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Influence of Dietary Titanium Dioxide Nanoparticles on the Biology and Antioxidant System of Model Insect, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae)

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ABSTRACT

The potential toxic effects of the widespread use of titanium dioxide (TiO_2) nanoparticles (NPs) on insects have been brought into question as their presence in the ecosystem is unavoidable. Hence, the toxic effects of the different TiO_2 NPs should be investigated by establishing experimental model insects. Here, we examined the effects of different concentrations of TiO_2 NPs (100, 500, 1000, 3000 and 5000 ppm) on the biological parameters and total protein amount, antioxidant enzyme activities, malondialdehyde (MDA) amounts in the hemolymph of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). We found that larval and pupal developmental times significantly increased at 100, 500, 1000 and 3000 ppm when compared with control and highest doses of TiO_2 NPs. However, adult longevity time was shortened at low concentrations of dietary TiO_2 NPs (100, 500 and 1000 ppm). Exposure with dietary TiO_2 NPs caused a significant increase in the total protein amount and content of MDA and glutathione S-transferase activity in the hemolymph at 100, 500 and 1000 ppm compared with control and other doses of TiO_2 NPs. While the activity of catalase increased by 1000, 3000, and 5000 ppm and superoxide dismutase activity increased at all doses of TiO_2 NPs when compared with control. Our results indicated that TiO_2 NPs has a dose-dependent toxic effects on the *G. mellonella* larvae and can enhance the stress resistant capacity of insects at low concentrations.

Key words: Titanium dioxide nanoparticles, *Galleria mellonella*, nanotoxicology, antioxidant enzymes, model insect.

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INTRODUCTION

The use of synthetic nanoparticles (NPs) in science and industry has been growing enormously in various commercially products because of their biological, chemical, and physical aspects which enable them to be an indispensable element for the technology. The most fascinating feature of these particles lies in their relatively smaller size and larger surface area. The word “nano”, derived from the Greek nanos which means dwarf, includes natural or manmade materials that are between 1 to 100 nm and NPs occur naturally in the environment (Buzea *et al*., 2007). NPs exhibit different behaviors than the materials that have the same composition, but a larger size. Two primary factors, such as surface and quantum effects, cause NPs to behave differently than bulk materials (Roduner, 2006; Buzea *et al*., 2007; Handy *et al*., 2008). These factors affect the material’s chemical reactivity along with mechanic, optic, electric, and magnetic properties (Buzea *et al.*, 2007). However, the potential toxic effects of the widespread use of synthetic NPs on living organisms have been brought into question as their presence in the ecosystem is inevitable given their extensive use in science and industry. Thus, this situation led to the emergence of the new term “nanotoxicity” in recent decades. Studies into the toxicological effects of NPs on living organisms (Klaper *et al.*, 2009; Chakravarthy *et al.*, 2012; Karthigarani and Navaraj, 2012; Li *et al*., 2012a, b; 2016; Pelclova *et al*., 2017) have been published but are still needed to continue for insects.

Here, our concern is about titanium dioxide (TiO_2) NPs, which exists in the form of white powders and are widely used in electronics (Robertson *et al*., 2010), cosmetics (Hu *et al*., 2010), water treatment (Lachheb *et al*., 2002), antibacterial products (Yu *et al*., 2007), self-cleaning (Carneiro *et al*., 2007), and air cleaning applications (Yu *et al*. 2007). TiO_2 NPs also used as a white pigment because it has a high refractive index (Ortlieb, 2010). According to particle size, purity, surface area and characteristics, crystalline shape, and chemical reactivity, several TiO_2 NPs are being produced today. Hence, adverse effects of TiO_2 NPs should be investigated by establishing experimental models to study their toxicity to environmentally relevant species. Some studies have been conducted relating to *in vivo* and *in vitro* effects of TiO_2 NPs on human and animals (Hussain *et al.*, 2005; Kannan *et al.*, 2011; Chakravarthy *et al.*, 2012; Dalai *et al*., 2013; Memarizadeh *et al*., 2014; Pelclova *et al.*, 2017). In brief, these studies showed that TiO_2 NPs caused adverse effects such as cytotoxicity, inflammation, and oxidative stress. However, TiO_2 NPs can be toxic or nontoxic depend on high or low concentrations on bio-organisms in particular insect (Hussain *et al*., 2005; Li *et al.*, 2012a, b, 2014, 2016; Dalai *et al.* 2013; Zhang *et al.*, 2014; Wang *et al.*, 2015). Wang *et al.* (2007) showed that different particle sizes of TiO_2 NPs (25 and 80 nm) accumulated in the liver, spleen, kidney, and lungs in mice and caused oxidative stress due to oral administration. Zhang *et al.* (2014) determined that feeding silkworm *B. mori* with TiO_2 NPs at low concentrations increased the feed efficiency of larval stage and increased the activities of trehalase, protease, and lipase. However a recent study found that high concentrations of TiO_2 NPs were toxic on the silkworm (Li *et al.*, 2016). Li *et al.* (2012a) investigated *Bombyx mori* L. (Lepidoptera: Bombycidae)

