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RESEARCH ARTICLE

Thiamethoxam-mediated alteration in multi-biomarkers of a model organism, *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Abstract

Thiamethoxam (TMX), a second-generation neonicotinoid, is extensively used to control numerous pests that infest crops. We investigated the effects of TMX (10, 20, 30, 40, and 50 μg/mL for 24, 48, 72, and 96 h) on biomarkers such as antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)); malondialdehyde (MDA), protein, lipid, and carbohydrate levels; micronucleus formation; and total hemocyte count in a model organism, *Galleria mellonella* L. SOD and CAT activities significantly decreased after 72 and 96 h of treatment at all TMX concentrations compared with control. MDA level increased following treatment with all TMX doses, with the exception of that following treatment with the lowest dose (10 μg/mL) at all tested treatment durations. Lipid and carbohydrate levels significantly decreased following treatment with high doses of TMX (40 and 50 μg/mL) after 48, 72, and 96 h. Micronucleated cell number significantly increased following treatment with all TMX doses at all tested treatment durations, except with 10 μg/mL of TMX for 24 h, when compared with control. During the first 72 h, total hemocyte count significantly decreased following treatment with 20-, 30-, 40-, and 50-μg/mL TMX; however, it was significantly reduced at all doses of TMX after 96 h. These results suggest that TMX can induce immunotoxicity, oxidative stress, and genotoxicity in a potential target and also in the model organism, *G. mellonella*. In addition, our study provides additional information regarding the prospective toxic effects of TMX.

Keywords Antioxidant enzymes · MDA · Micronucleus · Total hemocyte count · Thiamethoxam · Insects

Introduction

The use of neonicotinoids such as thiamethoxam (TMX), imidacloprid, and clothianidin has rapidly increased owing to the broad-spectrum insecticidal activity, relatively selective toxicity to insect pests, and low toxicological effects on non-target organisms of these neonicotinoids (Tomizawa and Casida 2003; Yan et al. 2016). Nevertheless, the use of neonicotinoid insecticides is suspected to be associated with negative effects on bees, which is why these insecticides have been banned by the European Union since 2013 (European Commission 2013).

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TMX is a second-generation neonicotinoid that has an agonistic effect on nicotinic acetylcholine receptors (nAChRs) and blocks neurotransmission in both insects and mammals (Tomizawa et al. 2000; Casida and Durkin 2013). Owing to the structural differences in nAChR receptors between mammals and insects, it has been suggested that the toxic effect of TMX on mammals is limited (Tomizawa and Casida 2003; Zhang et al. 2018).

Several studies on TMX have demonstrated that it induces changes in cell structures and apoptosis in the midgut of honeybees (Gregorc and Ellis 2011; Catae et al. 2018); in antioxidant responses in zebrafish (Yan et al. 2016) and rats (Keshta et al. 2016); in immune components in honeybee queens (Brandt et al. 2017); in hemato-biochemical parameters in cockerels (Gul et al. 2017); and in genotoxic biomarkers in snails and humans (Gad et al. 2016; Guo et al. 2018).

Biomarkers are powerful indicators for demonstrating the toxic effects of insecticides. Oxidative stress parameters such as superoxide dismutase (SOD), which scavenges superoxide radicals, and catalase (CAT), which removes H_2O_2, protect cells from oxidative stress. Malondialdehyde (MDA) is an important metabolite resulting from lipid peroxidation; thus,

any increase in MDA level is a certain indicator of damage to the cell membrane. Changes in the activities of these enzymes and an increase in MDA level are reliable indicators to determine the toxic effects of insecticides in insects. Changes in antioxidant enzyme activities and increases in MDA level, resulting from insecticide exposure, have been demonstrated in insects (Buyukguzel 2006; Buyukguzel 2009; Yucel and Kayis 2019).

Hemocytes play various roles in phagocytosis, nodule and capsule formation in cellular defense, the transport of hormones and nutrients, and detoxification of metabolites (Parakash 2008). Therefore, they are of great importance in insect physiology studies; changes in the number and structure of hemocytes have been used to demonstrate the effects of insecticides (Juhel et al. 2017). For example, imidacloprid, thiacloprid, and clothianidin change the total hemocyte number in insects (Kurt and Kayis 2015; Brandt et al. 2017; Yucel and Kayis 2019).

The micronucleus (MN) is an extra-nuclear body that forms during mitosis and is often used to show the genotoxic effects of toxic substances on DNA (Guo et al. 2018). It has been proven that pesticides can increase MN formation in certain cells of different organisms, including insect hemocytes (Karabay and Oguz 2005; Bolognesi et al. 2011; Kataria et al. 2016; Yucel and Kayis 2019).

