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Effect of the Neonicotinoid Insecticide Thiacloprid on Oxidative Stress, Genotoxic, and Immunotoxic Biomarkers in Greater Wax Moth, *Galleria mellonella*

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Abstract: The aim of this study was to investigate the effect of the neonicotinoid insecticide Thiacloprid on oxidative stress, genotoxic, and immunotoxic biomarkers in *Galleria mellonella*. The effects of neonicotinoid insecticide thiacloprid on antioxidant enzyme activities, malondialdehyde (MDA) levels, hemocyte number, micronucleus frequency of greater wax moth *(Galleria mellonella)* larvae at different doses (5, 10, 15, 20, 25, and 30 µg) and periods (24, 48, 72, and 96 hrs) were explored. Superoxide dismutase (SOD) activity increased significantly at 5, 10, and 15 µg thiacloprid doses compared to the control and negative control in all periods tested, while significantly decreased at 20, 25, and 30 µg doses. Catalase (CAT) activity showed significant increases at 5, 10 and 15 µg thiacloprid doses at 24 and 96h compared to the control and negative control. MDA concentrations showed significant increases in all periods compared to the control and negative control. At 24th, 48th, 72nd and 96th, total hemocyte count (THC) decreased significantly at all doses except 5 µg thiacloprid concentration. During all the tested periods, there was a significant increase in the number of micronuclei, particularly at high doses of thiacloprid (20, 25, and 30 µg) compared to both the control and negative control. Additionally, a positive correlation was observed between MDA and the number of micronuclei, while other markers showed a negative correlation with micronucleus (MN). These results suggest that high doses of thiacloprid induce significant increases in micronuclei formation and are positively correlated with MDA levels, indicating oxidative damage and genotoxicity caused by thiacloprid exposure in the tested organism. Overall, our findings suggest that the measured parameters can be considered reliable biomarkers to demonstrate oxidative damage from thiacloprid exposure.

Keywords: *G. mellonella*, SOD, CAT, Micronuclei, Total hemocyte counts.

Neonikotinoid İnsektisit Thiacloprid'in Büyük Balmumu Güvesi, *Galleria mellonella*'da Oksidatif Stres, Genotoksik ve İmmünotoksik Biyobelirteçler Üzerindeki Etkisi

Öz: Bu çalışmanın amacı, neonikotinoid insektisit Thiacloprid'in *Galleria mellonella* üzerindeki oksidatif stres, genotoksik ve immünotoksik belirteçler üzerindeki etkisini araştırmaktı. Neonikotinoid insektisit thiaclopridin farklı dozlarda (5, 10, 15, 20, 25 ve 30 µg) ve periyotlarda (24 saat, 48 saat, 72 saat ve 96 saat) büyük balmumu güvesi (*Galleria mellonella*) larvalarının antioksidan enzim aktiviteleri, malondialdehit (MDA) düzeyleri, hemosit sayısı ve mikronükleus frekansı üzerindeki etkileri araştırıldı. Süperoksit dismutaz (SOD) aktivitesi, test edilen tüm periyotlarda kontrol ve negatif kontrol ile karşılaştırıldığında 5, 10 ve 15 µg thiacloprid dozlarında önemli ölçüde artarken, 25 ve 30 µg dozlarında ise önemli ölçüde azaldı. Katalaz (CAT) aktivitesi, kontrol ve negatif kontrolle karşılaştırıldığında 24 ve 96 saatte 5, 10 ve 15 µg thiacloprid dozlarında önemli artışlar gösterdi. MDA konsantrasyonları kontrol ve negatif kontrole göre tüm dönemlerde önemli artışlar gösterdi. Toplam hemosit sayısı (THC), 24., 48., 72. ve 96. saatlerde 5 µg thiacloprid konsantrasyonu dışındaki tüm dozlarda önemli ölçüde azaldı. Tüm test edilen sürelerde, mikronukleus sayısında özellikle yüksek dozlarda (20, 25 ve 30 µg) thiacloprid kullanımına bağlı olarak kontrol ve negatif kontrolle karşı önemli bir artış görüldü. Ayrıca, MDA ile mikronukleus sayısı arasında pozitif bir korelasyon gözlemlendi, diğer belirteçler ise MN ile negatif bir korelasyon gösterdi. Bu sonuçlar, yüksek doz thiacloprid'in mikronukleus oluşumunda önemli artışlara neden olduğunu ve test edilen organizmada thiacloprid maruziyetine bağlı olarak oksidatif hasar ve genotoksisite ile pozitif bir ilişki olduğunu önermektedir. Genel olarak bulgularımız, ölçülen parametrelerin, thiacloprid maruziyetinden kaynaklanan oksidatif hasarı göstermek için güvenilir biyobelirteçler olarak kabul edilebileceğini göstermektedir.