nuclear polyhedrosis virus (BmNPV) resistance in insects after TiO_2 NPs exposure. Interestingly, TiO_2 NPs provided a decrease in reactive oxygen species (ROS) and caused an accumulation of nitric oxide (NO). In another study, Li *et al.,* (2012b) reported that TiO_2 NPs added to the diet at 5mg L^{-1}, significantly decreased biochemical dysfunctions in the hemolymph of fifth instar larvae of silkworms following exposure to phoxim insecticide. Similarly, Wang *et al*. (2015) showed that pretreatment with nano-TiO_2 attenuated the phoxim-induced midgut injury, increased body weight and survival, and decreased oxidative stress in the midgut of *B. mori*. On the other hand, Dalai *et al.* (2013) examined the toxicity of TiO_2 NPs (<25 nm) on *Ceriodaphnia dubia* (Cladocera: Daphniidae) and they reported that mortality increased at high doses. Furthermore, the expressions of the genes, including superoxide dismutase (SOD), catalase (CAT), and glutathione *S*-transferase (GST) were increased.

The latter results formed the basis of our current study concerning the biological and biochemical effects of TiO_2 NPs on the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). *G. mellonella* is found almost everywhere on earth and it is a pest for beekeeping. It is also used as a host for rearing biological control agents and evaluated in many different physiologic and toxicology studies owing to the various advantages of *G. mellonella* larvae for providing a model system in the laboratory (Dere *et al.*, 2015; Altuntaş *et al.*, 2016; Maguire *et al.*, 2016). It is also known that *G. mellonella* is an excellent model organism which can be used instead of mammalian species for *in vivo* toxicity and pathogenicity studies (Desbois and Coote, 2011; Maguire *et al*., 2016). For this reason, the nanotoxicity studies that are conducted on *G. mellonella* will help determine the possible effects on ecosystem and humans. Therefore, we aimed to determine the effects of dietary TiO_2 NPs on the life parameters, total protein amount, CAT, SOD and GST activities, and the content of malondialdehyde (MDA) of the *G. mellonella*.

MATERIALS AND METHODS

Insect culture

Galleria mellonella culture was prepared in glass jars (1 L) with honeycomb in the laboratory at 27 ± 1°C, 60 ± 5% RH, and constant darkness. Continuity of the culture was provided to emerge adult insects and newly hatched larvae.

Characterization and preparation of TiO_2 NPs

The sizes of TiO_2 NPs (Sigma-Aldrich Co.) were tested at The Scientific and Technological Research Council of Turkey (TUBITAK) by using a high resolution transmission electron microscope (model no: HRTEM, JEOL 2100) and scanning electron microscope (model no: JEOL/JSM-6510LV-INCA/EDS). The Zeta potential value of the particles was determined by using ZetaSizer Nano ZS (Malvern Instruments Inc., UK). Different concentrations of TiO_2 NPs (100, 500, 1000, 3000, and 5000 ppm) were added into double distilled water and treated with an ultrasonic homogenizer (amp: 60%, 15 min, Bandelin Sonoplus, HD 3200, Berlin, Germany) for the dispersion, stabilization, and preparation of the stock solution.

Biological assay

For bioassays, 1 ml of TiO_2 NPs at different concentrations of 100, 500, 1000, 3000, and 5000 ppm was sprayed into 1 gr of powderized honeycomb as a diet. Thus, TiO_2 NPs were easily attached on the surface of honeycomb. Then, the diet was left to dry for 1 day. Honeycomb in double distilled water was used for the control group. First instars of *G. mellonella* larvae were transferred individually into each sterile petri dishes (90 x 15 mm) with diet containing TiO_2 NPs, whereas diet without TiO_2 NPs served as a control. Development of first instar larvae was monitored daily until the last instars to determine the larval developmental time. Each last instars were also observed daily until pupation to determine the pupal developmental time. The time required for completion of pupal stage was recorded as the time of adult emergence per pupae. Adult longevity time (day) was also recorded in treated with different doses of dietary TiO_2 NPs and untreated groups. Each biological assay was replicated three times with twenty first instar larvae, selected from different populations at different times.