Proteins, lipids, and carbohydrates are structural molecules playing significant roles in the reproduction and expansion of insect populations. However, they are also crucial energy sources (Olson et al. 2000; Hogervorst et al. 2007); specifically, these molecules are used as energy sources in detoxification processes. Therefore, changes in their quantities are considered important biomarkers of toxic stress (Maryanski et al. 2002; Emre et al. 2013; Yucel and Kayis 2019). For example, insecticides cause depletions in the lipid levels of *Pimpla turionellae* (Sak et al. 2006) and *Spodoptera littoralis* (Rashwan 2013); and in the carbohydrate level of *Drosophila melanogaster* (Kissoum and Soltani 2016).

Galleria mellonella L. is a pest in the apiculture industry; however, it has also long been used as a host for the production of parasitoid and predator species in biological control programs (Ellis et al. 2013). Because insect and mammalian models of the innate immune response are positively correlated in many ways, many studies have been conducted on *G. mellonella* as an alternative model organism in the past two decades (Kavanagh and Reeves 2007; Tsai et al. 2016; Yucel and Kayis 2019). *G. mellonella* larvae offer a number of advantages over their mammalian counterparts: their production is labor and cost-effective; they do not require special laboratory conditions; and they require neither expensive equipment nor ethics committee permits (Tsai et al. 2016).

Previous studies on TMX have measured its effects on different parameters within different organisms. For example, Yan et al. (2016) focused on antioxidant enzyme activities in *Danio rerio*; Catae et al. (2014) looked at the cytotoxic effects of TMX on midgut and Malpighian tubules in *Apis mellifera*; and Calderón-Segura et al. (2012) investigated the effects of TMX on human micronucleus formation. Therefore, we focused on studying the effects of TMX on multiple biomarkers using a single-model organism, *G. mellonella*.

Materials and methods

Thiamethoxam

TMX (Actara 240 SC, Syngenta) was obtained from a local market in Adiyaman, Turkey.

Insect rearing

G. mellonella larvae were reared under laboratory conditions (30 °C ± 2 °C, 70% ± 5% RH, and all-day dark period) with Bronskill's (1961) diet.

Experimental design

In all experiments, the last instar larvae (250–300 mg) of *G. mellonella* were used. After determining the LD_{50} value of TMX according to Finney (1971), 10 μL of phosphate buffer solution (PBS) was used as a control and equal volumes of PBS containing 10, 20, 30, 40, and 50 μg/mL TMX were injected into the hemocoel of larvae using a Hamilton syringe. The larvae were kept on a synthetic diet for 24, 48, 72, and 96 h. At the end of each time duration, the larvae were weighed and kept at − 80 °C until the subsequent experiments were performed. Each experiment was performed with five replicates.

Homogenization

For the determination of protein, SOD and CAT activities, and MDA levels, four larvae (250–300 mg) were pooled together and homogenized at the rate of 1:20 (w/v) in phosphate-buffered saline (50 mM, pH = 7.4). Each replicate contained 0.001 g of phenylthiourea to prevent the melanization. The homogenate was centrifuged at 10,000×*g* and 4 °C, and the supernatant was used for the protein, antioxidant enzyme activities, and MDA analyses.

For the determination of total carbohydrate and lipid levels, one larva was homogenized in 2 mL of sodium sulfate (2%) with phenylthiourea (0.001 g) at 24000 rpm for 5 min. Eight milliliters of a chloroform/methanol mixture (1:2) was added into each tube, and then, the tubes were centrifuged at 9000×*g* for 10 min. The supernatant was used for subsequent analyses.

SOD activity

SOD activity was determined according to Sun et al. (1988). This method is based on the inhibition of the reaction between nitroblue tetrazolium and superoxide radicals by SOD. Fifty microliters of sample and 25 μL of xanthine oxidase were mixed with 1.425 mL of SOD reagent. The mixture was incubated for 20 min at room temperature; then, the reaction was stopped by adding copper chloride (50 μL). The SOD activity was measured at 560 nm spectrophotometrically and expressed as U/mg protein.

CAT activity

Catalase activity was determined according to the method of Aebi (1984). Two hundred microliters of sample was mixed with 3 mL of hydrogen peroxide (30 mM) and then shaken rapidly. The decreasing absorbance value was measured kinetically at 240 nm at 30-s intervals for 1 min. Catalase activity was expressed as U/mg protein.

MDA levels

MDA levels were measured by the thiobarbituric acid (TBA) assay following the method of Bar-Or et al. (2001). A 250-μL aliquot of the sample was mixed with 125 μL trichloroacetic acid (TCA) (25%) in a microcentrifuge tube, and the mixture was centrifuged at 15000 rpm for 10 min at 4 °C. After removing the supernatant, 200 μL of TBA (0.8%) was added to each tube. The mixture was incubated for 60 min in a water bath at 90 °C, and the absorbance of samples was measured at 535 nm against a blank sample. The level of MDA was expressed as nmol/mg protein.