Anahtar kelimeler: *G. mellonella*, SOD, CAT, Mikronükleus, Toplam hemosit sayısı.

1. Introduction

Pesticides are chemicals that are mostly used to combat pests in areas such as agriculture, forestry, veterinary medicine, and public health. Although they give very effective results in the fight against pests, they can cause negative effects on many living things, including beneficial insects, plants, and vertebrates, including humans (Meftaul et al., 2020; Rani et al., 2021).

Neonicotinoids are the most potent group of neuroactive insecticides due to their neurotoxic effects on the nicotinic acetylcholine receptor (nAChR) (Matsuda et al., 2020). Thiacloprid is among the most widely used synthetic neonicotineoids in the world to protect fruits, vines, vegetables, and ornamental plants from pests (Schwarzbacherová et al., 2019). Although the use of thiacloprid was forbidden in Europe in 2020 and in Turkey in June 2022, it continues to be widely used in the fight against pests in a lot of countries (e.g., Canada, China, Australia, United States, and other developed countries) of the world (Zhao et al., 2020; Yavuz-Türel et al., 2023).

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Recently, there has been a focus on studying lipid peroxidation and oxidative stress parameters in relation to pesticide exposure and highlighted the genotoxic and immunotoxic effects of neonicotinoid insecticides such as thiacloprid on *Galleria mellonella*, which is the subject of this study. Experimental studies have shown that there are significant changes in enzyme activities and glutathione redox system after pesticide exposure (Yang et al., 2012; Akbel et al., 2018, Yucel & Kayis, 2019). It is known that most pesticides cause deterioration or suppression of the immune system and, within the framework of this knowledge, in recent years researchers have focused on studies on oxidative stress-related immunotoxicity (Nishino et al., 2013; Pamminger et al., 2018).

Many parameters are used to show the toxic effects of insecticides and biomarkers are among the most important and reliable parameters. Superoxide dismutase (SOD) and catalase (CAT) enzymes, which are among the oxidative stress parameters, play a role in protecting cells from oxidative stress. Therefore, changes in the activities of these enzymes are accepted as important oxidative damage indicators due to pesticide exposure. The increase in malondialdehyde (MDA), another oxidative stress parameter, is accepted as one of the most reliable oxidative stress-related damage parameters (Surajudeen et al., 2014; Zepeda-Arce et al., 2017).

The increase in the amount of reactive oxygen species (ROS) in the cells cause the decrease in antioxidant enzyme activities which leads to the deterioration of the oxidative balance (Sies, 1991). Free radicals cause oxidative damage by providing reversible or irreversible oxidation of proteins in the cell (Grosicka-Maciąg, 2011). As a result of oxidative stress, protein structures are oxidized and changes occur in the structures of proteins and enzymes that make up the cytoskeleton. This situation causes the emergence of many diseases (Dalle-Donne et al., 2001).

Studies on the toxic effects of pesticides have shown that hemocyte count can also be used to show pesticide toxicity. Hemocytes have many crucial such as phagocytosis, cell defense, nodule and capsule formation, transport of hormones and nutrients, and detoxification of metabolites (Kurt & Kayis, 2015). Therefore, hemocytes are a vital titer during insect physiology. Changes in the structure and number of hemocytes are important titer used to show the effects of pesticide exposure. Studies have reported that the total number of hemocytes changes in insects after exposure to imidacloprid and thiacloprid insecticides (Ravaiano et al., 2018; Murawska et al., 2021).

Micronuclei are masses of cytoplasmic chromatin formed by the emergence of small nuclei arising from fragments of chromosomes or intact whole chromosomes during the anaphase stage outside of cell division. Their presence in cells is a reflection of the structural or numerical chromosomal abnormalities that occur during mitosis (Heddle et al., 1991; Bolognesi et al., 2006). As a result of studies on the determination of genotoxic effects due to pesticide exposure, it was reported that the micronucleus (MN) test is quite reliable in determining genotoxicity. Studies on the subject have shown that MN formation increases after pesticide exposure (Karabay & Oğuz, 2005; Arslan et al., 2010; Arslan et al., 2015; Guo et al., 2020).

Galleria mellonella L., belongs to the order Lepidoptera and is an agricultural pest that causes serious damage to honeybee colonies, especially in the larval stage (Ellis et al., 2013). Moreover, in the laboratory studies that are conducted on biological control, *G. mellonella* larvae are also used as host organisms in the production of parasitoid and predatory species. Since the natural immune response is positively related to insect and mammalian models in many ways, this pest has been the subject of studies as a model organism in recent years (Fernandes et al., 2017; Tosi et al., 2018). It has been widely used in infection pathogenesis research in recent years (Mukherjee et al., 2011; Cook & McArthur, 2013). Obtaining high amounts (~20-50 µL) of hemolymph fluid can be counted among the important reasons why *G. mellonella* larvae are preferred in research (Mukherjee et al., 2011; Cook & McArthur, 2013; Brandt et al., 2016).