Hemolymph collection

The amount of total protein, MDA and the activity of antioxidant enzymes were determined in the treated with different doses of dietary TiO_2 NPs and untreated larval hemolymph. For this purpose, hemolymph samples were collected from the last instars of *G. mellonella*. Before the hemolymph collection procedure, larvae were anaesthetized on ice for 10 minutes and sterilized with cotton including 70% ethanol. Subsequently, ten microliters of hemolymph from each individual larva were collected with a 10-µl glass micro capillary tube (Sigma, St. Louis, MO) and transferred to a micro centrifuge tube (1.5 ml) placed on ice. Hemolymph samples were immediately mixed with a cold homogenization buffer (1:2 v/v) and 0.001 mg 1-phenly-2-thiourea was added to each tube to avoid hemocyte aggregation. The samples were immediately stored at -80°C until assays. Before all analyses, samples were homogenized according to Dere *et al.*, (2015). Ten *G. mellonella* larvae (0.16 ± 0.01 g) were evaluated for each experimental and control assays in four replicates (n = 40 per treatment).

Total protein and MDA amount

The total protein amount in larval hemolymph was determined according to Bradford's method (Bradford, 1976) using bovine serum albumin for the standard curve and analyzed with a microtiter plate (SpectraMax M2) at 595 nm. The MDA content in larval hemolymph was determined by using a commercial test kit (Cayman Chemicals Co., 10009055, USA). This test was assayed according to Dere *et al*., (2015). In this method, 25 µL sodium dodecyl sulfate and coloring reagent, (TBA acetic acid, TBA sodium hydroxide), were added to 25 µL hemolymph and the mixture was boiled for 1 hour. It was then left on ice for 10 min. After cooling, the mixture was centrifuged at 1600xg, 10 min, 4°C. 150 µL supernatant was used to determine the content of MDA. Absorbance was measured at 532 nm with microtiter plate (SpectraMax M2) and the content of MDA was expressed as nmol/mg protein by using the extinction coefficient 1.56 x 105 $M^{-1}cm^{-1}$.

CAT activity

CAT (EC 1.11.1.6) activity in hemolymph was assayed using Dere *et al.*, (2015) method. In this method, a certain amount of phosphate buffer and hydrogen peroxide (H_2O_2) were added to hemolysate and analyzed for 3 min at 240 nm. Absorbance values were detected with an ultraviolet-visible spectrophotometer (Shimadzu UV-1601, Tokyo, Japan). Specific CAT activity was determined as the amount of decomposition of 1 mmol of H_2O_2 to water and oxygen per min per mg protein using the extinction coefficient value (e240 = 0.0394 $mM^{-1}cm^{-1}$).

SOD activity

SOD (EC 1.15.1.1.) activity was analyzed according to assay kit, following the manufacturer's protocols (Cayman Chemical Co., 706002, USA). According to the protocol, SOD activity was determined by 2-[4-iodophenyl]-3-[4-nitrophenol]-5-phenyltetrazolium chloride (INT) reacting with superoxide radicals, using xanthine and xanthine oxidase (XOD) at 450 nm (Dere *et al.*, 2015). Absorbance was read by a 96-well microtiter plate (Spectra Max M2). SOD enzyme activity was expressed as U/mg protein.

GST activity

GST (EC 2.5.1.18) activity (cytosolic and microsomal) was assayed with a commercial assay kit (Cayman Chemical, 703302). According to kit protocol, an increase absorbance was detected continuously at 340 nm for 5 min related to the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (Dere *et al.*, 2015). Analyses were performed in a 96-well microtiter plate, and enzyme activities were also determined as nmol /min/mg protein using the extinction coefficient e340 nm = 9.6 $mM^{-1}cm^{-1}$.

Statistical analysis

First, all means of analyses were checked against the normality of data distribution. Then one-way analysis of variance (ANOVA) was performed to compare means because the data were normally distributed. In addition, significant differences of means were defined using Tukey's Honestly Significant Difference (HSD) or Tamhane T2 post hoc tests according to the homogeneity of variances. SPSS software program (SPSS, version 18.0 for Windows, SPSS Science, Chicago, IL) was used for these analyses. Means were considered statistically significant when $p<0.05$.

