Protective effect of intravenous lipid emulsion treatment on malathion-induced ovarian toxicity in female rats

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Abstract. – OBJECTIVE: Malathion (MLT) is an organophosphate (OP) pesticide widely used in agriculture and for domestic purposes for several years. Intravenous lipid emulsion (ILE) has been reported to reduce toxicity caused by some lipid soluble agents. The aim of this study was to investigate the possible protective effects of ILE treatment on acute malathion toxicity in ovarian tissue of female rats.

MATERIALS AND METHODS: Twenty-one adult female Wistar rats (weighted 200-250 g) were divided into three groups; control (corn oil, gavage), MLT (one administration of 100 mg/kg/ by gavage), 20% ILE (one intravenous administration of 3 ml/kg) plus the MLT group. Blood samples were collected for biochemical tests. The ovaries were removed and fixed for histopathological and immunohistochemical analyses. Malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were investigated in ovarian tissues. Histopathological and immunohistochemical evaluations were performed through scoring ovarian tissue damage and bax/caspase-3 immunoreactivity, respectively.

RESULTS: SOD activity decreased in MLT group compared to the control group in tissue samples (p = 0.012). ILE treatment significantly increased SOD activity in MLT+ILE group compared to MLT group in tissue samples (p = 0.017). MLT treatment increased significantly caspase-3 and bax immunoreactivity while ILE decreased bax and caspase-3 immunoreactivity. However, no significant difference was found for MDA levels and GSH-Px activity in both blood and tissue samples and for histopathological results.

CONCLUSIONS: The present study revealed that acute oral MLT administration increased oxidative stress and apoptosis in the rats. ILE treatment partially decreased deleterious effects of MLT. Further controlled animal studies are required to define the role of ILE in acute OP poisonings.

Key Words:
Organophosphate, Malathion, Intravenous lipid emulsion, Ovary.

Introduction

Organophosphate (OP) pesticides are synthetic chemicals widely used since the mid-1940s for pest control[1]. They inhibit acetylcholinesterase, an enzyme hydrolyzing acetylcholine in cholinergic synapses and neuromuscular junctions. This inhibition causes acetylcholine to accumulate and leads to subsequent activation of cholinergic muscarinic and nicotinic receptors[1].

Malathion (S-(1,2-dicarbethoxyethyl) O,O-dimethyldithiophosphate) (MLT) is one of the most widely used OPs in agriculture, industry, and veterinary medicine because it has relatively lower acute toxicity compared to other OPs[2]. Several papers reported that it may have neurotoxic effects including various neuromotor, cholinergic, affective and cognitive disorders, when consumed at doses producing cholinesterase inhibition[3]. Owing to its lipophilic nature, MLT interacts with the cell membrane and results in disturbances in phospholipids bilayer structure of internal organs[4]. Recent studies have shown that OPs are likely to cause oxidative stress via disrupting the oxidant-antioxidant balance in the

body[5]. Increases in lipid peroxidation and decreases in antioxidant defense capacity are important results associated with OP toxicity[6]. MLT has toxic effects on experimental animals and exposed workers. It was observed that MLT is correlated with the dysfunction of several organ systems; among them are liver[7], testis[8] and brain[9].

Intralipid is the brand name of the first safe fat emulsion developed for human use and was introduced in 1962. Intravenous lipid emulsion (ILE) is always used in parenteral nutrition therapy. ILE is available in 10%, 20% and 30% concentrations[3]. ILE has proved to be an effective treatment for local anesthetic systemic toxicity in humans and animals and is promising as a novel antidote for a wide range of other lipophilic drug poisonings[10,11]. In this respect, we aimed to determine the acute effects of MLT on the ovarian tissue of female rats and to assess whether these effects could be improved by ILE treatment.

Material and Methods

Chemicals

MLT (Malaxon 65 EC®, Astranova Inc., Nigde, Turkey) and ILE (20% Clinoleic®, Baxter Healthcare Limited, Norfolk, England) were purchased. Other chemicals used in this study were described as needed.