The current study's main objective is to assess the potential impact of Thiacloprid on the overall health and physiological responses of *G. mellonella*. This study specifically aimed to investigate the impact of thiacloprid insecticide on hemocyte, micronucleus, and antioxidant enzyme activities in *G. mellonella* larvae since there is no previous research addressing this issue.

2. Material and Methods

2.1. Test Organism and Thiacloprid

In accordance with the method developed by Bronskill (1961), *G. mellonella* larvae were grown in a dark environment with a temperature of 28°C ± 2°C and a relative humidity of 60±5%. Experiments were carried out in four repetitions and last-stage (7^{th} instar, 200 – 250 mg) larvae weighing 250 ± 50 mg were used. The larvae were grown in glass jars using semi-synthetic nutrients for production. Thiacloprid (Formula: $C_{10}H_9ClN_4S$, Calypso Od 240) was obtained from a local market in Adiyaman, Türkiye.

2.2. Determination of LD50 value

In order to determine the thiacloprid LD_{50} value, the larvae that reached the final stage were selected and injections were made at different doses using a Hamilton brand syringe. Stock solution was prepared using Calypso Od 240 Thiacloprid as the first procedure. Different concentrations of solutions were prepared from the prepared stock solution by the dilution method. While preparing the stock solution, care was taken to mix it correctly so that there was no residue at the bottom. Experiments were performed in 4 repetitions using 10 larvae for each dose.

In order to determine the LD_{50} value, the larvae that died and survived at the end of 96 hours were recorded and photographed (Finney, 1971). Probit analysis method was applied and the LD_{50} value was determined as 38 µg. According to the obtained results, sublethal doses were determined as 10 µL (5 µg, 10 µg, 15 µg, 20 µg, 25 µg, and 30 µg) for each larva.

2.3. Experimental Design

Galleria mellonella larvae were divided into three groups as control, negative control, and thiacloprid. No treatment was provided to the larvae in the control group, whereas 10 µL of phosphate buffer solution (PBS) was injected into

the negative control group. Each larva was injected into the left hind leg (5 µg, 10 µg, 15 µg, 20 µg, 25 µg, and 30 µg) in 10 µL thiacloprid using a Hamilton brand microinjection needle into the experimental group. Larvae were weighed at the end of the 24th, 48th, 72th, and 96th hour periods and stored at 80 degrees until the experiments were carried out. For SOD, CAT, MDA, and protein analyses, four larvae were used for each dose (5 µg, 10 µg, 15 µg, 20 µg, 25 µg, and 30 µg) and per hour (24, 48, 72, and 96 h), including control and negative control groups.

Larvae stored at -80°C were taken into 10 mL plastic tubes and phenylthiourea was added to prevent melanization. Larvae were homogenized at 24,000 rpm in 50 mM phosphate buffer (1/5). After homogenization, it was centrifuged at 10,000 rpm for 30 minutes at +4 °C and the obtained supernatant were used in SOD, CAT, MDA, and protein analysis.

2.4. Total Protein Concentrations

The method developed by Lowry et al. (1951) was taken into account to determine the amount of total protein. In the first stage, aliquots of 300 µL of supernatant and 3 mL of solution were placed in test tubes. Samples were then incubated at room temperature for 15 min. After incubation, Folin-Ciocalteu reagent (300 µL) was added to the tubes. Finally, after 30 min, the absorbance value of the mixture in the tubes was read on a 750 nm spectrophotometer (Shimadzu UV-1800). As a standard, 0.1% bovine serum was used and the total protein amount was determined as mg/100 mg of insect.

2.5. Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) enzyme activity was determined according to the method of Sun et al. (1988). In this context, first, 1.425 mL of reagent (Stock reagent was formed by mixing 0.3 mM of xanthine solution, 0.6 mM of EDTA solution, 150 µM of NBT solution, 400 mM of Na_2CO_3 solution and 1 g/liter of BSA solution) was added to the sample and blank tubes. Then, 0.05 mL sample was put into the sample tube.

After this, 0.025 mL of xanthine oxidase was added to the sample and blank tube, mixed, and left at room temperature for 20 min. After this step, the reaction was stopped by adding 0.04 mL of $CuCl_2$ to the sample and blank tubes. In the last step, 0.05 mL sample was added to the blank tube and absorbance values were determined at 560 nm with the help of spectrophotometer (Shimadzu UV-1800). SOD activity was expressed as U/mg protein.

2.6. Catalase (CAT) Activity

The determination of CAT enzyme activity was performed according to Aebi (1984). Here, 200 µL of sample and 3 mL of 30 mM hydrogen peroxide were mixed. The resulting mixture was shaken quickly. The decreasing absorbance values were measured kinetically at 240 nm for 1 min at 30 second intervals. CAT activity was given as U/mg of protein.