RESULTS

Characterization of TiO_2 NPs

TiO_2 NPs have an anatase crystalline structure according to the X-ray diffraction pattern. The particle size of TiO_2 NPs was calculated by using the Scherrer equation. We found that the size of TiO_2 NPs is about 25 nm based on this equation. The Zeta potential value, which is a measurement of the pushing or pulling of particles on each other in aqueous medium, was also calculated for TiO_2 NPs as +26.1 millivolt

(mV). The result showed that the stability of the TiO_2 NPs are less. This value is used for the calculation of the electro-kinetic properties of metal oxide NPs in aqueous suspension. It is expressed as negative or positive mV. The value is directly affected by suspension stability, aggregation of NPs and the surface morphology of the particle. The morphology of TiO_2 NPs was also observed by using HRTEM. These analyses showed that the structural shapes of TiO_2 NPs were hexagonal.

Effects on biological parameters

Larval and pupal developmental time prolonged at all concentrations of dietary TiO_2 NPs except for doses of 5000 ppm when compared with untreated groups (x^2= 8.040, df= 5, 340, P= 0.015; x^2= *16*.421, df= 5, 301, P= 0.006). However, adult longevity time was shortened at doses 100 and 500 ppm TiO_2 NPs when compared to untreated larvae. On the other hand, adult longevity did not change in higher doses of TiO_2 NPs with respect to control (x^2= 13.356, df= 5, 279, P= 0.02, Table 1).

Table 1. Biological effects of TiO_2 NPs on the *G. mellonella*.

TiO_2 NPs (ppm)	Larval developmental time (day)	Pupal Developmental Time (day)	Adult Longevity Time (day)
0	28.2 ± 0.36c	7.5 ± 0.31c	20.3 ± 0.65 ac
100	44.8 ± 2.97a	22.4 ± 3.06a	16.9 ± 1.18 b
500	44.9 ± 3.25a	19.9 ± 2.65a	18.8 ± 0.89 bc
1000	39.0 ± 2.63b	15.8 ± 2.19b	19.0 ± 1.02 bc
3000	40.7 ± 3.03b	16.6 ± 2.73b	22.1 ± 0.81 a
5000	31.8 ± 1.75c	11.3 ± 1.62bc	19.6 ± 0.79 ac

*Average for three assays. each with 20 larvae per treatment. Means within a column followed by the same letter are not significantly different (P≥0.05. 2-independent samples test: Mann-Whitney U).

Effects on total protein and MDA amount

The total protein amount in larval hemolymph of *G. mellonella* fed with dietary different doses of TiO_2 NPs is given in Fig. 1. The amount of total protein in control was 46.66 µg/µL and did not change significantly at 3000 and 5000 ppm. The total protein amount of larval hemolymph was showed a significant increase at 100, 500 and 1000 ppm of TiO_2 NPs exposure (F= 3.870; df= 5, 18; P= 0.015) when compared with untreated larval hemolymph. The effects of dietary TiO_2 NPs on the MDA amount in larval hemolymph are also represented in Fig. 1. The amount of MDA in control was 3.05 µM. TiO_2 NPs treatment had the most significant effect on MDA level a greater than two-fold increase at 100 ppm when compared to untreated larvae (F= 29.274; df= 5, 18; P= 0.000). The same trend is also evident at 500 and 1000 ppm doses when compared to that of control. However, exposure to TiO_2 NPs in diet did not significantly change the amount of MDA in larval hemolymph at 3000 and 5000 ppm doses when compared with control.

Effects on antioxidant anzyme activities

Results relating CAT activity in hemolymph of the last instars after exposure to TiO_2 NPs in diet is given in Fig. 2. CAT activity in the hemolymph of untreated larvae

was detected as 0.11 mmol/min/mg protein. CAT activity increased at 1000, 3000, and 5000 ppm doses whereas no statistically difference in activity were observed at 100 and 500 ppm doses of TiO_2 NPs exposure when compared with untreated larvae. Exposure to the highest dose of 5000 ppm of TiO_2 NPs in diet result in greater than four-fold increase in CAT activity in larval hemolymph when compared with control (F= 261.638; df= 5, 18; P= 0.000).

