{c}	7.99±0.53	1.95±0.19
MAL100	6.76±0.37^{b}	6.92±0.73^{c}	7.00±0.58^{b}	4.48±0.15^{b}	7.18±0.55	1.37±0.07
MAL200	6.37±0.11^{b}	5.6±0.33^{bc}	6.61±0.21^{b}	5.28±0.25^{bc}	6.91±0.6	1.74±0.25

Data were expressed as mean ± SE (n = 8), Different superscript letters (a, b, and c) indicate a significant difference ($P < 0.05$) among groups (column).

TABLE 4: Effects of oral administration of Malathion (50, 100 and 200 mg/ kg, b.w) on interleukin-1 beta contents (pg/mg pro) in the rat tissues

	Tissues					
	Liver	**kidney**	**Lung**	**Heart**	**Testis**	**Blood**
Control	445.73±20.12^{c}	346.16±14.59^{ab}	310.23±13.33^{a}	359.06±7.77^{bc}	255.2±18.17^{a}	72.42±8.38^{a}
MAL50	436.01±19.36^{bc}	448.91±10.69^{bc}	434.73±35.05^{b}	431.37±21.46^{c}	464.35±18.84^{b}	59.34±4.5^{ab}
MAL100	350.45±19.5^{a}	525.02±35.6^{c}	330.7±19.97^{ab}	293.06±21.31^{ab}	415.45±20.52^{b}	50.79±6.35^{b}
MAL200	362.78±13.35^{ab}	314.96±21.99^{a}	316.38±30.3^{a}	250.9±7.77^{a}	400.34±6.71^{b}	52.28±7.42^{b}

Data were expressed as mean ± SE (n = 8), Different superscript letters (a, b, and c) indicate a significant difference ($P < 0.05$) among groups (column).

TABLE 5: Effects of oral administration of Malathion (50, 100 and 200 mg/ kg, b.w) on tumor necrosis factor alpha contents (pg/ mg pro) in the rat tissues

	Tissues					
	Liver	**kidney**	**Lung**	**Heart**	**Testis**	**Blood**
Control	448.77±11.06^{c}	447.23±19.53^{c}	370.67±14.25	352.05±13.43^{a}	352.5±18.77	100.82±15.91^{a}
MAL50	351.03±12.23^{ab}	366.24±6.04^{ab}	351.82±24.95	374.6±18.53^{a}	345.68±41.18	73.91±7.44^{ab}
MAL100	315.97±12.78^{a}	412.49±9.27^{bc}	344.24±60.94	337.28±26^{ab}	328.95±21.29	92.03±7.42^{ab}
MAL200	394.03±11.8^{b}	328.63±15.34^{a}	349.70±5.12	253.23±17.27^{b}	299.49±11.74	72.62±9.62^{b}

Data were expressed as mean ± SE (n = 8), Different superscript letters (a, b, and c) indicate a significant difference ($P < 0.05$) among groups (column).

= 175.33±10.49 U/mg protein, Heart = 199.57±10.93 U/ mg protein, Blood = 74.42±0.18 U/mg protein, $P<0.05$). MAL50 significantly reduced SOD activity in the lung tissue ($P< 0.05$).

Lipid peroxidation marker (MDA level)

Commonly known as a marker of oxidative stress, MDA is one of the end-products generated by the peroxidation of polyunsaturated fatty acids. MAL-treated rats showed higher MDA levels in the liver, kidney, lung, and heart tissues compared with the control group (Liver = 6.37±0.11 nmol/mg protein, Kidney = 5.6±0.33 nmol/mg protein, Lung = 6.61±0.21 nmol/mg protein, Heart = 5.28±0.25 nmol/mg protein, $P < 0.05$) (Table 3).

Pro-inflammatory cytokines (IL-1β and TNF-α)

The content of IL-1β in the kidney (MAL50= 448.91±10.69 and MAL100 = 525.02±35 pg/mg protein), lung (only MAL50 = 434.73±35.05 pg/mg protein), and testis (MAL50, 100, and 200 = 464.35±18.84, 415.45±20.52, 400.34±6.71, respectively) were significantly higher in comparison to untrained rats (Table 4). MAL200-treated rats showed higher IL-1β levels in the liver (362.78±13.35 pg/mg protein), kidney (314.96±21.99 pg/mg protein), heart (250.9±7.77 pg/ mg protein), and blood tissues (52.28±7.42 pg/mg protein) (Table 4).

