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RESEARCH REPORT

Biochemical biomarkers of *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae) in exposure to TiO_2 nanoparticles

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Abstract

Biochemical biomarkers and bioassays, due to their assumed immediate response after acute exposure of the organism to the stressor, are useful tools to gauging anthropogenic impacts. The toxicity of TiO_2-nanoparticles (TiO_2-NPs) on the *Glyphodes pyloalis* Walker was assessed and the LC_{50} value obtained as 660.85 mg/L. The *in vivo* responses of *G. pyloalis* to sub-lethal concentrations of TiO_2-NPs were surveyed by monitoring the activity of general esterases (EST), peroxidase (POD) and glutathione S-transferase (GST), as biochemical biomarkers. Activity of these biomarkers affected by exposure to TiO_2-NPs and this could lead to the mortality or sub-lethal impacts. The effect of TiO_2-NPs concentrations on the activity of these enzymes was correlated to the exposure time. The activity of EST and GST was significantly decreased compared to the control, after 24 h of treatments. By increasing exposure time, the expression of EST and GST was significantly increased. More POD expression was occurred at low concentrations (i.e. LC_{20} and LC_{30}); however, at high concentrations, less POD activity obtained. It can be concluded that these enzymes are good early indicator of toxicity and in conjunction with acute toxicity studies allow adverse effects of TiO_2-NPs to be predicted and managed.

Key Words: TiO_2-NPs; biomarker; *Glyphodes pyloalis*; esterases; glutathione S-transferase; peroxidase

Introduction

Nanoparticles (NPs) are particles with a diameter between 1 and 100 nm (Buzea *et al*., 2007). They show unique physico-chemical properties which differ from those of their respective bulk materials; such as large surface area, charge and shape (Handy *et al*., 2008). NPs can be utilized in the agricultural practices and this can be result in both risks and benefits for the ecosystem. Therefore, the evaluation of the safety of NPs in the environment looks very important (Kahru *et al*., 2008; Cattaneo *et al*., 2009).

Due to unique characteristics of manufactured NPs of titanium dioxide (TiO_2); such as chemically and biologically inert, stable toward corrosion and photocatalytic property, they are widely produced and consumed. Hence, the potential uses of TiO_2-NPs in various fields subjected them to the ecotoxicological studies (Linhua *et al*., 2009).

Cytotoxicity, phytotoxicity, lung inflammation and oxidative stress in mammals, plants and microorganisms have been reported as side effects of TiO_2-NPs (Ferin *et al.,* 1992; Warheit *et al*., 2007; Wang *et al*. 2007, 2009; Clemente *et al*., 2012). Despite the fact that TiO_2 has been classified as innocuous to the organisms (WHO, 1996); recently the International Agency for Research on Cancer (IARC) has classified this material as “possibly carcinogenic to humans” (IARC, 2010).

Till now, the vast majority of nanoecotoxicological studies with TiO_2-NPs have been focused on their toxicity to aquatic organisms (Clemente *et al*., 2012). Lovern and Klaper (2006) reported that *Daphnia magna* exposed to filtered TiO_2-NPs showed 100 % mortality at 10 mg/L; whereas, sonicated TiO_2-NPs caused only 9 % mortality at 500 mg/L. Federici *et al*. (2007) concluded that TiO_2-NPs caused sub-lethal toxicity in rainbow trout (*Oncorhynchus mykiss*) involved oxidative stress, organ pathologies, and the induction of antioxidant defense system such as reduced glutathione (GSH). Zhu *et al*. (2008) -

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compared the toxicity of several metal oxide NPs with their bulk counterparts to early developmental stages of zebrafish (*Danio rerio*) and showed that neither TiO_2-NPs nor bulk TiO_2 caused any toxicity to zebrafish embryos and larvae. Klaper *et al*. (2009) demonstrated that GST and catalase (CAT) are appropriate early biomarkers for prediction of physiological impacts and future toxicity of NPs to *Daphnia*. Linhua *et al*. (2009) showed that superoxide dismutase (SOD), CAT and POD activities and lipid peroxidation (LPO) levels in various tissues of carps varied with the concentration and exposure time.