2.7. Malondialdehyde (MDA)Concentrations

The determination of MDA concentrations was performed according to the method developed by Bar-Or et al. (2001). Accordingly, 125 µL of 20% trichloroacetic acid was added to 250 µL sample and centrifuged at 15000 rpm for 30 min. After the sample was centrifuged, 200 µL of 0.8% thiobarbituric acid mixture was added on it and it was placed in a hot water bath at 90°C with mixing. After the samples were cooled down to room temperature, the absorbance was recorded using a spectrophotometer (Shimadzu UV-1800) at 525 nm against the blank and the values were shown in nmol/mg protein.

2.8. Total Hemocyte Counts

Total hemocyte count were counted at different times as four repetitions. A total of 128 larvae, 32 larvae for each replicate, were studied. The method developed by Jones (1962) was used for determining THC. The larvae were kept on ice with cold application to slow down their movements. Then, the hind legs of the larvae were pierced with the help of a lancet and 5 µL of hemolymph fluid was drawn with a pipette. Then, this hemolymph fluid was added to tubes containing of Tauber-Yeager solution (Tauber and Yeager, 1936) at a rate of 1:10. Then, it was mixed by pulling and releasing it several times with the help of a micro pipette. 10 µL of the obtained mixture was taken and placed in a Neubauer hemocytometer (Improved Neubauer Hemocytometer; Superior, Germany). THC was counted with an Olympus CX21 light microscope and THCs were expressed as number of cells per ml of hemolymph.

2.9. Total Micronucleus Count

The Venier et al. (1997) method was employed for micronuclei identification. Briefly, three larvae were punctured with a sterile needle and their hemolymph was spread onto a glass slide. After drying in air for 15 mins, the smear was fixed with methanol for 5 mins before being stained with Giemsa (10% in Sorensen Buffer, pH 6.8) for 10 mins. The slides were then rinsed in distilled water and a total of 1,000 hemocytes were counted per slide in each replicate. This procedure was repeated five times.

2.10. Statistical Analysis

With the help of Finney's (1971) probit analysis, the LD_{50} value of thiacloprid was determined at 96h. SPSS 13.0 program was used to analyze the data. Mean, standard error, and significance values were calculated for all the replicates. Results were given as mean ± SE. Levene test was used to determine a homogeneous distribution of the data. As a result of Levene test, it was determined that the data showed homogeneous distribution, one-way analysis of variance was used to determine the differences between groups. Student-Newman-Keuls (SNK) analysis was used to evaluate the differences between the means. Whether there was a relationship between continuous variables was evaluated using Pearson's correlation analysis. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Antioxidant Enzyme Activities and MDA Concentrations

It was determined that SOD enzyme activity increased significantly at 5, 10, and 15 µg thiacloprid doses compared to the control and negative control groups in all tested periods, whereas it decreased significantly at high doses of thiacloprid (20, 25, and 30 µg) according to the controls ($P < 0.05$). CAT activity at 5, 10, and 15 µg thiacloprid doses at 24 and 96 h showed significant increase compared to the

control and negative control groups, while there was no significant difference in CAT activity at low thiacloprid doses at 48 and 72 h (Table 1). At high doses of thiacloprid (25 and 30 µg) in all periods tested, CAT activity was significantly reduced compared to controls ($P < 0.05$). Depending on the increase in thiacloprid concentration, the total MDA amount increased significantly in all periods compared to the control and negative control ($P < 0.05$) (Table 1). While a positive correlation was found between SOD and CAT activities, it was observed that the relationship between these enzyme activities and MDA was negative (Table 2).

3.2. Total Hemocyte Counts

At the 24 h, THC was calculated as 231 × 10^4 in the control group and 237 × 10^4 in the negative control group, increased significantly at the lowest dose of 5 µg thiacloprid concentrations (270 × 10^4). Significant reductions occurred in THC at all other thiacloprid doses tested. At 48, 72, and 96 h, THC decreased significantly ($P < 0.05$) at all doses except 5 µg thiacloprid concentration (Fig. 1). There is a negative correlation between total hemocyte count and MDA and micronucleus (Table 2).