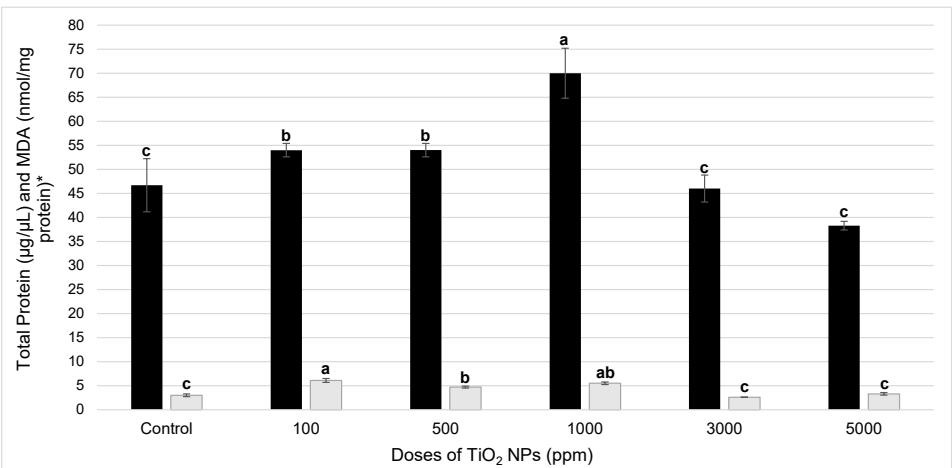

Fig. 1. Effects of TiO_2 NPs on the total protein and MDA amount in hemolymph of last instars of *G. mellonella.* *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-c) indicate statistically significant differences (P<0.05; Tukey-HSD test). Black bars represent the total protein amount, grey bars represents the MDA amount.

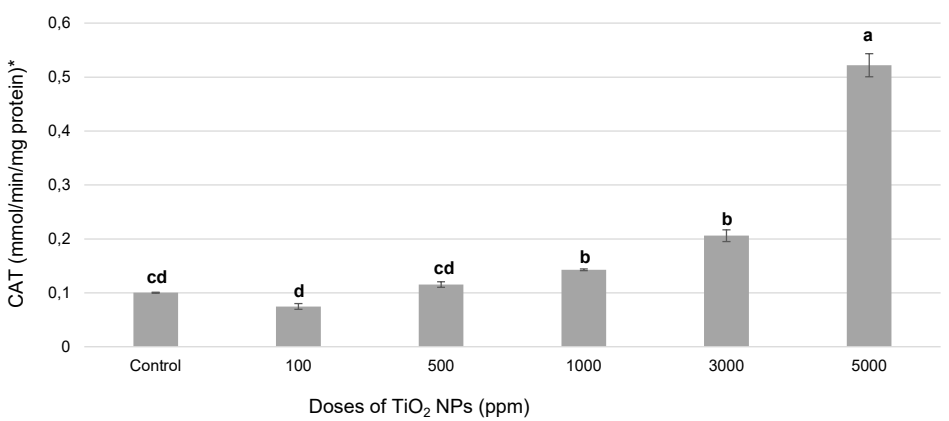

Fig. 2. Effects of TiO_2 NPs on CAT activity in hemolymph of last instars of *G. mellonella*. *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-d) indicate statistically significant differences (P<0.05; Tukey-HSD test).

SOD activities associated with the exposure of dietary TiO_2 NPs doses in hemolymph of the last instar *G. mellonella* larvae is showed in Fig. 3. These results

revealed that SOD activity in larval hemolymph increased at all doses of TiO_2 NPs when compared to the untreated group. In particular, a great increase of SOD activity was determined in the hemolymph of larvae at 3000 ppm doses of TiO_2 NPs when compared with control and other doses. In contrast, a drastic decrease in SOD activity was observed in the highest dose of TiO_2 NPs with respect to other treatment doses ($F= 61.594$; $df= 5, 18$; $P= 0.000$).

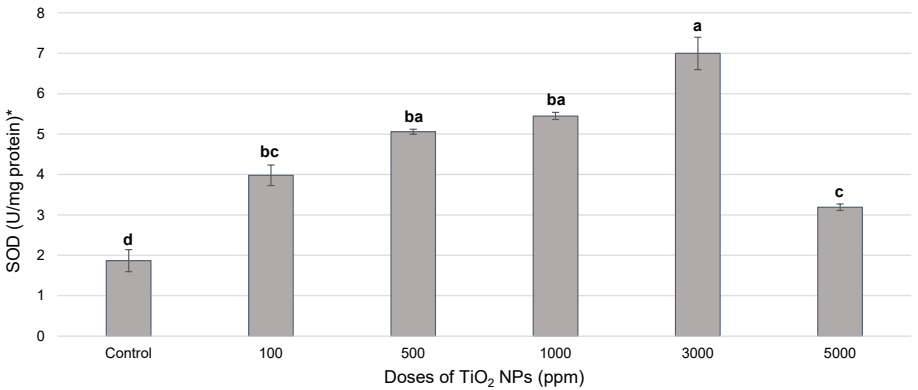

Fig. 3. Effects of TiO_2 NPs on SOD activity in hemolymph of last instars of *G. mellonella*. *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-d) indicate statistically significant differences ($P<0.05$; Tukey-HSD test).