Animals and Treatment

Adult female Wistar albino rats (weighed 200-250 g) were used in the study. Before the treatment, the animals were held at room temperature (22 ± 1 °C) and at 40-50% humidity. The light pattern was set to be 12 hour day and 12 hour night. They were left free in terms of eating and drinking. The rats were kept under observation for one week and they were physically examined daily. This work was performed in the experimental research unit after obtaining the permission of the local Ethics Committee (2014 HADYEK 77).

Rats were randomly divided into three groups: (1) control group (corn oil), (2) MLT treatment group (100 mg/kg), and (3) MLT + 20% ILE 3 ml/kg treatment group. All groups consisted of seven rats. MLT was administrated via gavage for once. Corn oil (vehicle of MLT) was given in the same way to rats in the control group. ILE was intravenously administrated for once.

Rats were sacrificed by decapitation under 50 mg/kg i.m. ketamine hydrochloride (Eczacıbası,

Istanbul, Turkey) anesthesia, 12 hours after the last oral dose administration. The ovaries were removed and left ovary was fixed in 10% formalin for histological and immunohistochemical analysis. The right ovary was removed in all animals for biochemical analysis of MDA level, SOD and GSH-Px activity.

Biochemical Analysis

Evidence of oxidative stress was determined based on ovarian tissue homogenates by measuring the levels of malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities. All assays were carried out at room temperature. The ovarian tissues were first washed with a cold isotonic saline solution. Then, tissues were homogenized using a homogenizer (IKA Ultra-Turrax t 25 Basic, Staufen, Germany) at 3,000 rpm for 3 min. Homogenates were then filtered and centrifuged. Supernatants obtained were used to determine the enzymatic activities.

Measurement of MDA level: Levels of MDA were measured as described by Esterbauer et al[12]. This method is based on the reaction of MDA with thiobarbituric acid (TBA) at 90-100 °C. After the MDA and TBA reaction, a pink pigment with an absorption maximum at 532 nm is produced. Results were expressed as nanomoles per gram protein (nmol/ g protein).

Measurement of *GSH-Px* activity: GPx activity was determined according to the method described by Paglia and Valentine[13]. In this method, the enzymatic reaction was initiated by the addition of H_2O_2, and the change in absorbance at 340 nm was monitored with a spectrophotometer.

Measurement of SOD activity: Total (Cu/Zn and Mn) SOD activity was determined using the method described by Sun et al[14]. This method is based on the inhibition of nitroblue tetrazolium (NBT) reduction. One unit of SOD is defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. Both GPx and SOD activities are expressed as units per gram protein (U/ g protein).

Histopathological Examination

The ovaries were removed and fixed in 10% formaldehyde solution at room temperature, then dehydrated through ascending grades of alcohol, cleared in xylene and embedded in paraffin blocks. Ovary tissue sections (4-5 µm in thickness) were stained with hematoxylin and eosin (H&E). Histological evaluation was performed

under light microscopy by the same pathologist, who was blinded to the study. The sections were viewed and photographed using a Zeiss Axio light microscope (Zeiss Axio Lab A1, Jena, Germany) and were scored from 0 to 3 according to their severity, where 0 represents absence of any pathologic finding, and 1, 2, and 3 represent respective percentages of involved areas as < 33%, 33%-66%, and > 66% of the ovary, respectively, as previously described[15]. The average scores for follicular degeneration, vascular congestion, hemorrhage, edema and inflammatory cell infiltration were calculated in each group, and total tissue damage score was determined by the sum of these scores.