Table 1. Effect of thiacloprid on antioxidant enzyme activities and malondialdehyde level of *G. mellonella* larvae (0.00: Control group, 0.00*: Negative control group)

	Thiacloprid (µg)	24 h (mean±SE)	48 h (mean±SE)	72 h (mean±SE)	96 h (mean±SE)
SOD (U/mg protein)	0.00	4.450±0.094b	4.505±0.111b	4.490±0.083c	4.750±0.067c
	0.00*	4.382±0.080b	4.512±0.051b	4.477±0.133c	4.651±0.062c
	5.00	8.041±0.417a	8.071±0.088a	6.321±0.077b	5.534±0.106b
	10.00	8.328±0.127a	7.828±0.187a	6.279±0.214b	8.579±0.121a
	15.00	8.407±0.194a	8.244±0.117a	8.196±0.268a	8.549±0.067a
	20.00	3.486±0.113c	3.475±0.158c	2.993±0.114d	2.899±0.099d
	25.00	3.744±0.074c	3.354±0.086c	2.552±0.148d	2.533±0.092e
	30.00	2.302±0.075d	3.348±0.123c	2.599±0.104d	2.178±0.159f
CAT (U/mg protein)	0.00	0.019±0.0004b	0.022±0.0004a	0.023±0.0011a	0.026±0.0009b
	0.00*	0.019±0.0006b	0.022±0.0009a	0.023±0.0009a	0.028±0.0006b
	5.00	0.023±0.0008a	0.022±0.0005a	0.026±0.0005a	0.035±0.0006a
	10.00	0.024±0.0009a	0.023±0.0011a	0.025±0.0008a	0.036±0.0006a
	15.00	0.025±0.0004a	0.025±0.0007a	0.026±0.0009a	0.036±0.0006a
	20.00	0.024±0.0017a	0.024±0.0005a	0.016±0.0012b	0.017±0.0011c
	25.00	0.012±0.0006c	0.017±0.0009b	0.016±0.0008b	0.017±0.0005c
	30.00	0.011±0.0006c	0.013±0.0011c	0.013±0.0007b	0.016±0.0011c
MDA (nmol/mg protein)	0.00	11.386±0.393d	11.141±0.383d	10.240±0.158e	10.774±0.203d
	0.00*	11.370±0.516d	11.078±0.603d	10.742±0.310e	10.651±0.233d
	5.00	12.965±0.299c	14.492±0.562c	13.855±0.420d	18.029±0.459c
	10.00	13.123±0.239c	14.857±0.371c	14.952±0.186c	18.290±0.524c
	15.00	17.681±0.394b	15.741±0.623c	21.078±0.338b	19.576±0.423c
	20.00	17.569±0.400b	23.289±0.733b	21.460±0.253b	25.617±0.713b
	25.00	25.906±0.618a	31.220±0.594a	32.443±0.421a	33.998±0.680a
	30.00	26.432±0.392a	31.318±0.555a	32.569±0.342a	34.896±0.371a

Data were expressed as mean ± SE. Different letters (a, b, c, d, and e) in the same column indicate statistical differences between groups at the P<0.05 level.

Table 2. Pearson correlation (two-tailed) coefficients (r) among tested biomarkers of *G. mellanella* exposed to thiacloprid (N = 32)

24 h	SOD	CAT	MDA	THC
CAT	0.703**			
MDA	-0.501**	-0.665**		
THC	0.323	0.251	-0.690**	
MN	-0.559**	-0.634**	0.853**	-0.692**
48h				
CAT	0.529**			
MDA	-0.574**	-0.697**		
THC	0.147	0.234	-0.646**	
MN	-0.251	-0.276	0.627**	-0.721**
72 h				
CAT	0.862**			
MDA	-0.502**	0.738**		
THC	0.412*	0.664**	-0.795**	
MN	-0.591**	-0.805**	0.833**	-0.789**
96 h				
CAT	0.908**			
MDA	-0.548**	-0.647**		
THC	0.233	0.467**	-0.803**	
MN	-0.650**	-0.786**	0.903**	-0.803**