Total protein levels

Total proteins were determined according to the method of Lowry et al. (1951). Three-hundred-microliter aliquots of supernatant were placed into test tubes, and 3 mL of a reactive solution that were prepared according to the method of Lowry et al. (1951) was added to the tubes. Samples were incubated for 15 min at room temperature. Finally, 300 μL of the Folin–Ciocalteu reagent was added to the tubes. After 30 min, the absorbance value of each mixture was measured spectrophotometrically at 750 nm. Bovine serum albumin (0.1%) was used as a standard and the amount of total protein was expressed as mg/100 mg.

Total lipid and carbohydrate levels

Total lipid and carbohydrate levels were determined according to Van Handel's (1985a, 1985b) methods.

For lipid analysis, 200 μL of supernatant was kept in a water bath at 90 °C until the chloroform/methanol solution has been completely evaporated. Forty microliters of concentrated sulfuric acid was added to the dried supernatant, and then, the mixture was kept in a water bath for 2 min at 90 °C. After cooling, 960 μL of vanillin phosphoric acid solution was added and mixed. The mixtures were incubated at room temperature for 30 min; then, the absorbances were measured spectrophotometrically at 525 nm. Soy oil (0.1%) was used as a standard and the total amount of lipid was expressed as mg/100 mg.

For carbohydrate analysis, 200 μL of supernatant was kept in a water bath at 90 °C until the chloroform/methanol solution had completely evaporated. After cooling, 950 μL of anthrone solution was added. The sample was kept in a water bath for 15 min at 90 °C. The absorbances of samples were measured spectrophotometrically at 625 nm. Glucose (0.1%) was used as a standard and the total amount of carbohydrate was expressed as mg/100 mg.

Micronucleus assay

The method of Venier et al. (1997) was used to identify micronuclei. Briefly, three larvae were pierced with a sterile needle and the hemolymph was spread on a glass slide. After air-drying for 15 min, the smear was fixed with methanol for 5 min; then, the slides were stained with Giemsa (10%) for 10 min. Subsequently, the slides were rinsed in distilled water. A total of 1000 hemocytes were scored per slide in every replicate. This assay was performed in five replicates.

Total hemocyte counts

The total hemocyte count was calculated using the method of Jones (1962). Four *G. mellonella* larvae were anesthetized by cold treatment and pierced with a sterile needle. The resulting hemolymph was mixed with the Tauber–Yeager solution (Tauber and Yeager 1936) at a rate of 1:10; then, 10 μL of the dilution was placed in a Neubauer hemocytometer and hemocytes were counted using a light microscope (Olympus CX21). Total hemocyte counts were calculated according to Jones (1962), with five replicates.

Statistical analysis

The LD_{50} value of TMX at 96 h was determined using Finney's probit analysis (Finney 1971). Means, standard errors, and significance levels were calculated from five independent replicates. The results are presented as means ± SE.

Homogeneity of variance, checked using Levene's test, showed homogeneous data subsets. Therefore, treatment effects were tested using one-way ANOVA and the Student–Newman–Keuls (SNK) test was conducted to evaluate the

differences between means using SPSS 13.0. The significance level was set at 0.05. The relationships between TMX and the amounts of proteins, lipids, and carbohydrates; SOD and CAT activities; MDA levels; micronucleus formation; and total hemocyte counts were assessed using Pearson's correlation.

Results

Antioxidant enzyme activities and MDA level

Antioxidant enzyme activities and MDA levels of the different treatments are shown in Table 1. After 24 h, only the 50-μg TMX dose resulted in a decrease in SOD activity (df = 5; F = 15.923; $P < 0.05$). At higher doses of TMX (40 and 50 μg), SOD activity significantly decreased compared with control after 48 h (df = 5; F = 33.157; $P < 0.05$). All TMX doses caused a decrease in SOD activity when compared with control after 72 h (df = 5; F = 26.848; $P < 0.05$) and 96 h (df = 5; F = 36.459; $P < 0.05$). Similar to SOD, CAT enzyme activity was significantly reduced in response to increased concentrations of TMX. Except for the lowest dose of TMX (10 μg) after 24 h (0.063 ± 0.001) (df = 5; F = 244.594; $P > 0.05$), all doses of TMX caused a reduction in CAT activity when compared with control at all tested treatment durations (Table 1).

MDA levels increased significantly in all of the tested doses of TMX in all of the tested treatment durations when compared with control, with the exception of the 10-μg dose after 24, 48, and 72 h (Table 1). The negative correlation between MDA levels and antioxidant enzyme activities was observed at all tested treatment durations (Table 3).

Protein, lipid, and carbohydrate levels

There were no significant differences between the total protein levels of the control and TMX-treated larvae after 24, 48, 72, and 96 h (2.194, 2.342, 2.511, and 2.680 mg/100 mg, respectively) ($P > 0.05$) The primary energy sources (lipids and carbohydrates) were significantly affected by TMX. The amounts of lipids and carbohydrates decreased significantly at the highest dose of TMX (50 μg) after 24 h of treatment (df = 5, F = 9.959, $P < 0.05$; and df = 5, F = 50.863, $P < 0.05$, respectively). At 48 h, total lipid levels decreased significantly at all doses of TMX, except for the dose of 30 μg compared with control (df = 5; F = 16.145; $P < 0.05$). Total carbohydrate levels also decreased significantly at TMX doses of 20, 40, and 50 μg compared with control (df = 5; F = 92.871; $P < 0.05$). Furthermore, total lipid and carbohydrate levels also decreased significantly at TMX doses of 20, 30, 40, and 50 μg compared with control after 72 h (df = 5; F = 49.225; $P < 0.05$ and df = 5; F = 152.193; $P < 0.05$, respectively) and after 96 h (df = 5; F = 88.050; $P < 0.05$ and df = 5; F = 127.881; $P < 0.05$, respectively) (Table 2).