Immunohistochemical Analyses

Immunohistochemical staining was carried out based on previously described methods[16]. In short, five-micrometer serial sections were collected on poly-L-lysine coated slides (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 56 ℃. Tissue sections were deparaffinized in xylene and rehydrated in ethanol series. Then, sections were treated in a microwave oven in 10 mM citrate buffer (pH 6.0) twice for 5 minutes each and cooled for 20 min. After washing three times in phosphate buffered saline (PBS), endogenous peroxidase activity was quenched by 3% hydrogen peroxide in PBS for 20 minutes, and washed again three times in PBS. To block non-specific binding sections were incubated in a blocking serum (Ultra V Block, ScyTek Laboratories, West Logan, UT, USA) for 10 minutes. Subsequently, sections were kept in a humidified chamber overnight at 4 °C using the rabbit polyclonal active-Caspase 3 (ab13847, Abcam, UK) and rabbit polyclonal anti-Bax (ab7977, Abcam, Germany) primary antibodies. Sections were washed with PBS at room temperature, and incubated first with biotinylated polyvalent antibodies (ScyTek Laboratories) and then peroxidase-labeled streptavidin (ScyTek Laboratories). Immunohistochemical analysis was performed using a horseradish peroxidase-labeled streptavidin biotin (SensiTek HRP) kit (ScyTek Laboratories) based on the manufacturer's instructions. Bound peroxidase was developed using 3-amino-9-ethylcarbazol (AEC) chromogen (ScyTek Laboratories). After being counterstained with Mayer's hematoxylin (ScyTek Laboratories), sections were mounted using Permount (Fisher Chemicals, Springfield, NJ, USA) on glass slides. As control, sections were treated only with normal rabbit serum. The concentrations of rabbit serum and the primary antibody used were the same. All samples for each individual antibody were subjected to the same protocol. Photomicrographs were taken with a Leica microscope (Leica DM2500, Nussloch, Germany).

Evaluation of Immunohistochemical Analyses

Evaluation of the immunohistochemical labeling was performed using H-SCORE analyses as previously described[16,17]. Caspase-3 and Bax immunoreactivities of the ovarian tissues were semi-quantitatively evaluated based on the following categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining), and 3+ (intense staining)[18]. An H-SCORE was calculated for each tissue as follows: per cent cells stained at each intensity category was calculated and then was multiplied by the weighted intensity of the staining based on the following formula: H-SCORE = $\sum Pi(i+1)$; where 'i' represents the intensity scores, and 'Pi' is the corresponding percentage of the cells. Five randomly selected areas were studied under a light microscope on each slide (40× objectives). Two investigators, who were unaware of the type and source of the tissues, determined the per cent cells at each intensity within these areas. The average score of both observers was used.

Statistical Analysis

Statistical analyses were performed using IBM-SPSS 20 program. Data were presented as mean ± standard deviation (SD). One-way ANOVA test was used to compare groups in terms of H-score values, and Tukey test was used as a post-hoc test. $p < 0.05$ was considered statistically significant.

Results

Biochemical Results

Tissue MDA, SOD and GSH-Px values in all groups were given in Table I. SOD activities in ovarian tissue were significantly lower in the MLT group compared with the control group ($p = 0.012$). ILE treatment significantly increased SOD activity in the MLT+ILE group compared with the MLT group ($p = 0.017$). MDA levels in ovarian tissue in MLT group was significantly higher than that in the control and MLT+ILE group but the difference was not sig-

Table I. MDA levels, SOD and GSH-Px activities in tissue samples.

	Control	MLT	MLT+ILE
MDA (nmol/g)	2.4 ± 0.3	3.4 ± 1.8	2.8 ± 1.1
GPx (U/g)	228 ± 31	177 ± 67	225 ± 42
SOD (U/mg)	0.8 ± 0.2	0.3 ± 0.1*,#	0.6 ± 0.1

MDA: malondialdehyde; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase. Values are expressed as mean ± SD.*p = 0.012, control vs. MLT #p = 0.017, MLT+ILE *vs*. MLT.

nificant (p = 0.222 and p = 0.300, respectively). The difference among the study groups for GPx activity in tissue samples was not significant.