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

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

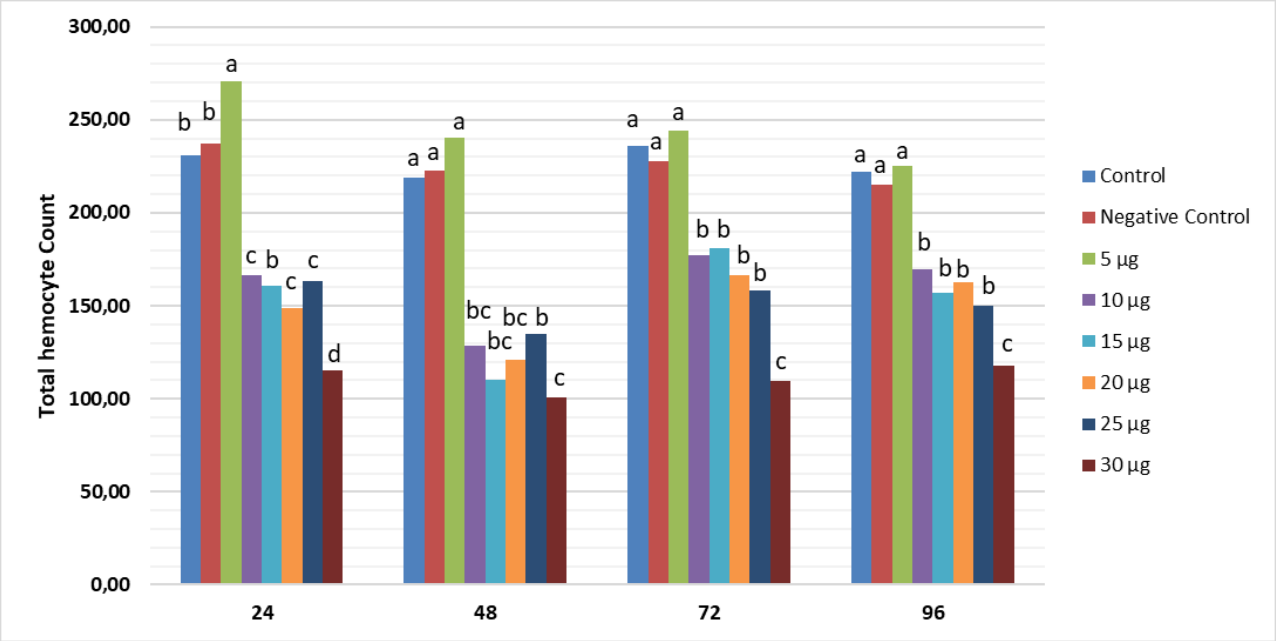

Figure 1. Total hemocyte counts

3.3. Total Micronucleus Count

The effect of thiacloprid on the total micronucleus count in *G. mellonella* larvae is presented in Figure 2. The number of micronuclei increased significantly in all tested periods, especially at high doses of thiacloprid (20, 25, and 30 µg) compared to the control and negative controls. Simultaneously, a positive correlation was found between MDA and the number of micronuclei, while there was a negative correlation between other markers and MN (Table 2).

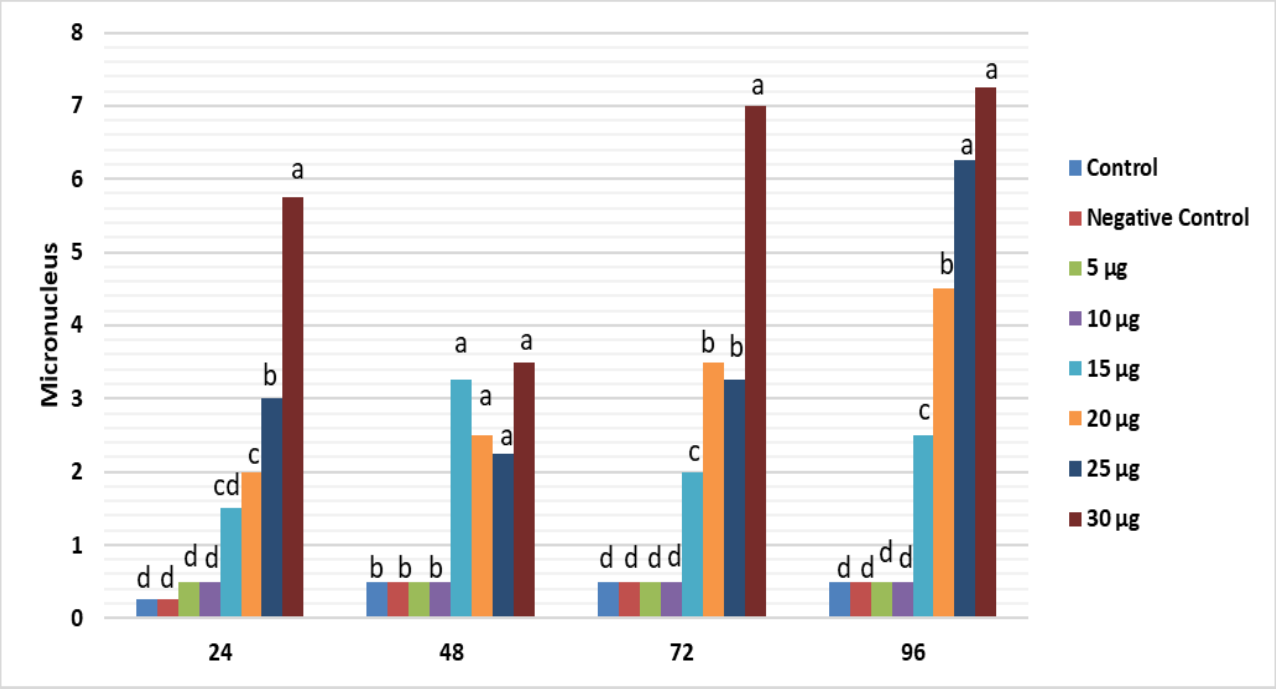

Figure 2. Total micronucleus counts

4. Discussion

Neonicotinoids are a new class of pesticides with a new mode of action. These have been previously referred to as nitro-quanidines, neonicotinyls, neonicotinoids, chloronicotins, and more recently chloronicotinyls. Just as synthetic pyrethroids resemble and model natural pyrethrins, nicotinoids resemble and are modelled after natural nicotine (Bass & Field, 2018). The introduction of neonicotinoids as new pesticides represents a milestone in pesticide research over the past three decades. Neonicotinoids represent the fastest growing class of insecticides introduced to the market since the commercialization of pyrethroids (Nauen & Bretschneider, 2002). One of the newer classes of insecticides, neonicotinoids, as agonists, preferentially act on nAChRs in insects. These receptors belong to the group of ligand-gated ion channels, which are responsible for providing rapid excitatory cholinergic transmission (Tomizawa &