Table 1 Effects of TMX on antioxidant enzymes and malondialdehyde content of *G. mellonella* L. larvae

	TMX (μg/mL)	24 h	48 h	72 h	96 h
SOD (U/mg protein)	0.00	16.250 ± 0.591^{a}	14.260 ± 0.500^{ab}	14.441 ± 0.390^{a}	13.125 ± 0.350^{a}
	10.00	16.194 ± 0.958^{a}	13.613 ± 0.399^{b}	11.422 ± 0.411^{c}	11.648 ± 0.392^{b}
	20.00	15.699 ± 0.532^{a}	15.505 ± 0.503^{a}	11.699 ± 0.381^{c}	11.563 ± 0.423^{b}
	30.00	15.076 ± 0.518^{a}	15.031 ± 0.536^{ab}	10.704 ± 0.345^{bc}	8.824 ± 0.143^{c}
	40.00	13.971 ± 0.225^{a}	10.215 ± 0.247^{c}	9.811 ± 0.263^{c}	8.238 ± 0.416^{c}
	50.00	9.856 ± 0.598^{b}	9.639 ± 0.355^{c}	9.602 ± 0.198^{c}	7.990 ± 0.336^{c}
CAT (U/mg protein)	0.00	0.065 ± 0.001^{a}	0.063 ± 0.001^{a}	0.064 ± 0.001^{a}	0.057 ± 0.001^{a}
	10.00	0.063 ± 0.001^{a}	0.056 ± 0.001^{b}	0.027 ± 0.001^{b}	0.028 ± 0.001^{b}
	20.00	0.033 ± 0.001^{b}	0.032 ± 0.001^{c}	0.026 ± 0.001^{bc}	0.023 ± 0.001^{c}
	30.00	0.035 ± 0.001^{b}	0.027 ± 0.001^{d}	0.023 ± 0.001^{d}	0.022 ± 0.000^{c}
	40.00	0.033 ± 0.001^{b}	0.028 ± 0.000^{d}	0.023 ± 0.001^{cd}	0.021 ± 0.000^{c}
	50.00	0.031 ± 0.001^{b}	0.025 ± 0.001^{d}	0.020 ± 0.000^{e}	0.017 ± 0.000^{d}
MDA (nmol/mg protein)	0.00	3.99 ± 0.16^{e}	4.28 ± 0.19^{e}	4.42 ± 0.20^{e}	4.50 ± 0.17^{f}
	10.00	4.13 ± 0.30^{e}	4.12 ± 0.24^{e}	4.27 ± 0.20^{e}	6.22 ± 0.13^{e}
	20.00	13.25 ± 0.51^{d}	13.03 ± 0.48^{d}	12.73 ± 0.43^{d}	12.77 ± 0.21^{d}
	30.00	14.66 ± 0.42^{c}	14.68 ± 0.37^{c}	19.22 ± 0.42^{c}	22.68 ± 0.31^{c}
	40.00	25.85 ± 0.39^{b}	29.33 ± 0.49^{b}	27.70 ± 0.52^{b}	31.03 ± 0.42^{b}
	50.00	31.38 ± 0.75^{a}	34.39 ± 0.38^{a}	37.26 ± 0.35^{a}	38.64 ± 0.47^{a}

Data were expressed as mean ± SE of five replicates ($N = 5$). Different letters (a, b, c, d, e, and f) indicate statistical differences between groups at the $P < 0.05$ level at the same column

Table 2 Effects of TMX on protein, lipid, and carbohydrate amounts of *G. mellonella* L. larvae