Tissue GSH-Px and SOD activities decreased and MDA levels increased in the MLT group compared with the control and the MLT+ILE group. Although ILE treatment reduced MDA level and improved GSH-Px and SOD activity, the differences were not significant.

Histopathological Results

Microscopic images of histopathological changes in all groups were shown in Figure 1. Also, histopathological findings of ovarian tissue samples were summarized in Table II. When the tissue damage scores were comparatively evaluated, it was seen that pathological findings were minimum in the control group. Tissue damage scores demonstrating vascular congestion, hemorrhage, edema, inflammatory cell infiltration and follicular degeneration were slightly, but not significantly, higher in the MLT group compared to the control group (p = 0.310). Co-administration of MLT and ILE significantly decreased all of the pathological finding scores in the MLT+ILE group compared to MLT group, but this decrease was not significant (p = 0.690).

Immunohistochemical Findings

Bax and caspase-3 proteins were immunohistochemically stained and H-score results were presented in Figure 2. Immunoreactivity was not observed in negative control staining (Figures 2 and 3). H-score analysis revealed that bax and caspase-3 immunoreactivities significantly increased in the MLT group compared to the control and the MLT+ILE group (p < 0.001 and p < 0.01, respectively). Also, the bax and caspase-3 activities in the MLT+ILE group were significantly lower compared to the control group (p < 0.01) (Figures 2 and 3).

Discussion

Although OPs are commonly used as insecticides in agriculture all over the world, their specific effect on the female reproductive system is not sufficiently clear. In the present study, we studied biochemical, histological and immunohistochemical alterations caused by acute MLT toxicity on rat ovarian tissue and ameliorative effects of ILE treatment on these alterations.

Acute, subchronic and chronic toxicity studies indicated that MLT is highly toxic to mammals.
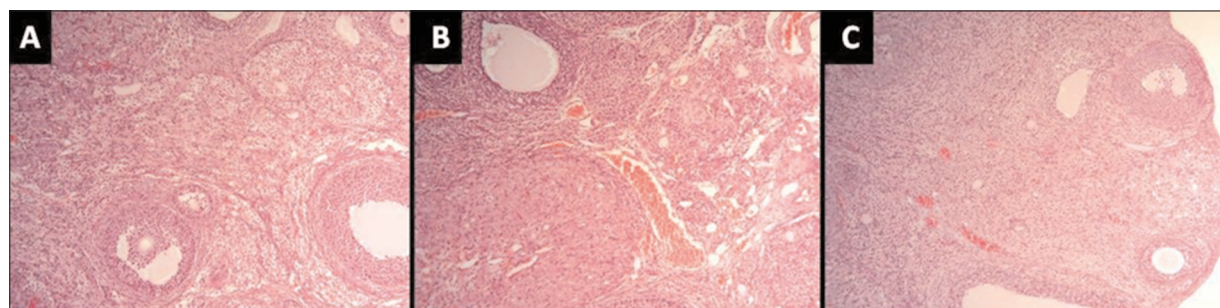
Figure 1. **A,** In the control group, ovarian stroma had a normal appearance. **B,** Ovarian tissues of the MLT group demonstrated pathological changes as indicated by the appearance of diffuse congestion, interstitial edema, and follicular degeneration. **C,** In the MLT+ILE group, milder congestion and hemorrhage were observed. (HE, ×10 objective).