Casida, 2005). However, it has been reported to have side effects on non-target organisms such as hepatotoxicity (Toor et al., 2013), neurotoxicity (Lonare et al., 2014), low sperm fertility (Bal et al., 2012), genotoxicity (Şekeroğlu et al., 2013; Kataria et al., 2016), and hormone disorders (Şekeroğlu et al., 2014). Clotianidine, imidacloprid and thiamethoxam, which are neonicotinoid insecticides, are banned for outdoor use in the EU countries due to their high toxicity especially for bees (EFSA, 2018).

Several studies have demonstrated that thiacloprid has lethal effects on various species (Brandt et al., 2016; Codling et al., 2016; Tosi et al., 2018). In this study, the LD50 value was determined for the first time by injecting thiacloprid into last instar larvae of *G. mellonella*. Our experimental results confirmed that thiacloprid caused dose-related mortality in *G. mellonella*, consistent with previous findings (Brandt et al., 2016; Mora-Gutiérrez et al., 2021).

In this study, the activities of SOD and CAT enzyme activities decreased significantly with an increase in thiacloprid dose, while MDA levels increased. Similar findings were reported by Büyükgüzel (2009) and Emre et al. (2013) in *G. mellonella* and by Aslanturk et al. (2011) in *Lymantria dispar*, showing a decrease in SOD and CAT enzyme activities as well as an increase in MDA concentrations due to pesticide dosage escalation. However, other studies have indicated that there is no significant change in oxidative stress parameters depending on the pesticide dose and application time in *Perna viridis* and *Cyprinus carpio* (Cheung et al., 2001; Yonar, 2013). The inhibition of SOD and CAT enzyme activities due to the pesticide use can result in the generation of ROS within cells, ultimately leading to oxidative stress (Lushchak, 2016). The dose-dependent effects of thiacloprid on the activities of SOD and CAT enzymes as well as the increase in MDA levels suggest that thiacloprid induces oxidative stress in *G. mellonella*.

According to the results of SOD and CAT enzyme activity in *G. mellonella*, their activities are affected by the dose of thiacloprid and their response varies depending on the specific time period of exposure. The dose-dependent effects of thiacloprid on the activities of SOD and CAT enzyme activities, as well as the variations in response based on the specific time period of exposure, indicate the complex nature of oxidative stress induction in *G. mellonella* due to thiacloprid. Depending on the increase in thiacloprid concentration, MDA level showed significant increases in all periods compared to the control and negative control. SOD is an enzyme that activates the breakdown of superoxide anion into oxygen and hydrogen peroxide. It removes oxygen by catalyzing a dismutation reaction which is important in reducing the harmful effects of oxidative stress. In the case of low SOD enzyme activity, this reaction occurs extremely slowly which leads to increased damage due to oxidative stress (Yang & Lee, 2015; Wang et al., 2019; Winterbourn, 2020). Although the enzyme activities of SOD and CAT increased at low doses and then decreased, it can be interpreted that these radicals inhibit the enzyme itself at high concentrations; although, there is an increase in enzyme activity for the removal of free radicals. While a positive correlation was found between SOD and CAT enzyme activities in all periods, the relationship between these enzymes and MDA levels was found to be negative. MDA, which is found *in vivo* in animals and humans in body fluids and in different tissues, is a product of lipid peroxidation (Pamplona et al., 2005; Baltacıoğlu et al., 2007; Weissman et al., 2011). MDA is widely used as a marker for evaluating oxidative stress in various organisms (Esterbauer & Cheeseman, 1990) and it is one of the lipid peroxidation products found *in vivo* in body types and different tissues in humans and animals (Pamplona et al., 2005; Weissman et al., 2011). Since MDA, which is produced as a result of the breakdown of fatty acids, easily reacts with lipoprotein, protein, DNA and RNA groups, it is widely used in the assessment of oxidative stress (Esterbauer & Cheeseman, 1990). In previous studies, it has been reported that pesticide exposure causes an increase in MDA levels, which is in line with the results obtained in our study (Zhang et al., 2018; Kayis et al., 2019; Yucel & Kayis, 2019).