	TMX (μg/mL)	24 h	48 h	72 h	96 h
Protein (mg/100 mg)	0.00	2.194 ± 0.076^{a}	2.342 ± 0.160^{a}	2.511 ± 0.064^{abc}	2.680 ± 0.110^{a}
	10.00	2.089 ± 0.072^{a}	2.379 ± 0.170^{a}	2.673 ± 0.142^{abc}	2.564 ± 0.075^{a}
	20.00	2.134 ± 0.142^{a}	1.941 ± 0.038^{a}	2.289 ± 0.135^{c}	2.566 ± 0.127^{a}
	30.00	2.179 ± 0.161^{a}	2.377 ± 0.123^{a}	2.438 ± 0.154^{bc}	2.715 ± 0.091^{a}
	40.00	1.939 ± 0.087^{a}	2.483 ± 0.128^{a}	2.925 ± 0.035^{a}	2.754 ± 0.079^{a}
	50.00	2.201 ± 0.100^{a}	2.439 ± 0.163^{a}	2.758 ± 0.050^{ab}	2.501 ± 0.127^{a}
Lipid (mg/100 mg)	0.00	6.320 ± 0.442^{a}	7.041 ± 0.339^{a}	7.441 ± 0.259^{a}	8.155 ± 0.252^{a}
	10.00	6.226 ± 0.295^{a}	5.960 ± 0.221^{b}	7.114 ± 0.193^{a}	8.043 ± 0.200^{a}
	20.00	5.981 ± 0.086^{a}	5.799 ± 0.300^{b}	5.457 ± 0.182^{b}	6.140 ± 0.263^{b}
	30.00	5.448 ± 0.160^{a}	6.351 ± 0.263^{ab}	5.673 ± 0.154^{b}	5.669 ± 0.069^{b}
	40.00	5.850 ± 0.141^{a}	4.978 ± 0.172^{c}	4.558 ± 0.188^{c}	4.854 ± 0.111^{c}
	50.00	4.202 ± 0.184^{b}	4.242 ± 0.122^{d}	3.904 ± 0.196^{d}	3.768 ± 0.132^{d}
Carbohydrate (mg/100 mg)	0.00	8.522 ± 0.350^{a}	8.272 ± 0.191^{a}	9.339 ± 0.220^{a}	8.838 ± 0.221^{a}
	10.00	8.074 ± 0.274^{a}	7.756 ± 0.265^{ab}	8.846 ± 0.191^{a}	8.384 ± 0.166^{a}
	20.00	7.651 ± 0.191^{a}	7.047 ± 0.232^{b}	6.838 ± 0.298^{b}	6.662 ± 0.466^{b}
	30.00	8.045 ± 0.357^{a}	7.828 ± 0.220^{ab}	6.714 ± 0.162^{b}	6.133 ± 0.185^{b}
	40.00	7.660 ± 0.356^{a}	4.257 ± 0.228^{c}	3.759 ± 0.186^{c}	2.904 ± 0.222^{c}
	50.00	2.724 ± 0.264^{b}	2.293 ± 0.339^{d}	2.837 ± 0.194^{d}	1.826 ± 0.083^{d}

Data were expressed as mean ± SE of five replicates ($N = 5$). Different letters (a, b, c, and d) indicate statistical differences between groups at the $P < 0.05$ level at the same column

Micronucleus formation

TMX caused an increase in micronucleus formation in hemocytes. All tested doses of TMX significantly increased the number of micronucleated cells at all tested treatment durations except for 10 μg TMX after 24 h (1.00 ± 0.32) when compared with the control (0.20 ± 0.20) (df = 5; $F = 30.678$; $P > 0.05$) (Fig. 1). There was a high positive correlation between MDA level and micronucleus formation at all tested treatment durations (Table 3).

Total hemocyte count

Compared with control, TMX caused a decline in THC after 24, 48, and 72 h, except for the 10-μg dose of TMX; meanwhile, all doses of TMX caused a significant reduction in THC compared with control after 96 h (df = 5, $F = 12.957$; $P < 0.05$) (Fig. 2). THC was negatively correlated with MDA level (Table 3).

Discussion

G. mellonella antioxidant enzyme activities, energy source levels, and THCs decreased significantly; while lipid peroxidation levels and micronucleus formation increased significantly in response to TMX, in a dose-dependent manner (Table 1).

Previous studies have shown significant changes in antioxidant enzyme activities in insects exposed to various pesticide formulations (Buyukguzel 2009; Aslanturk et al. 2011; Kayis et al. 2015). The increase in antioxidant enzyme activities is an