TNF-α content (pg/mg pro) in the rat tissues is shown in Table 5. Based on the results, MAL200 significantly attenuated TNF-α content in all tissues except for the lung and testis (liver = 394.03±11.8 pg/mg protein, kidney = 328.63±15.34 pg/mg protein, heart =253.23±17.27 pg/

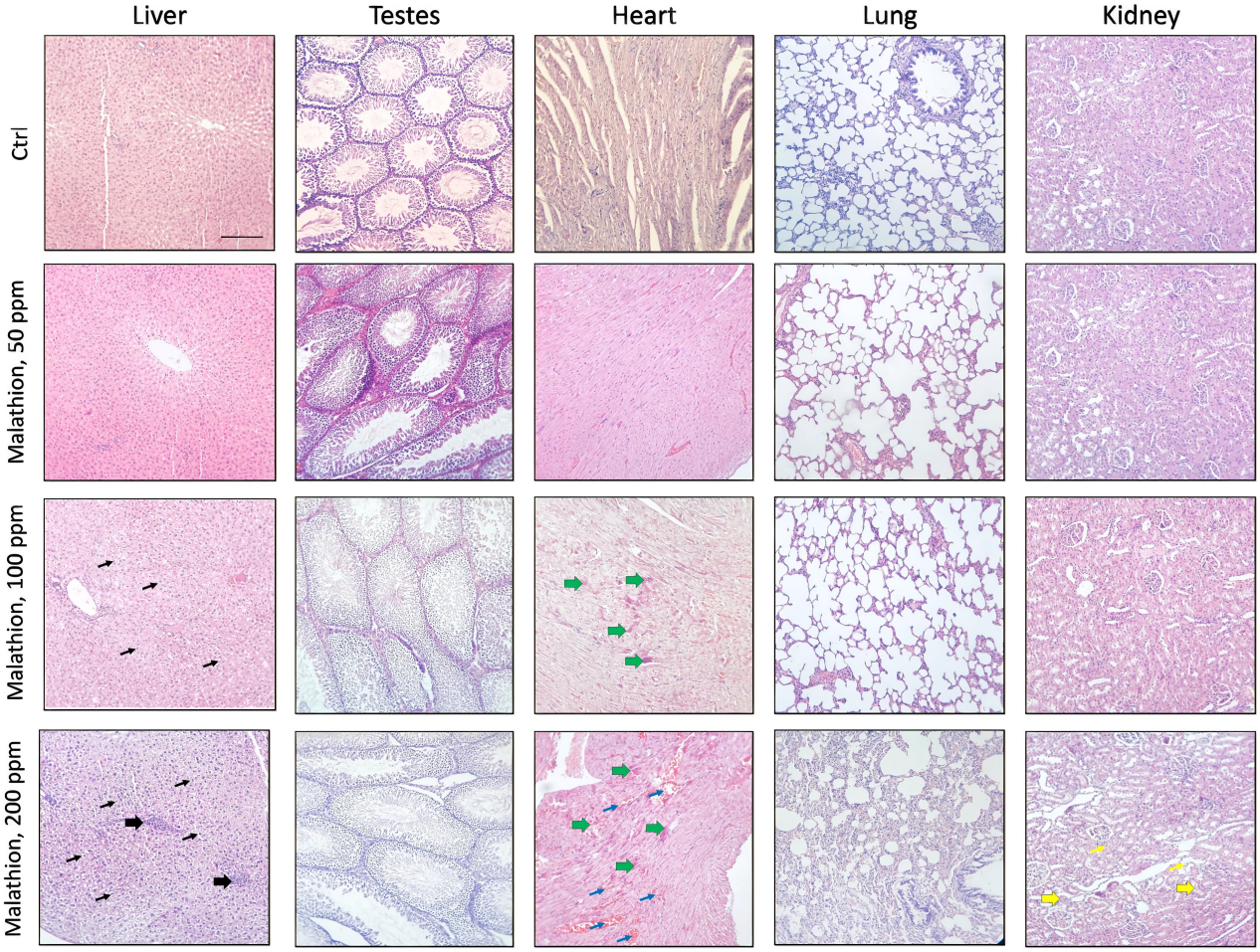

FIGURE 1. Light microscopy observation of rat tissue. Liver H&E results showed vacuolar (ballooning) degeneration of hepatocytes (thin black arrows) and infiltration of inflammatory cells (Black thick arrows). Kidney sections of MAL-treated rats showed tubular cast formation (yellow thick arrows) and tubular cell necrosis (yellow thin arrows) at the highest dose of this treatment (13.5 mg/ kg, BW). Heart sections of MAL-treated rats showed severe hyperemia and hemorrhage in group C (blue arrows) and necrosis of myocardial cells in groups B & C (thick green arrows), (Ctrl) control; (A) MAL 50 mg/ kg, BW; (B) MAL 100 mg/ kg, BW; (C) MAL 200 mg/ kg, BW , H&E stain, 200X. Scale bar: 100 µm.

mg protein, blood = 72.62±9.62 pg/mg protein, $P<0.05$).

Histopathological damages

All H&E-stained liver, testis, kidney, heart, and lung sections from different experimental groups were evaluated histologically. The histopathological micrographs of normal tissues are shown in the figures (ctrl). Micrographs relating to the liver sections of MAL (100-200 mg/kg) post-treatment showed a severe fatty change and the hydropic degeneration of hepatocytes (Figure 1). Moreover, portal hepatitis and infiltration of polymorphonuclear inflammatory cells were evident at the highest dose of MAL (200 mg/kg). The histopathological evaluation of the kidney which was exposed to the highest dose of MAL (200 mg/kg) showed moderate proximal and distal tubular cell necrosis, and the classical architecture of the kidney deteriorated. Tubular cast formation was further seen in this treatment group (Figure 1). The cardiac samples of groups treated with malathion (100-200 mg/kg) indicated cardiac myocyte necrosis (in both doses) and severe hyperemia (only in 200 mg/kg) (Figure 1). In addition, the toxicity of these agents increased in a dose-dependent manner. In all treatment groups, histopathological evaluation of testes and lungs revealed normal tissues without significant histopathological changes (data not shown).

Discussion

In this study, we investigated antioxidant enzyme activity (SOD, CAT), MDA level, and expression of TNF-α and IL-1β in MAL-treated rats. Our findings showed that SOD (liver, kidney, heart, and blood tis-