Table II. Distribution of histopathological findings in groups.

| Histopathological findings	Control (n = 7)	MLT (n = 7)	MLT+ILE (n = 7)
Vascular congestion	0.75	1.2	1.1
Hemorrhage	0.50	0.8	0.6
Edema	0.25	0.5	0.4
Inflammatory cell infiltration	0.50	0.7	0.5
Follicular degeneration	0	0.5	0.2
Tissue damage score	2.0	3.7	2.8

MLT can adversely affect mammals through oral, dermal and respiratory exposure[19]. Some aggressive tissue damaging factors may impair oxidant/antioxidant balance in favor of oxidants in a process called oxidative stress. OPs can trigger oxidative stress, resulting in the production of free radicals and leading to alterations in antioxidants or reactive oxygen species (ROS) scavenging enzymes[21]. Because of its lipophilic properties, OPs interacts with the cell membrane and disrupts phospholipids bilayer structure of most internal organs[4]. Thus, OPs may elevate lipid peroxidation (LPO) level through directly interacting with cellular membranes and ROS generation. Main toxicity of OPs in acute exposure is through its irreversible binding to enzyme acetylcholinesterase and preventing its activity, thereby leading to accumulation of acetylcholine which ultimately leads to acute muscarinic and nicotinic effects[22].

There are some recent *in vivo* studies reporting OPs-induced oxidative stress in humans and animals[23,24] as well as some *in vitro* studies in animals[25]. MDA is an end product of lipid peroxidation and has been used to determine oxidative stress level of different tissues. SOD and GSH-Px are the main endogenous antioxidant defense enzymes. Several studies revealed that MLT increased MDA level and decreased SOD and GSH-Px activity[26-28]. We found that acute MLT exposure significantly decreased SOD activity in ovarian tissue compared to control group ($p = 0.012$) (Table I), indicating an impairment in the anti-oxidative system. ILE treatment after MLT exposure restored SOD activity considerably ($p = 0.017$). However, MDA levels and GSH-Px activities in ovarian tissue were not significantly different between control and treatment groups (Table I).

ILE was introduced in 1961 as a component of total parenteral nutrition. Then, it became a drug delivery vehicle and finally an adjunct in the resuscitation of cardiovascular collapse caused by local anesthetics[29]. Its administration has been strongly recommended to patients who developed cardiac arrest due to an overdose of local anesthetics such as bupivacaine[30-32]. ILE has also been shown to be useful in the treatment of neurotoxicity and cardiotoxicity resulting from a variety of cardiovascular and psychoactive drugs. Animal studies showed the usefulness of ILE in various toxicities arising from verapamil[33], propranolol[34], amiodarone[35] and clomipramine[36]. However, studies describing the effect of ILE on oxidative stress are rare. In one of those rare studies, oxidative stress parameters in parenterally nourished human preterm infants were not affected by ILE[37]. On the other hand, Basarslan et al[3] reported that ILE seriously reduced total oxidant status (TOS) in rats with MLT-induced neurotoxicity and stated that IFE is a promising safe therapy for acute MLT intoxication.