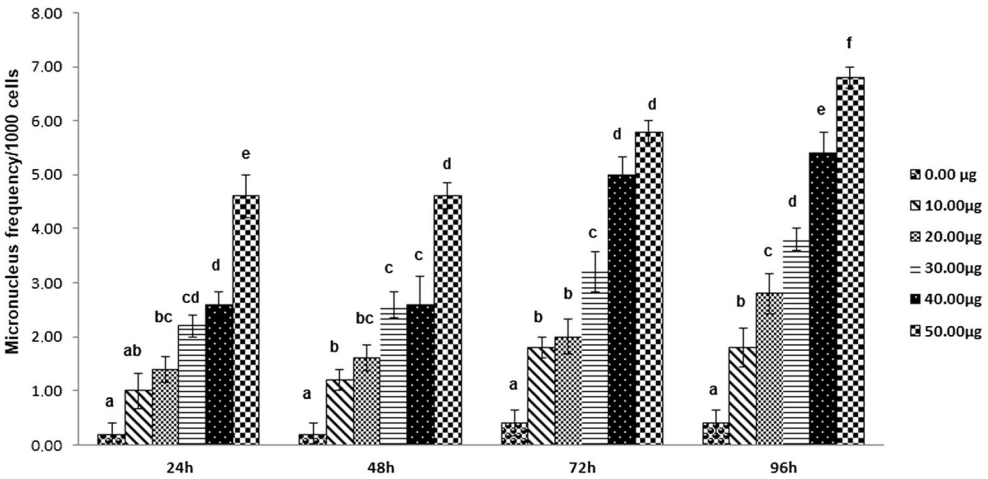

Fig. 1 Effects of TMX on micronucleus formation in hemocytes of *G. mellonella* L. ($N = 5$). Data were expressed as mean ± SE of five replicates. Different letters (a, b, c, d, e, and f) indicate statistical differences between groups at the $P < 0.05$ level at the same duration

Table 3 Pearson's correlation (two-tailed) coefficients (*r*) among tested biomarkers of *G. mellonella* exposed to TMX

	Protein	Lipid	Carbohydrate	SOD	MDA	CAT	THC
	24 h						
Lipid	− 0.129						
Carbohydrate	− 0.059	0.760**					
SOD	− 0.012	0.626**	0.847**				
MDA	− 0.115	− 0.676**	− 0.737**	− 0.778**			
CAT	0.030	0.534**	0.449*	0.503**	− 0.816**		
THC	0.093	0.361	0.299	0.360	− 0.641**	0.889**	
Micronucleus	− 0.008	− 0.754**	− 0.816**	− 0.824**	0.877*	− 0.689**	− 0.524**
	48 h						
Lipid	0.005						
Carbohydrate	− 0.163	0.869**					
SOD	− 0.446*	0.675**	0.819**				
MDA	0.175	− 0.791**	− 0.925**	− 0.760**			
CAT	0.035	0.586**	0.595**	0.344	− 0.794**		
THC	0.009	0.622**	0.672**	0.435*	− 0.818**	0.850**	
Micronucleus	0.144	− 0.669**	− 0.792**	− 0.543**	0.838**	− 0.735**	− 0.710**
	72 h						
Lipid	− 0.193						
Carbohydrate	− 0.375*	0.908**					
SOD	− 0.371*	0.708**	0.779**				
MDA	0.341	− 0.916**	− 0.965**	− 0.743**			
CAT	− 0.157	0.674**	0.636**	0.865**	− 0.599**		
THC	− 0.214	0.865**	0.899**	0.677**	− 0.934**	0.585**	
Micronucleus	0.342	− 0.874**	− 0.910**	− 0.776**	0.932**	− 0.688**	− 0.890**
	96 h						
Lipid	0.060						
Carbohydrate	− 0.036	0.940**					
SOD	− 0.108	0.811**	0.845**				
MDA	− 0.020	− 0.946**	− 0.961**	− 0.896**			
CAT	0.107	0.728**	0.674**	0.732**	− 0.695**		
THC	0.053	0.643**	0.628**	0.616**	− 0.631**	0.757**	
Micronucleus	− 0.047	− 0.917**	− 0.933**	− 0.872**	0.948**	− 0.769**	− 0.666**

**Correlation is significant at the 0.01 level (2-tailed)

*Correlation is significant at the 0.05 level (2-tailed)

important reaction to xenobiotic-mediated toxicity that occurs in response to oxidative stress due to exposure to insecticides (Al-Barty 2014; Muthusamy and Rajakumar 2016). Nevertheless, Cheung et al. (2001) and Yonar (2013) stated that this cannot be assumed to be a general rule because enzyme activities may increase or decrease, depending on the concentration of the stress factor or the duration of administration.

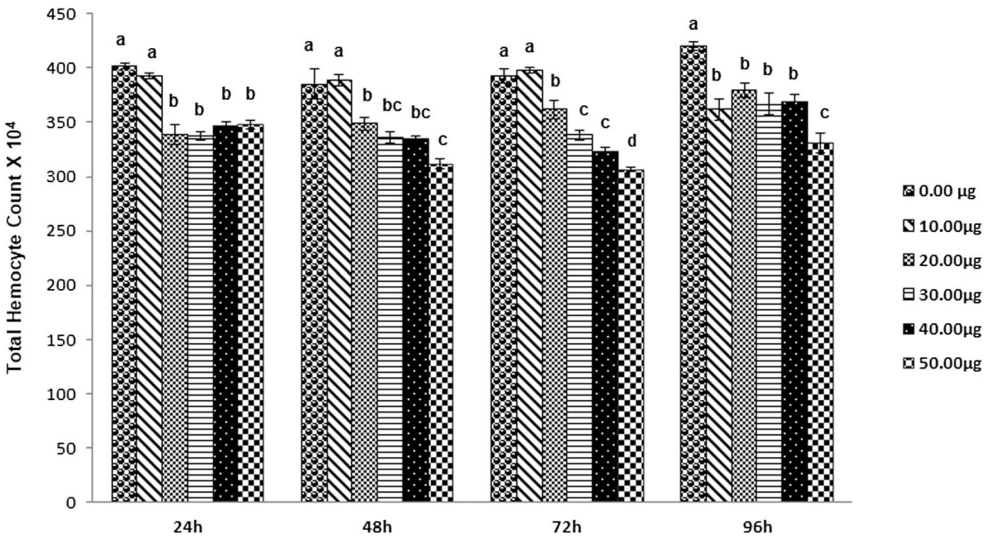

Fig. 2 Effects of TMX on total hemocyte count of *G. mellonella* L. ($N = 5$). Data were expressed as mean ± SE of five replicates. Different letters (a, b, c, and d) indicate statistical differences between groups at the $P < 0.05$ level at the same duration