Jianhui *et al*. (2005) formulated dimethomorph with sodium dodecyl sulfate (SDS)/TiO_2/Ag nanomaterial as a photodegradable nanofungicide. Guan *et al*. (2008) also used the same nanomaterials to formulation of imidacloprid. Furthermore, they produced W/TiO_2/avermectin photodegradable microcapsules (Guan *et al*., 2010). Despite the investigation on nanoecotoxicological studies with aquatic organisms; so far, no published reports are available on acute toxicity and biochemical alteration (*i.e.,* enzyme activities) caused by exposure of insect to TiO_2-NPs. Thus, due to potential for application of TiO_2-NPs to formulation of photodegradable pesticides; we aimed to make the ecotoxicological assessment of TiO_2-NPs exposure to an insect pest model species, *Glyphodes pyloalis*.

ESTs are detoxification enzymes which are involved in the insect physiology, metabolism, and xenobiotic detoxification (Ishaaya, 1993). Glutathione S-transferases (GSTs) are multifunctional enzymes in the phase II of pesticides metabolism (Ezemonye and Tongo, 2010). POD is an antioxidant enzyme which catalyzes the oxidation by hydrogen peroxide of a number of xenobiotics (Linhua *et al*., 2009). Because of the lack of knowledge about responses of insect EST, GST and POD to TiO_2-NPs, these enzyme activities were evaluated in *G. pyloalis* treated with TiO_2-NPs at different exposure times to predict the impact of these NPs at sub-lethal levels.

Materials and methods

Chemicals

Glycerol, ethanol, triton X-100, H_2O_2, bovine serum albumin, α-naphthyl acetate (α-NA), β-naphthyl acetate (β-NA), Reduced Glutathione (GSH), 1-choloro-2,4-dinitrobenzene (CDNB), 1,2-Dichloro-4-nitrobenzene (DCNB), Bromophenol blue, Ethylene diamine tetraacetic acid (EDTA), Tris and acetic acid were purchased from Merck (Germany). Titanium isopropoxide ($Ti(i\text{-}Pro)_4$) and guaiacol were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Fast blue RR salt was bought from Fluka (Buchs, Switzerland).

Insects

The *Glyphodes pyloalis* was collected from infested mulberry orchards in the vicinity of Rasht, Iran. Mass rearing of insects was carried out in the laboratory, under controlled conditions with 25 ± 2 °C, 70 ± 10 % RH, and 16:8 L:D. Newly-ecdysed fifth instar larvae of *G. pyloalis* were used for bioassay experiments.

Synthesis of TiO_2-NPs

TiO_2-NPs were prepared according to the method of Trung *et al*. (2003) by hydrolyzing titanium isopropoxide which was added drop by drop into stock solution (i.e. ethanol and acetic acid in a ratio of 8:3 v/v with glycerol) at 10 °C, followed by rigorous stirring under an argon atmosphere for 3 h. Then, the solutions were heated at 60 °C for 5 h or until the gelling reaction was completed. The dried precipitates were heated at 400 °C for 10 h, at the heating rate of 1 °C/min.

Bioassay with TiO_2-NPs suspension

The toxicity of TiO_2-NPs suspension in water was assayed to newly-ecdysed fifth instar larvae of *G. pyloalis* using the leaf dip bioassay (Memarizadeh *et al*., 2011). Five serial dilutions of TiO_2-NPs suspensions (250, 500, 800, 1000 and 1200 mg/L) were prepared and then sonicated for 30 min in a bath type sonicator (100 W, 40 kHz) to disperse the nanoparticles. Mulberry leaf discs (diameter 3.5 cm) were immersed in the dilutions for 45s. After drying of leaf discs, petioles of them were placed in a vial containing water to provide moisture. Up to 5 synchronized fifth instar larvae of *G. pyloalis* were placed on each treated leaf disk. Mortality was assessed after the treated larvae were maintained at 25 ± 2 °C, 70 ± 10 R.H. for 48 h. Each experiment was replicated ten times. The criterion for death was that a larva did not move when prodded with a camel's hair brush.