sues) and CAT activities (all tissues), and MDA level (liver, kidney, lung, and heart) in the MAL-treated rats were significantly higher than that of the control group.

Organophosphate agents such as MAL have a toxic effect on antioxidant defense systems and oxidative degradation of lipids (Brocardo et al., 2007). Furthermore, emerging evidence suggests that MAL can increase the generation of reactive oxygen species (ROS) and oxidative stress in the biological system (Fortunato et al., 2006). Acute (a single injection) and chronic (treatment for 28 days) use of MAL (25, 50, 100, and 150 mg/kg), as noted by *Fortunato et al*, results in induced oxidative stress and activity of antioxidant enzymes such as SOD and CAT in rat brain regions (Fortunato et al., 2006). The results of our study suggest that MAL-induced oxidative stress resulted in increased activity of CAT and MDA, which is consistent with the results obtained by *Edwards et al.* where other OPs such as methyl parathion (C8H10NO5PS) and parathion (C10H14NO5PS) increased the levels of MDA through oxidative stress in human liver carcinoma cells (Edwards et al., 2013). Other organophosphates such as diazinon can increase MDA levels in the testis tissue of male rats (Anbarkeh et al., 2014). Similar to our results, *Akhgari et al*.reported a significant increase in CAT and SOD activities in liver and blood cells, but we used lower dosages and shorter exposure times (Akhgari et al., 2003). *Possamai et al.* reported that intraperitoneal administration of sub-chronic MAL increased liver CAT activity only at lower doses. The administration method and MAL doses are the most important differences between their study and ours (Possamai et al., 2007). It has also been reported that MAL increases the levels of MDA and reduces SOD and CAT activities in human erythrocytes *in vitro* (Durak et al., 2009). We reported a significant increase in SOD levels in the liver, kidney, heart, and blood and a major reduction in SOD levels in the lung (MAL50). Previous studies have indicated that MAL changes SOD activities; changes in enzyme levels might differ depending on tissue and duration of exposure (Ahmed et al., 2000; Yarsan et al., 1999). Our results suggested that lower dosages and shorter exposure time of malathion can induce oxidative stress and modulate SOD and CAT activities in certain tissues. This up-regulation in antioxidant activity appears to be a protective cell response against the toxic effect of MAL.