Pesticides may enter the redox cycle directly and may cause oxidative stress by increasing the amount of reactive oxygen species (ROS). In addition, they can cause oxidative stress without entering the redox cycle by inhibiting the antioxidant enzymes (Lushchak 2016). In our study, only high doses of TMX caused a decrease in SOD activity after 24 and 48 h; meanwhile, all TMX doses decreased SOD activity significantly after 72 and 96 h. CAT activity was positively correlated with SOD activity (Table 3). It was reduced in all TMX doses at all treatment durations, except for the lowest dose (10 μg) of TMX for 24 h.

SOD is the first line of defense against ROS, and it is induced as the main cellular response to superoxide anion production (Ighodaro and Akinloye 2018). The superoxide radical is formed by the addition of an electron to molecular oxygen and is dismutated by the SOD enzyme, leading to the formation of hydrogen peroxide. The hydrogen peroxide formed by SOD enzyme activity is converted to water and molecular oxygen by catalase (Nordberg and Arnér 2001; Wickens 2001). However, any excess amount of ROS can cause direct inhibition of antioxidant enzyme activities (Yonar 2013; Han et al. 2016; Yan et al. 2016). Such decreases in antioxidant enzyme activities are caused by the oxidation of the cysteine residues in antioxidant enzymes by ROS (Dimitrova et al. 1994; Bagnyukova et al. 2006).

In our study, the SOD activity may have been inhibited by excess ROS, when it was present at high concentrations. Given the positive correlation between SOD and CAT enzyme activities, CAT activity may have also been directly inhibited by ROS; it is also possible that decreased CAT activity may involve the reduction of H_2O_2 production as a result of inhibition of SOD activity. Catalase activity is directly regulated by H_2O_2 concentration, and because of the high K_m values of CAT for H_2O_2, the enzyme is inefficient at low concentrations of H_2O_2. In insects, low concentrations of H_2O_2 are scavenged by ascorbate peroxidase (APOX) and dehydro-ascorbate reductase (DHAR) (Summers and Felton 1993; Mathews et al. 1997). Zhang et al. (2018) reported that high doses of fluoxastrobin cause the formation of excess amounts of reactive oxygen derivatives, resulting in lipid peroxidation due to the inhibition of SOD and CAT enzymes by their substrates. Gultekin et al. (2000) showed that chlorpyrifos-ethyl administered in vitro increased ROS production and therefore decreased SOD and CAT enzyme activities. Yan et al. (2016) and Keshta et al. (2016) demonstrated that TMX reduces both SOD and CAT activities in fish and rats, respectively. Similarly, findings that antioxidant enzyme activities decrease in response to different pesticides in different organisms (Ge et al. 2015; Lushchak 2016; Noshy et al. 2017; Velisek and Stara 2018) agree with the results of our study.

If an organism's antioxidant defense system is insufficient, then, ROS may cause lipid peroxidation. MDA, a product of lipid peroxidation, may cause a loss of membrane permeability and function, apoptosis, and necrosis in the cell (Toroser et al. 2007). Moreover, MDA levels can also reflect the level of lipid peroxidation indirectly (Kanbur et al. 2008; Zhang et al. 2014). In the present study, all doses of TMX (except for 10 μg), administered for up to 72 h, caused a dose-dependent increase in MDA levels in *G. mellonella*. Given that antioxidant enzyme activities (SOD and CAT) are negatively correlated to MDA levels (Table 3), then, increased MDA levels may be related to increased levels of ROS, causing a decrease in antioxidant enzyme activities, which are ultimately induced by TMX. Yucel and Kayis (2019) demonstrated that MDA levels are significantly increased in *G. mellonella* exposed to imidacloprid. A similar increase in MDA level was observed in *Danio rerio* (Ge et al. 2015; Shuklaa et al. 2017; Zhang et al. 2018) after imidacloprid, TMX, and fluoxastrobin treatments.

Energy molecules, such as proteins, lipids, and carbohydrates, are affected by ROS arising from toxic substances; the synthesis and utilization of these molecules may alter under toxic stress (Saleem et al. 1998). These molecules play central roles in growth, development, and reproduction; however, they are also used as sources of energy during the biotransformation of toxic substances (Olson et al. 2000; Hogervorst et al. 2007). For example, the energy derived from these molecules is used to produce enzymes and heat shock proteins involved in detoxification and removal of toxic substances (Maryanski et al. 2002).