Treatment

The newly-ecdysed fifth instar larvae of *G. pyloalis* were treated by different concentrations of TiO_2-NPs suspensions (0, 290, 380, 475, 565 and 665 mg/L, equivalent to control, LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50}, respectively). Treatments were conducted according to the bioassay method. Over 3 days after treatments, representative samples were taken from survived larvae. The collected samples were placed in a deep freezer at -20 °C until biochemical assays were performed.

Preparation of samples

For determining EST activity, one larva was homogenized in 150 µl of 0.1 M phosphate buffer, pH 7.0 containing 0.05 % (v/v) Triton X-100 using a glass hand-held homogenizer on ice. After homogenization, they were centrifuged at 12,000×*g* for 15 min at 4 °C and resulted supernatant was used in the assay. For GST assay, enzyme preparation was similar to that previously mentioned for EST; however without Triton X-100. For POD assay, each larva was homogenized in 150 µl of 50 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA. Once homogenized, they were centrifuged at 12,000×*g* for 15 min at 4 °C and resulted supernatant was used in POD assay.

Determining POD activity

The 1 mL reaction mixture was consisted of 450 µl of 50 mM phosphate buffer (pH 7.4) containing 45 mM guaiacol, and 100 µl of prepared supernatant. Adding 450 µl of 50 mM phosphate buffer (pH 7.4)

Table 1 Log dose probit-mortality data for TiO_2-NPs against *G. pyloalis* after 48 h

	n	LC_{10} (95% CI)[a]	LC_{20} (95% CI)[a]	LC_{30} (95% CI)[a]	LC_{40} (95% CI)[a]	LC_{50} (95% CI)[a]	Slope ±SE	χ^2 (df)[b]
G. pyloalis	250	290.86 (151.02- 394.95)	385.51 (237.99- 492.06)	472.34 (326.8- 582.86)	561.88 (422.32- 683.51)	660.85 (525.57- 810.02)	3.59± 0.42	6.25 (3)

[a]The LC values are expressed as part per million (ppm) and their 95 % confidence intervals (95 % CI)
[b]The value of $p > \chi^2$ larger than or equal to 0.05 indicates a significant fit between the observed and expected regression lines

containing 225 mM H_2O_2, the reaction was initiated. Then, the oxidation of guaiacol was followed at 470 nm with a spectrophotometer (Cary 3) using an extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ (Bergmeyer, 1974). The POD activity was expressed as $\mu mol.min^{-1}mg\ protein^{-1}$.

Determining EST activity

Esterase activity was determined based on the van Asperen (1962) method. α-NA and β-NA were used as substrates. 12 µl of supernatant were added to per well of a microplate which containing 113 µl phosphate buffer (pH 7.0). After 3 min, adding 50 µl of 1.8 mM substrate solution, the reaction was initiated. Following the addition of 50 µl of the fast blue RR salt, absorbance at 450 and 540 nm were measured in a microplate reader (Awareness Technology Inc., Florida, USA) for α-NA and β-NA, respectively. The formation of the α-naphthol- and β-naphthol-fast blue RR dye complex was converted to a specific activity using standard curves, which were obtained from different concentrations of α-naphthol and β-naphthol mixed with fast blue RR salt (0.075 %), respectively (Miller and Karn, 1980). The EST activity was expressed as $nmol.min^{-1}mg\ protein^{-1}$.

Determining GST activity

GST assays were conducted according to the method of Habig *et al*. (1974), using CDNB and DCNB as substrates. 15 µl supernatant, 100 µl of 1.2 mM substrate solution and 100 µl of 10 mM GSH were added to per well of a microplate. Enzyme activity was determined by continuously monitoring the change in absorbance at 340 nm for 5 min at 25 °C with a microplate reader (Awareness Technology Inc.). The GST activity was expressed as $\mu mol.min^{-1}mg\ protein^{-1}$.

Determining protein concentration

Protein concentrations were estimated by the Bradford (1976) method, using bovine serum albumin as standard.

Statistical analysis

Three replicates were conducted for all the biochemical assays and data were subjected to analysis of variance (ANOVA). Statistical analyses were performed at $p = 0.05$ by Tukey's test using the SAS software.

Results

Bioassay results

Table 1 shows the median lethal concentration (LC_{50}), sub-lethal endpoints and the 95 % confidence limits which calculated from probit regression using the POLO-PC computer program (LeOra, 1987) and based on Finney (1971).