The influence of dietary TiO_2 NPs showed similar changes in total protein amount and GST activity in hemolymph of larvae (Fig. 4.). Therefore, a significant increase of GST activity was determined at 100, 500 and 1000 ppm when compared to control. However, GST activity did not change statistically in higher doses of TiO_2 NPs in larval diet when compared with untreated larvae ($F= 15.375$; $df= 5, 18$; $P= 0.000$).

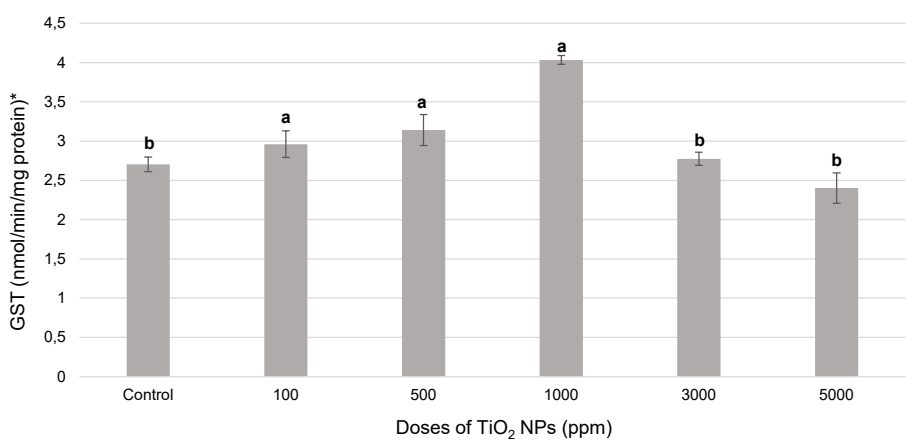

Fig. 4. Effects of TiO_2 NPs on GST activity in hemolymph of last instars of *G. mellonella*. *Values in the figure are Mean + SE from four replicates with 10 larvae per treatment. Different letters (a-c) indicate statistically significant differences ($P<0.05$; Tukey-HSD test).

DISCUSSION

Nanotoxicology has become the revolutionary subbranch of toxicology and has been gaining importance in agriculture, particularly in pest management recently. Sizes of NPs are the most effective factor for their toxic properties (Buzea *et al*., 2007; Handy *et al*., 2008). In principle, smaller sizes of NPs easily penetrate into the cells (Panariti *et al*., 2012) and this effect could then cause toxicity by generating reactive oxygen species (ROS) in organisms (Gurr *et al*., 2005; Bhattacharya *et al*., 2008; Shukla *et al*., 2011; Unnithan *et al*., 2011). Reports on the toxicity of nanoparticles are now continuously growing in number (Ghosh *et al*., 2010; Chakravarthy *et al*., 2012; Karthigarani and Navaraj, 2012; Pelclova *et al*., 2017). Generally, NPs show greater toxicity than their larger counterparts because of their specific particle number and surface area per unit mass (Buzea *et al*., 2007). TiO_2 has also been classified as a possible carcinogenic by the World Health Organization (WHO) (IARC, 2010) in spite of the fact that previous studies about silkworm reported that TiO_2 was non-toxic at low doses of application into diet (Zhang *et al*., 2014; Wang *et al*., 2015; Li *et al*., 2016). Zhu *et al*. (2009) also indicated that TiO_2 NPs toxicity changed in a dose-wise manner in *Daphnia magna* Straus (Cladora: Daphniidae). Two recent studies revealed that silkworm (*B. mori*) growth and developmental time were promoted and silkworm ecdysteroidogenesis was stimulated at low doses of dietary TiO_2 NPs (Shi *et al*., 2017). However, in the same study it was reported that high concentrations of TiO_2 NPs showed negative biological effects in *B. mori* with inhibited growth and development (Li *et al*., 2016). Contrary to this previous study, in our study showed that low doses of dietary TiO_2 NPs prolonged larval and pupal period and shortened the adult longevity in *G. mellonella*. Also, this biological parameters of *G. mellonella* did not change at higher doses. Our findings indicated that biological effects of TiO_2 NPs may be depend on digestion and absorption process of nutrition in the midgut of *G. mellonella* larvae.