In previous studies, it has been reported that MDA levels increase due to the increase in pesticide concentration which is in line with the results obtained in our study (Rashwan, 2013; Kayis et al., 2019). Reactive oxygen species can result from aging, radiation, metabolism, and exposure to insecticides (Yan & Sohal, 1998; Dokuyucu et al., 2014) leading to reduced antioxidants and disrupting the oxidative balance (Sies, 1991). This imbalance causes oxidative stress and the generation of free radicals that induce reversible or irreversible oxidation of proteins within cells (Prokai et al., 2007; Rao & Møller, 2011).

In the current study, after 24 hours, the THC following administration of a 5 µg dose of thiacloprid was significantly higher than the total hemocyte count in the Control group. Conversely, the most significant decrease in total hemocyte count occurred 24 hours after applying a 30 µg dose of thiacloprid compared to other doses. Furthermore, it was observed that the total hemocyte count reached its lowest point after administering a 30 µg dose at 48, 72 and 96 hours. These findings indicate that thiacloprid exposure in Greater Wax Moth, *Galleria mellonella*, leads to changes in hemocyte count and suggests immunotoxic effects of thiacloprid in this insect species. This study provides evidence that exposure to the neonicotinoid insecticide thiacloprid induces oxidative stress, genotoxicity, and immunotoxicity in *G. mellonella*. Many researchers have conducted research on the effect of sublethal doses of insecticides on hemocytes (Prakash, 2008; Kurt & Kayis, 2015; Kayis et al., 2019). Insects have two types of immune systems, cellular and humoral, that can respond quickly to disease-causing factors (Gupta, 1986; Gillespie et al., 1997). Humoral immunity is a defense system formed by antimicrobial proteins or peptides produced in the adipose tissue of mammals, in some of the blood cells of insects, which ensures the elimination of many bacterial species (Cytrynskaa et al., 2007). This disruption includes inhibition of cellular immune reactions, apoptosis mechanisms, endocrine glands (Sharma et al., 2003; Pandey et al., 2007), and hematopoietic function in larvae leading to decreases in hemocyte numbers (Zhu et al., 2012). The impact of pesticide exposure on the oxidative balance, generation of free radicals, and disruption of cellular defense systems highlights the potential health risks associated with pesticide use.

After 24, 48, 72, and 96 hours of administering a 15 µg dose of thiacloprid, the total micronucleus number increased significantly. The highest level of total micronucleus number was reached after administering a 30 µg dose of thiacloprid. There was also a significant difference between this dose and the others. It is known that exposure to low concentrations of pesticides can lead to effects such as the formation or induction/inhibition of enzymes involved in protein or DNA attachment (Kocaman et al., 2014). These findings highlight the potential genotoxic effects of thiacloprid exposure and further emphasize the need for careful regulation and assessment of pesticide use to mitigate potential health risks and environmental impacts. In the study conducted by Kayis et al. (2019), it was reported that the number of micronuclei increased significantly depending on the insecticide dose, in line with the results obtained in our study. In genotoxic toxicology, it is important that breaks or damages in chromosomes cause mutations and this is associated with cancer (Fenech, 2000). MN test used in the determination of genotoxicity is one of the most reliable techniques and has been used in recent years to determine genetic changes in organisms for many aquatic and terrestrial organisms (Arslan et al., 2010; Tsarpali & Dailianis, 2012; Arslan et al., 2015). A decrease in antioxidant enzyme levels was found in all lymphoid organs and plasma as a result of acute and subacute exposure to thiacloprid, as well as the thiacloprid/deltamethrin combination, in the rats used in the study. Considering these results, the researchers suggested that the oxidative stress induced by pesticide exposure may be partly related to the carbnonyl content of polymorphinucleated leukocytes or lipid peroxidation in addition to nitric oxide (NO) levels that cause an increase in myeloperoxidase activity. It was stated that peroxynitrite and hydroxyl radical production also increased due to the increase in NO activity and these caused oxidative stress and DNA damage (Barthwal et al., 1999). The findings of the current study indicate that exposure to thiacloprid and other pesticides can lead to a decrease in antioxidant enzyme levels and induce oxidative stress, potentially leading to DNA damage.

5. Conclusion

Thiacloprid has been shown to have lethal effects on different species in various studies and our study adds to the growing body of evidence on its toxic effects, particularly at low doses. Considering the results, it can be said that thiacloprid caused significant changes in THC, micronucleus, antioxidant enzyme activities, and MDA levels in *G. mellonella* larvae; therefore, the indicated parameters are reliable biomarkers that can be used in demonstrating oxidative damage due to pesticide exposure. Thiacloprid disrupted the normal hemostatic mechanism of larvae by increasing free radical production, leading to changes in antioxidant enzyme activities. Since the use of *G. mellonella* as a model organism has increased significantly in recent years and since it is an extremely useful model organism for mammals, the results obtained from the study are important in terms of demonstrating the potential effects of thiacloprid on mammals.

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