POD activity

The level of POD differed significantly among treatments (Fig. 1). 24 h after *G. pyloalis* larvae treatment with LC_{20} and LC_{30} concentrations of TiO_2-NPs, the highest POD levels were observed. Results showed that by increasing the sub-lethal concentrations from LC_{10} to LC_{30} and by increasing exposure time, POD activity was significantly increased compared to the control. LC_{50} concentration of TiO_2-NPs significantly decreased POD activities. However, they were not significantly changed at LC_{40} concentration in comparison to the control group (Fig. 1).

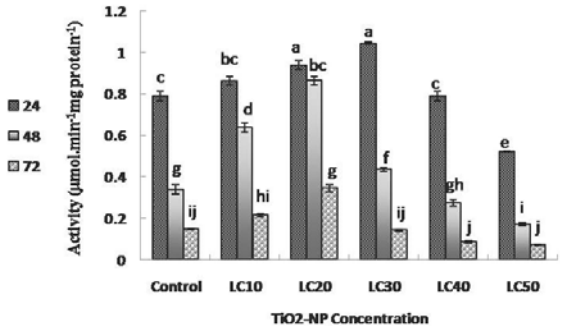

Fig. 1 Peroxidase (POD) activity in *G. pyloalis* exposed to sub-lethal concentrations of TiO_2-NPs at different time intervals. Different letters indicate that the specific activity of enzymes is significantly different from each other by Tukey's test ($p < 0.05$).

EST activity

EST activities of *G. pyloalis* treated with sub-lethal concentrations of TiO_2-NPs are presented in

Figure 2. Analysis of variance showed that esterase activity, using both α-NA and β-NA, significantly affected by: 1) the TiO_2-NPs concentrations, 2) time of exposure to TiO_2-NPs and 3) interplay effect of concentration and exposure time. This means that the effect of TiO_2-NPs concentrations on the esterase activity is correlated to the length of exposure time. Assessment of EST activity in treatments after 24 h showed that TiO_2-NPs led to inhibition of these enzymes and thus decreased their activities. However, when α-NA was used as substrate, by increasing the time of exposure to LC_{10}, LC_{20} and LC_{30} concentrations, the EST activities significantly increased (Fig. 2).

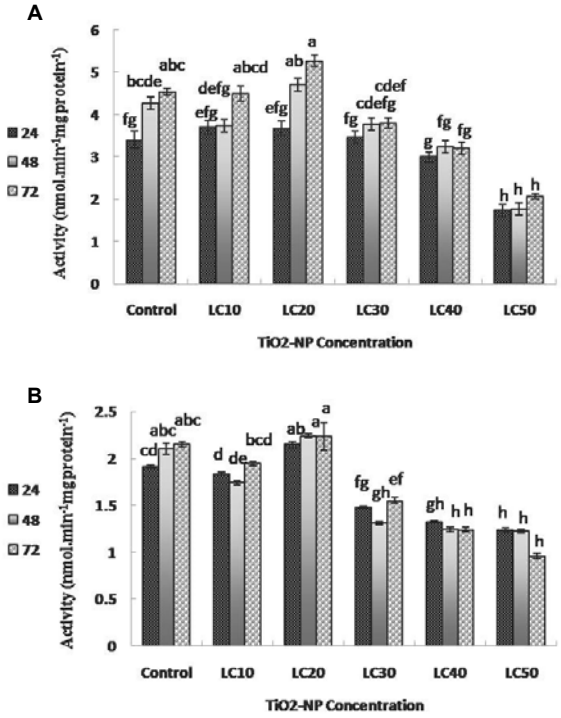

Fig. 2 Comparison of esterase activity in *G. pyloalis* exposure to different concentrations of TiO_2-NPs over 3 days, using α-NA (A) and β-NA (B) as substrates. Means followed by similar letters showed no significantly difference from each other by Tukey's test ($p < 0.05$).