Our results also revealed that MAL treatment significantly increased the level of IL-1β in the testis, kidney, and lung tissues while reduced in blood, liver, and heart. Experimental studies have proved that sub-chronic MAL-induced tissue damage results in stimulating inflammatory cytokines released in hepatocytes, such as IL-1β (Badr, 2020). Moreover, *Ince et al.* (2017) reported increased IL-1β expression in the liver of the MAL-induced toxicity rat model (Ince et al., 2017). In contrast, a significant decrease in the blood level of IL-1β expression was observed in rats acutely intoxicated with MAL, which is sim

ar to our results (Zabrodskii et al., 2015). Herein, we reported, for the first time, an increased IL-1β expression in other tissues such as the kidney, testis, and lung in rats treated with MAL.

In our study, MAL caused a significant decrease in the TNF-α level in the liver, kidney, heart, and blood tissues. TNF-α is one of the most important cytokines involved in host innate and adaptive immune response, tissue injury, cell proliferation, and programmed cell death (apoptosis) (Pober and Min, 2006). Toxicity and biological effects of MAL have been reported in several studies suggesting that their effects may be related to the increase in pro-inflammatory molecules such as TNF-α. Contrary to our results, many studies have shown that OPs induce inflammatory reactions and increase cytokines release. For instance, neurobehavioral impairments in rats exposed to MAL have been reported as a consequence of elevated TNF-α (Mohammadezadeh et al., 2018). According to the literature, TNF-α and IL-1β can induce apoptosis signaling pathways in organophosphate-treated microglial cells (Tan et al., 2013). Furthermore, it has been reported that acute organophosphorus pesticide poisoning (diazinon) can elevate the expression of TNF-α in the liver and spleen of mice (Hariri et al., 2010). *Tian et al.* (2015) showed that the expression of pro-inflammatory cytokines, IL-6, and TNF-α increased in the amygdala of sub-acute chlorpyrifos-treated rats after 24 and 72 hours (Tian et al. 2015). On the other hand, *Ayub et al.(2003)* showed that MAL was able to suppress nitrite production and TNF-α generation in LPS-induced cells (Ayub et al., 2003). There exist other reports that show the effect of organophosphorus toxins on the reduction of cytokines. For example, *Moser etal.* (2015) reported that overstimulating muscarinic and/or nicotinic receptors in immune cells by acetylcholine inhibitor pesticides in high doses

reduced the secretion of inflammatory cytokines (Moser et al., 2015). *Alluwaimi and Hussein* (2007) reached similar conclusions in terms of reduction in inflammatory cytokines by 1/5 LD_{50} of diazinon (a nonsystemic organophosphate insecticide). (Alluwaimi and Hussein, 2007). It has been detected that malathion inhibits the acetylcholinesterase activity of most eukaryotes; therefore, malathion may reduce inflammatory cytokines such as TNF-α and IL-1β immune cells' response in liver tissues. The results of Moser et al. (2015) show that the reduced IL-1β and TNF-α observed in liver tissues is not unexpected. According to this result, the decrease in IL-1β can be attributed to the effect of malathion on muscarinic or nicotinic receptors in the immune cells of liver tissue (Moser et al., 2015).

Conclusion

Our results indicated that even 14 days of MAL toxicity increased antioxidant enzymes activity (SOD and CAT) and lipid peroxidation (MDA level) synchronously in many tissues of rats. The most potency of MAL-induced tissue damage was also observed, after a short time, with cell degeneration, hyperemia, hemorrhage, inflammation, and cell necrosis in the liver, kidney, and heart tissues. Testes are probably less sensitive to the ratio of MAL-induced oxidative stress. Moreover, the suppression effect of MAL on inflammatory cytokines suggests that further studies are required to evaluate different doses and duration times on inflammatory responses.

Conflict of interest

There is no conflict of interest among the authors.

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