Previous studies[38,39] revealed the adverse effects of OPs on male and female reproductive systems in rats by affecting the histological func-
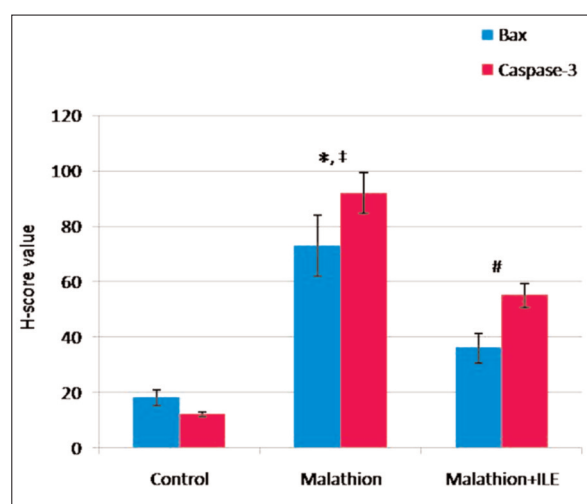
Figure 2. H-score values of bax and caspase-3 immunoreactivity. *$p < 0.001$, compared to the control group; ‡$p < 0.01$, compared to the MLT+ILE group; #$p < 0.01$ compared to the control group.
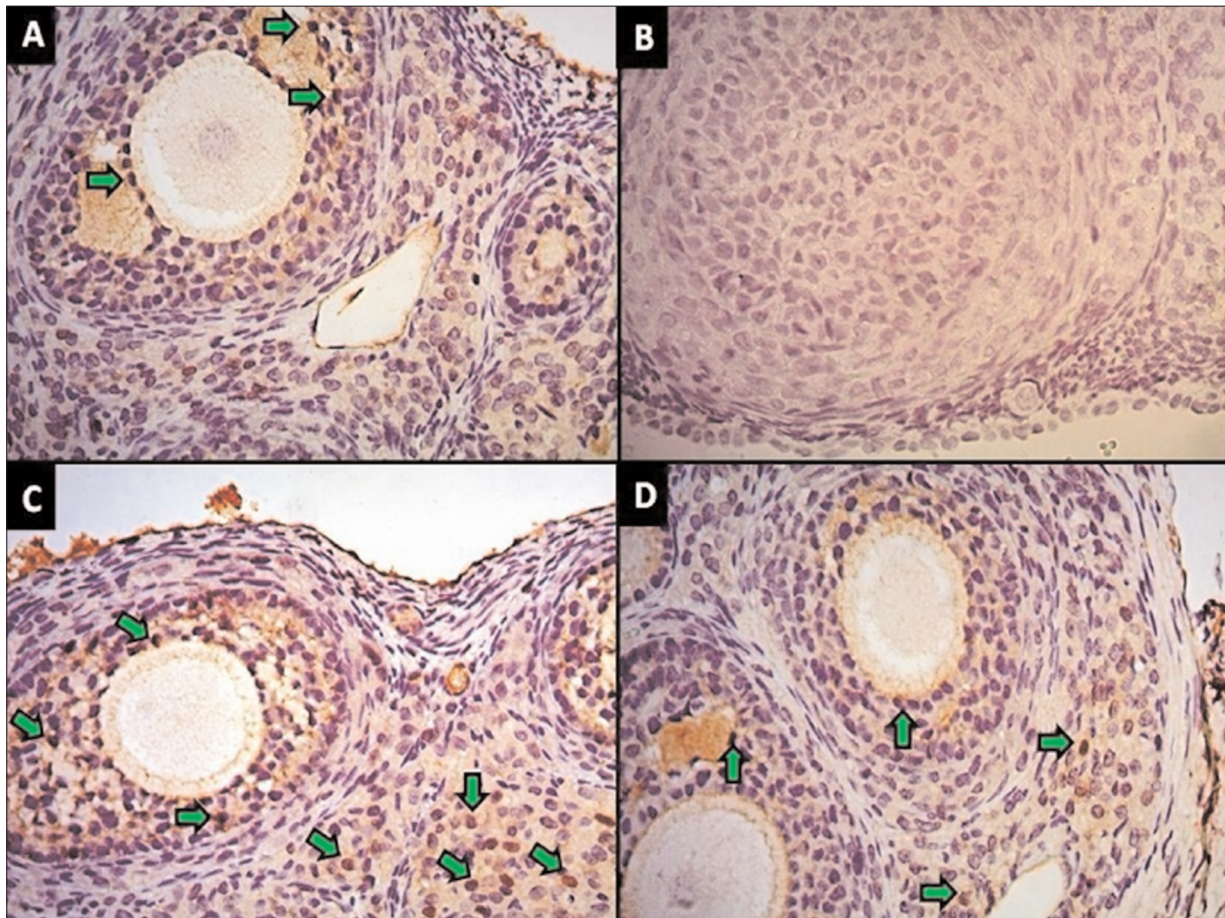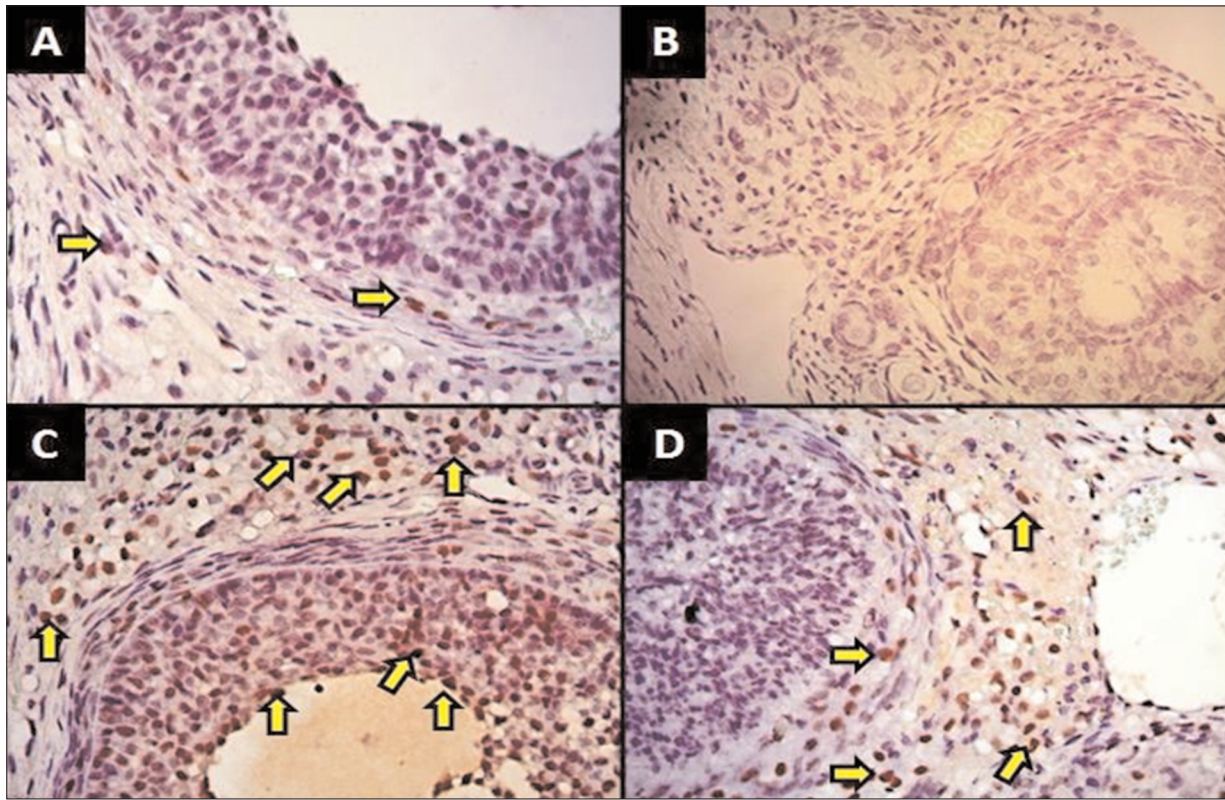
Figure 3. Evaluation of apoptosis by Bax (immunohistochemical method). **A,** Bax immunoreactivity in the control group. The green arrows indicate the Bax immunopositive granulose cells in secondary follicle. **B,** Negative control tissue. **C,** Strong Bax immunoreactivity observed in the MLT group. The green arrows indicate the Bax immunopositive granulose cells in primary follicle and stromal cells. **D,** In the MLT+ILE group, Bax immunoreactivity was significantly lower in comparison to the MLT group The green arrows indicate the Bax immunopositive granulose cells in primary and secondary follicle, and stromal cells ×40 magnifications).