In the previous toxicity studies, high concentrations of TiO_2 NPs caused the generation of ROS in various human cells (Gurr *et al*., 2005; Bhattacharya *et al*., 2008; Shukla *et al*., 2011), reproductive toxicity on the various freshwater organisms (Zhu *et al*., 2009). Whereas in some studies showed that low concentrations of TiO_2 NPs induced protein and carbohydrate metabolisms (Li *et al.* 2012a, b, 2014) and stimulated the important resistance genes (GPx, SOD, heat shock protein 21 (HSP21)), and induced AChE activity to the support the antioxidant response (Li *et al*., 2012a). Therefore, these previous findings are consistent with our data that the amount of total protein, MDA level, and the activities of CAT, SOD, and GST enzymes in larval hemolymph of *G. mellonella* varied depending on the concentrations of TiO_2 NPs. These results also may be related to some factors of TiO_2 NPs, such as size, surface area, particle chemistry, and crystallite structure (Buzea *et al*., 2007). In particular the toxicity of TiO_2 NPs may be depend on their bulk form's property. Because aggregates increase the size of the NPs and cause them to exhibit microparticulate properties (Buzea *et al*., 2007). For this reason, the zeta (ζ) potential value of TiO_2 NP is 26.1 mV indicating that the stability of the NPs in the aqueous solution can be weak and it strengthens the possibility that our material can aggregate.

Previous studies showed that low or optimum concentration of dietary TiO_2 NPs increased protein synthesis, midgut protease activity and improved the absorption and utilization of amino acids of fifth instar larva of silkworm *B. mori* (Zhang *et al*., 2005; Li *et al.*, 2012b, 2016). Similarly, in the present study, the highest amount of protein in larval hemolymph was detected as 69,7 µg/µl at 1000 ppm of TiO_2 NPs exposure, then an insignificant decrease was detected at the two highest concentrations of 3000 and 5000 ppm when compared to control (Fig. 1). This case may be related to the adaptive response of the organism against the toxicity of TiO_2 NPs and it is likely that the amount of various proteins, such as metal binding proteins, heat shock proteins (HSPs), and metallothioneins (MTs), may increase to block toxicity. However, the conservancy in the amount of total protein at doses higher than 1000 ppm may be due to the lipoproteins that are used for healing the antioxidant damage which is created by toxic materials. It is also likely that TiO_2 NPs may cause an increase in the intracellular calcium (Ca^{2+}) concentrations and this fact may in turn result in oxidative stress in organisms (Panariti *et al.*, 2012). The increase of Ca^{2+} binding proteins that regulate the activities of enzymes and structural proteins depends on Ca^{2+} releases (Lee *et al.*, 2002), which is an adaptive response for toxicity. Therefore, the increase of Ca^{2+}-binding proteins may affect the increase in the total protein amount in the hemolymph of *G. mellonella* at 1000 ppm dose of TiO_2 NPs. Another scenario is that the increase in free Ca^{2+} in response to cellular stress may promote autophagy that damages proteins and organelles (Panariti *et al.*, 2012). In our study, the main reason for the decrease in the total protein amount at 3000 and 5000 ppm doses of TiO_2 NPs might depend on protein damage by the autophagy process.

Lipid peroxidation in hemolymph was estimated by measuring the amount of an active aldehyde that is known as MDA which is of great importance for toxicity studies in non-metabolized form. Increasing the MDA level is a very important factor for toxicity studies (Ghosh *et al*., 2010; Karthigarani and Navaraj, 2012). Previous studies revealed that exposure to TiO_2 NPs increased the MDA level in human bronchial epithelial cells and rats (Gurr *et al.*, 2005; Unnithan *et al*., 2011). In our study, we also observed that TiO_2 NPs induced MDA content in hemolymph of the model organism, *G. mellonella*, and an increase was evident at doses 100 to 1000 ppm (Fig. 3) following a decrease again at 3000 and 5000 ppm. Undoubtedly, higher doses of TiO_2 NPs were more effective on *G. mellonella* than lower doses. This might be a response of the organism against the toxicity of TiO_2 NPs and may also be related to the activity of the antioxidant enzymes. It is likely that MDA content elevated by the increase of H_2O_2 initially, then later began to decline with the activity of CAT at especially 1000, 3000, and 5000 ppm doses of TiO_2 NPs, because CAT is an important antioxidant enzyme which responds to scavenge H_2O_2 concentration (Dorval *et al.*, 2003). It is well known that CAT is found in nearly all living organisms and catalyzes to separate H_2O_2 to water and molecular oxygen (Chelikani *et al*., 2004). The most striking effect observed here was a tremendous increase in CAT activity when last instars of *G. mellonella* were treated with 5000 ppm dose of TiO_2 NP. This activity of CAT at the highest dose treatment might be related to an adaptive response of the larvae to

an increase in H_2O_2, because CAT regulates H_2O_2 concentration in living organisms during oxidative stress conditions (Fornazier *et al*., 2002).