In the present study, all doses of TMX (except for 10 μg) caused dose-dependent decreases in lipid and carbohydrate levels after 48, 72, and 96 h of treatment in *G. mellonella*. Similar decreases in lipid and carbohydrate levels were observed in *Oreochromis mossambicus*, *Spodoptera littoralis*, *P. turionellae*, and *Leptinotarsa decemlineata* by Raj and Joseph (2015), Rashwan (2013), Sak et al. (2006), and Fotouhi et al. (2015), respectively, after insecticide stress. In this study, lipid and carbohydrate levels were positively correlated with SOD and CAT activities, while they were all negatively correlated with MDA. Given the observed correlations between lipid and carbohydrate levels and oxidative stress biomarkers, we find it likely that the decreased levels of lipids and carbohydrates may be related to the synthesis of detoxification enzymes or to the damage of lipids and carbohydrates by ROS (Birben et al. 2012).

Micronucleus formation reflects damage to the genetic material; thus, it has been used as a biomarker for the genotoxic effects induced by environmental contaminants (Shimizu 2011; Guo et al. 2018).

There are very few studies on MN formation in insects in response to insecticides (Uckan and Sak 2010; Kurt and Kayis 2015; Kalita et al. 2016; Yucel and Kayis 2019). Studies have been shown that neonicotinoids such as imidacloprid (Stivaktakis et al. 2010; Ansoar-Rodriguez et al. 2015; Kataria et al. 2016; Yucel and Kayis 2019), acetamiprid (Kocaman and Topaktas 2007; Cavas et al. 2014), and

clothianidin (Calderón-Segura et al. 2015) cause an increase in MN formation in different organisms.

Although there may not be enough evidence to conclude confidently that TMX has a genotoxic effect (Hertner 1995; Sinha and Thaker 2013), studies conducted in recent years have shown that TMX induces DNA damage and MN formation in human neuroblastoma cells, in mice, and in the white garden snail (Salema et al. 2014; Gad et al. 2016; Senyildiz et al. 2018). In the present study, a significant increase in the abundance of micronucleated cells was observed at all tested doses (except for 10 μg for 24 h) of TMX. Yan et al. (2016) stated that increased ROS levels and excess MDA levels are the main agents of DNA damage and there is a positive correlation between MDA levels and micronucleus formation. We found that MDA levels are positively correlated with micronucleus formation; and antioxidant enzyme activities are negatively correlated to micronucleus formation. We suggest that the increase in abundance of micronucleated cells is due to DNA damage caused by increased levels of ROS that cannot be eliminated due to decreased activities of antioxidant enzymes and increased MDA level.

In addition to being an important component of the immune system, hemocytes play important roles in the detoxification of toxic substances and the transport of hormones and metabolites (Parakash 2008).

TMX caused a significant reduction in THCs of *G. mellonella* larvae, especially at doses higher than 10 μg. Similar decreases in THCs were observed in *Apis mellifera* exposed to imidacloprid, thiacloprid, and clothianidin (Brandt et al. 2017); and in *Apis dorsata* and *G. mellonella* exposed to imidacloprid (Perveen and Ahmad 2017; Yucel and Kayis 2019).

Pesticides can inhibit hematopoietic function (Zhu et al. 2012) and mitotic activity (Rajak et al. 2015) and promote apoptosis (Gregorc and Ellis 2011; Wu et al. 2015) in insect cells. In addition, studies have shown that TMX may cause structural deformation of insect cells (Oliveira et al. 2013; Catae et al. 2014). Because insects have an open circulatory system, circulating hemocytes can be targeted by toxic substances entering their body. The decrease in THCs may be due to either necrosis resulting from cellular damage or the induction of apoptosis, resulting from elevated levels of ROS due to TMX exposure. In addition, Salema et al. (2014) reported that the cytotoxic effect of TMX on mice is due to high levels of MDA. This agrees with the negative correlation between THC and MDA levels in our study; thus, the decrease in THC may be due to elevated levels of MDA.

Conclusions

According to present data, TMX causes significant alterations on oxidative, biochemical, genotoxic, and immunotoxic biomarkers in *G. mellonella*. These effects may be related to the disruptive effect of TMX on balance between the production of free radicals and antioxidant defense system. All of the changes in the biomarkers could also be used for further inspection of toxic effects of TMX in various organisms.

In the light of present findings, it can be concluded that TMX has a potential risk to non-target organisms such as parasitoid and predator insects, and honeybees. Even the observed findings within the model organism, *G. mellonella*, may reflect the potential toxic effects of TMX on mammalian organisms. Further investigations on the genetic effects of TMX at the molecular level should be conducted to determine the role of specific genes in the synthesis of antioxidant enzymes and the effects of TMX on cellular pathways leading to apoptosis and necrosis, thus leading to a better understanding in the toxicity mechanism of TMX.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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