tions of the organs. A study designed to investigate the effects of Diazinon, an OP, on rat pituitary-gonad axis and ovary showed no effect of 14-day oral Diazinon application on the mean number of primary, secondary and graaffian follicles. In addition, no difference was found[40] between groups with respect to histopathological changes including vascular congestion, hemorrhage, edema, inflammatory cell infiltration and follicular degeneration. In another study[41] using methyl parathion and male rats, on the other hand, the weight of seminal vesicle and prostate were found to decrease in a dose-dependent manner. A significant reduction in the weight of testes, epididymis, seminal vesicle and ventral prostate was reported by another study[42] in which MLT was orally administered to rats at 50, 150 and 250 mg/kg/body weight/day rates for 60 days. Koc et al[43] reported that MLT decreased the size of the ovary in adult Wistar albino rat depending on the doses used. In another study, MLT was given as 500 mg/kg body weight (BW) dose to adolescent male mice at the pubertal age for three days and no histological changes appeared in the testis compared to control groups[44]. MLT has also been reported to reduce sperm motility and viability and to increase the number of abnormal sperms[8,45,46].

Here, we observed that MLT caused histopathological changes in ovarian tissue including vascular congestion, hemorrhage, edema, inflammatory cell infiltration and follicular degeneration (Figure 1). ILE treatment partially improved these problems, but the effect did not

Figure 4. Evaluation of apoptosis by caspase-3 (immunohistochemical method). *A,* Caspase-3 immunoreactivity in the control group. The yellow arrows indicate the Bax immunopositive techa cells in secondary follicle and stromal cells. *B,* Negative control tissue. *C,* Strong caspase-3 immunoreactivity observed in the MLT group. The yellow arrows indicate the Bax immunopositive granulose cells in primary follicle and stromal cells. *D,* In the MLT+ILE group, caspase-3 immunoreactivity was significantly lower in comparison to the MLT group. The yellow arrows indicate the Bax immunopositive techa cells in primary follicle and stromal cells (×40 magnifications).

reach significance level (Table II). This finding could be due to the duration of exposure to MLT and application doze.

Apoptosis is programmed cell death process and plays an important role in the regulation of growth and development. Apoptosis has been known to take place from early developmental stages to adult stages and is involved in homeostasis of multicellular organisms, disease development and different stimuli in different systems[47]. Apoptosis is regulated by many modulators such as ions (e.g. calcium), genes (e.g. c-myc, Bcl-2/Bax, Fas and DR5), proteins (e.g. p53, caspases and IAPs) and even organelles (mitochondria and endoplasmic reticulum)[47]. OPs lead to oxidative stress, causing damage to cellular DNA and activation of apoptosis-related p53, caspase 3 and 9 genes. Thus, they have the potential to cause genetic alterations and cellular toxicity[48]. Impairments of several cellular processes associated with DNA repair and regulation dur-

ing apoptosis are mediated by caspase-3 activation[21]. OP poisoning has been reported to induce apoptosis via caspase-3 activation[49]. Four weeks of Diazinon administration was reported to increase bax and caspase-2 activity in cardiac tissue of rats[50]. Similarly, chlorpyrifos was concluded to cause apoptosis in rat cortical neuron[51]. Different immunolabeling patterns with caspase-3 and -9 were obtained in endometrial epithelium and stroma specimens from methyl parathion (MPT) treated and untreated rat groups[21]. In addition, subchronic exposure to dichlorvos was also reported to lead to similar differences in endometrium tissue[52].

There were only limited data available concerning histopathological and immunohistochemical changes caused by MLT in the ovary. Whether MLT had toxic effects in ovarian tissue remained largely unclear. In the present study, apoptosis was evaluated using bax and caspase-3 activity, and a significant difference was found

between treatment groups. In MLT group, a significant increase in apoptosis was observed compared to control group and MLT+ILE group ($p < 0.001$ and $p < 0.01$, respectively). Decreased apoptosis in MLT+ILE group ($p < 0.01$) is an important finding.

Conclusions

To our best knowledge, this is the first report evaluating the effects of MLT on ovarian tissue. The results revealed that the acute MLT exposure induced ROS production and apoptosis in ovarian tissue. Although ILE treatment partly improved these findings, further studies investigating the effects of ILE on the toxicity of OPs are required.

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Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper

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