GST activity

GST activities of *G. pyloalis* treated by sub-lethal concentrations of TiO_2-NPs are presented in Figure 3. When CDNB was used as substrate, increasing TiO_2-NPs concentrations led to significant reduction in GST activities after 24 h; however, this reduction was steeper than when DCNB was used in the assay. Furthermore, as depicted in Figure 3, by increasing the exposure time of *G. pyloalis* to LC_{20}, LC_{30} and LC_{40} of TiO_2-NPs, GST activity significantly increased. Results of analysis of variance showed that using both CDNB and DCNB, GST activity significantly affected by: 1) the TiO_2-NPs concentrations, 2) exposure time of larvae to TiO_2-NPs and 3) interplay effect of concentrations and exposure time. This means that the increasing TiO_2-NPs concentrations over exposure time could be enhance the conjugation of GSH to these NPs and thus the enzyme activity was affected.

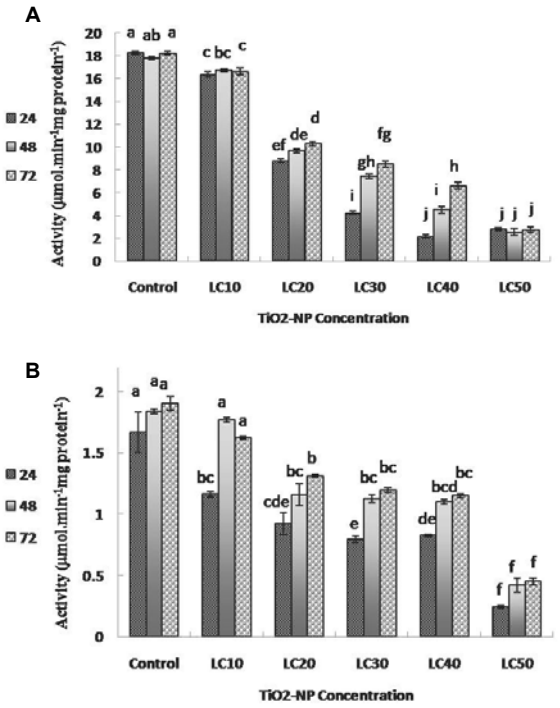

Fig. 3 Comparison of GST activity in *G. pyloalis* exposure to different concentrations of TiO_2-NPs over 3 days, using CDNB (A) and DCNB (B) as substrates. Means followed by similar letters showed no significantly difference from each other by Tukey's test ($p < 0.05$).

Discussion

Since the growth in use of NPs and nanotechnology in various fields mainly in agriculture is inevitable, ongoing study of nanotoxicity will be necessary in order to avoid their unpredictable complications (Clemente *et al*., 2012). To minimize the adverse environmental effects of manufactured and engineered nanomaterials, it is critical to have a good understanding of their toxic potential (Handy *et al*., 2008).

The dose-mortality data for TiO_2-NPs generated in the present study showed a LC_{50} value of 660.85 mg TiO_2-NPs suspension $liter^{-1}$ in *G. pyloalis* larvae. Following the bioassay results, the *G. pyloalis* larvae were exposed to 290, 380, 475, 565 and 665 mg/L TiO_2-NPs as LC_{10}, LC_{20}, LC_{30}, LC_{40} and LC_{50}, respectively. The experiments were designed to allow sub-lethal physiological effects of TiO_2-NPs. The exposure time of 3 days was chosen to enable some biochemical responses of treated insects.

Lovern and Kapler (2006) reported an LC_{50} of 5.5 ppm in *D. magna* which exposed to filtered TiO_2-NPs for 48 h. Kim *et al*. (2010) reported a 70 % mortality rate in *D. magna* exposed to 5 mg/L of Sigma Aldrich TiO_2-NPs for 21 days. Wang *et al*. (2009) were estimated 80 mg/L of TiO_2-NPs as LC_{50}

and showed that TiO_2-NPs are more toxic than their bulk counterparts to the *Caenorhabditis elegans*.

Acute toxicity assays will not be able to provide sufficient information on the interaction of nanomaterials and organisms. Whereas, sub-lethal effects can be indicated the impacts of NPs on the physiology and survival of organisms. Biochemical biomarkers as indicators of sub-lethal effects of a stressor can be used to early warning of population level impacts (De Coen and Janssen, 1997; klaper *et al*., 2009). Biomarkers indicative of neurotoxicity (EST), oxidative stress (POD) and phase II biotransformation of xenobiotics (GST), as well as general mortality have been linked to population-level endpoints (Jemec *et al*., 2007; Paskerova *et al*., 2012). Usage of these biomarkers in risk assessments is suitable diagnostic tool for the detection of specific contaminants well before real adverse effects can occur (Nascimento *et al.,* 2008).