SOD is phase I enzyme that is found in nearly all cells exposed to oxygen (Bayır, 2005) and catalysis the dismutation of superoxide radicals to O_2 and H_2O_2 (Karthigarani and Navaraj, 2012). Li *et al*. (2012a) reported that adding TiO_2 NPs to an artificial diet at optimal doses significantly promotes SOD, CAT, and GPx expression in *B. mori* larvae under BmNPV infection. The results of this study support previous study that a dose-wise correlation was evident in SOD activity up to 3000 ppm of TiO_2 NPs exposure, with a tremendous decrease then at 5000 ppm. This increase may be related to the over production of ROS at lower doses. Otherwise, the cause of decreased SOD activity in higher doses may be related to increased CAT activity at 5000 ppm of TiO_2 NPs exposure. SOD activity is also used as a bio-indicator of toxicity showing the scavenging ability of ROS and the overwhelming of the antioxidant defense system (Vander *et al.*, 2003). Thus, the decrease in SOD activity at 5000 ppm dose of TiO_2 NPs might be related to an overproduction of ROS and decreasing the defensive ability of the antioxidant system (Karthigarani and Navaraj, 2012). It is a well-known fact that CAT and SOD are the main enzymes, which prevent cells from oxidative stress (Ayar-Kayalı and Tarhan, 2004). Collectively, our results indicate that CAT activity is an effective antioxidant enzyme against nanoparticle toxicity.

GST, which is also responsible for the radical detoxification mechanism in organisms (Fournier *et al*., 1992), is the major antioxidant enzyme which provides cells and tissue protection against the adverse effects of ROS. GST activity at the highest doses of 3000 and 5000 ppm did not differ from those estimated in control in our study. A relationship also appeared between MDA content and GST activity at the latter doses which may be an adaptive response of the organism against toxicity. It is expected that the activity of GST may increase at these doses depending on the MDA content, because GST is the major detoxifying enzyme synthesized in response to catalyze the conjugation of GSH during oxidative stress (Barbehenn *et al.*, 2013). As a physiological response, this activity provides the detoxification of endogenous compounds such as peroxided lipids (Oakley, 2011). Similar to SOD, GST activity might also be affected by the overproduction of ROS and inhibited at higher doses of TiO_2 NPs. Long *et al.* (2006) also reported that free radical species which are generated by TiO_2 NPs reduce antioxidant enzymes including GST. Likewise, it has been declared that other NPs such as cerium oxide, single-wall carbon nanotubes (SWCNTs), and semiconductor quantum dots (QDs), considerably reduce the level of antioxidants (Park *et al*., 2008). Similar to our results, Klaper *et al.* (2009) suggested that GST and CAT activities can be used to determine the physiological effects and toxicity of NPs. On the other hand, considerable elevations in the activity of GST at 100, 500, and 1000 ppm may be an attempt to counteract the elevation of MDA level as a defense mechanism against the accumulation of lipid peroxidation products in cells and physiological response mechanism against TiO_2 NPs toxicity for cellular detoxification. Connecting all these results we suggested that low doses of TiO_2 NPs can improve the antioxidant capacity to provide of physiological resistance in insects. The most striking difference in our

study was also in CAT activity, particularly at 5000 ppm dose of TiO_2 NPs. Collectively, we can emphasize that antioxidant enzyme activities, particularly CAT activity, can be used as a bio-indicator to evaluate NPs induced-oxidative stress in insects.

CONCLUSION

In conclusion, this paper is the first study that investigates the biological effects of TiO_2 NPs on biology and antioxidant capacity of *G. mellonella*'s. Therefore, our study provides important information about nanotoxicity on the insect. In general, biological data show that *G. mellonella* larvae are significant targets for TiO_2 NPs exposure and need to be included when evaluating the toxicological impact of NPs chemicals in the environment. Our study also suggests that TiO_2 NPs caused alterations to the total protein amount, the content of MDA, and the enzyme activity of *G. mellonella*. For this reason, we recommend common use of this insect as a model test organism for environmental effects of nanomaterials.

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