Pollutants may increase the intracellular formation of reactive oxygen species (ROS) which have been reported to affect the physiology, growth, and survival of organisms (Filho, 1996; Pandey *et al*., 2003). POD is the key enzyme in antioxidant defense systems to convert the resulting free radicals H_2O_2 to water and oxygen (Linhua *et al*., 2009). Over 3 time points of treatments, by increasing the concentration of TiO_2-NPs from LC_{10} to LC_{30}, the POD activities were increased and then at LC_{40} and LC_{50} concentrations were decreased. Therefore, results of the present study demonstrate that enhanced activities of POD at low concentrations could lead to the elimination of ROS. This also could be an indication that exposure to low concentrations would yield less mortality in these treatments.

Because of the importance of general ESTs in the insect physiology, metabolism and detoxification of xenobiotics, in this study the possible role of these enzymes as a biomarker to determination of NPs toxicity was investigated (Ishaaya, 1993; Memarizadeh *et al*., 2013). Results of the present work showed that by increasing TiO_2-NPs concentration ESTs activities were decreased due to their inhibition. Also the LC_{50} concentration of TiO_2-NPs had the highest inhibition on the EST activity, when α-NA was used as substrate. Since, an effective inhibition of α-esterases occurs at LC_{50} concentration; these enzymes may be as a good indicator of the TiO_2-NPs toxicity.

GSTs are multifunctional enzymes of the phase II biotransformation system which play a key role in metabolism of a broad variety of xenobiotics and endogenous compounds (Ezemonye and Tongo, 2010; Ezeji *et al*., 2012; Memarizadeh *et al*., 2013; Zamani *et al*., 2014). GSTs are able to conjugate the xenobiotics and their metabolites to the tripeptide glutathione (GSH) and making them soluble for easy excretion (Oakley, 2011). In addition to detoxification role of these enzymes, GSH also has antioxidant properties. Thus, GSH as a general stress indicator is a more useful diagnostic tool. Our study revealed that the GST activity significantly affected by exposure to TiO_2-NPs. So, increased TiO_2-NPs concentrations led to significant reduction in GST activities. Results also showed that the LC_{50} concentration of TiO_2-NPs had the highest impact on the GST activity.

Data demonstrated that activities of detoxification and antioxidant enzymes altered by exposure *G. pyloalis* to TiO_2-NPs and this can be lead to the mortality or sub-lethal impacts. Furthermore, GST, EST and POD activities mainly affected by LC_{50} concentration and thus, this concentration is a good early indicator of TiO_2-NPs toxicity.

The sub-acute toxicity of TiO_2-NPs to carp (*Cyprinus carpio*) was assessed by Linhua *et al*. (2009). They showed that 100 and 200 mg/L TiO_2-NPs caused the significant decrease in SOD, CAT and POD activities suggesting that the fish exposed to TiO_2-NPs suffered from the oxidative stress.

As each biomarker may have different sensitivities depending on the mode of action of the chemical (Jemec *et al*., 2007); the use of multiple biomarkers to evaluate toxicity of NPs for insects will provide the most suitable tool to indicate the potential impacts of them.

Conclusion

The results of this study suggest that sub-lethal effects of TiO_2-NPs to *G. pyloalis* are related to concentration and length of exposure time. EST, GST and POD as biochemical biomarkers are useful diagnostic tools to determination of NPs toxicity. POD activity in different treatments indicates that these particles are causing oxidative stress; especially at a lower concentration than the LC_{50} concentration. Therefore, the expression of POD in low sub-lethal concentrations of TiO_2-NPs in addition to the inhibition of EST and GST at high concentrations are appropriate early biomarkers for impacts of TiO_2-NPs in insects. Eventually, application of biochemical biomarkers in conjunction with acute toxicity studies of NPs will be more informative in predicting physiological impacts and future toxicity.

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