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Research Article

Partial biochemical risk assessment of carbon nanotubes and carbon nanotubes/titanium dioxide nanoparticles on *Glyphodes pyloalis* (Lepidoptera: Pyralidae)

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Abstract: Cellular energy allocation (CEA) test was performed in order to investigate the effects and costs of bare carbon nanotubes (CNTs) and CNTs in combination with titanium dioxide nanoparticles (CNTs/TiO_2-NPs) on *Glyphodes pyloalis* Walker after 24, 48 and 72 hours of exposure to 100, 200, 300, 400 and 500 ppm of the treatments. Results showed the negative correlation between total lipid amounts and concentrations of treatments (*i.e*. CNTs and CNTs/TiO_2-NPs) as well as exposure time. Contrary to CNTs treatments, carbohydrate contents were affected by both of CNTs/TiO_2-NPs concentration and time of exposure. Results showed that the effect of bare CNTs in the enhancement of glycogen content appeared significantly faster than that of CNTs/TiO_2-NPs. Increasing time of exposure to all concentrations of CNTs, except for 100 ppm, prevented enhancement of protein content. The effect of bare CNTs on the reduction of protein contents was faster and greater than that of CNTs/TiO_2-NPs. The results indicated that *G. pyloalis* cannot regulate internal CNTs and CNTs/TiO_2-NPs concentrations efficiently without considerable impact on the energy reserves (Ea). The comparison of energy consumed (Ec) in treated larvae showed that CNTs/TiO_2-NPs reflected the higher energy demand of the stress response than CNTs. Generally, CEA was significantly decreased as the concentration of CNTs treatments increased. More reduction in CEA amount of all treatments by CNTs/TiO_2-NPs than that of the control is also probably considered as a cost to deal with detoxification when the concentration increased and at all the tested time points. Therefore, CEA test might be considered as an early biochemical biomarker for assessing immediate response of organisms after acute exposure to stressors and thus could be applied to risk assessment of nanomaterials.

Keywords: CEA test, CNTs/TiO_2-NPs, biochemical biomarker; risk assessment, lesser mulberry pyralid

Introduction

Some of the human past bitter experiences in the application of new sciences and technologies without regard to their environmental impacts have led to paying attention to the fate of nanomaterials in nature. Attention to the production and application of synthetic nanomaterials in the various fields such as agriculture and food has increased in recent years (Adana, Fen, Dergisi, and Tunçsoy, 2018; Gottschalk and Nowack, 2011; Kah *et al.,* 2016). Different properties and effects of synthetic

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nanomaterials compared to the same materials in bulk, can pose new health risks to humans and environment. It needs to be examined how defense mechanisms of organisms would be able to respond adequately to these engineered particles which may have characteristics never encountered before (Elsaesser and Howard, 2012; Schwirn and Völker, 2016). Following the publication of last virgin of guidance on the risk assessment of the application of nanosciences and nanotechnologies in the food and feed chain for human and animal health by European Food Safety Authority (EFSA)'s Scientific Committee; the hope is that comprehensive knowledge will be available on environmental effects of nanoparticles (Hardy *et al.,* 2011; Hardy *et al.,* 2018).

CNTs are tubes made of carbon with diameters ranging from below 1 nm to 10 s of nm. Certain properties of these nanoparticles, such as cost-effective, light-weight and high surface area-make them particularly suitable for a wide range of industrial applications (Venkataraman *et al.,* 2019). Generally, CNTs surface modifications which is caused by oxidation or treatment with surfactants to facilitate their aqueous solution could increase possible environmental exposures to these nanoparticles (Venkataraman *et al.,* 2019). In a similar way in the environment, some natural coatings could increase the dispersability of CNTs in aquatic solutions by covering the hydrophobic surface (Jackson *et al.,* 2013). Different ecotoxicological studies showed that exposure to CNTs led to oxidative stress, induced reactive oxygen species (ROS) production and also reduction in levels of intracellular ATP.

Titanium dioxide (TiO_2) as one of the most important transition metal oxides has potential application for environmental photocatalysis purifications. (Rodríguez *et al.,* 2017). Our previous studies showed that TiO_2-NPs could have lethal and sub lethal effects on *G. pyloalis* which are related to the treatment concentration and length of exposure time to each concentration (Memarizadeh *et al.,* 2014a and 2014b). Combination of TiO_2-NPs with CNTs could form hybrid structure with enhanced photocatalytic activities compared with bare TiO_2-NPs (Nguyen *et al.,* 2014). The role of CNTs in mentioned combination is providing a large surface area support and also outstanding charge transfer abilities for TiO_2-NPs as a photocatalytic catalyst (Nguyen *et al.,* 2014).

The vast majority of the nano-ecotoxicological studies have focused on the toxicity of the bare form of CNTs in aqueous or algal ecosystems. However, in nature, CNTs due to very strong adsorption affinity, most commonly are found in the conjunction with various contaminants (Schwirn and Völker, 2016; Jang and Hwang, 2018). Therefore, the interactions of CNTs with other hazardous compounds (such as toxic metals) can influence the behavior of toxic materials in the environment. Although the mechanisms of this issue has not been fully elucidated but the effects of interaction of CNTs with other pollutants on the behavior and toxicity of both CNTs and the other pollutants should be studied (Abega *et al.,* 2019).

Biochemical biomarkers were utilized to determine the possible risks for human and ecological exposures to the nanoparticles. It is an important and challenging issue to know and determine the behavior of biochemical biomarkers in the exposure to combination of CNTs and TiO_2-NPs. Furthermore, it is essential to pay more attention to adverse effects of nanoparticles on insect species. The mechanisms by which organisms control the possibly extrinsic stressors can be studied from different aspects. For instance, the energy reserves available for metabolism (Ea) which is the total amount of energy acquired from available total lipid, total protein, glucose and glycogen content in addition to the energy consumption (Ec) which is the activity of electron transport system which can be measured by altering enzyme production in an organism (De Coen and Janssen, 1997) can be used as early indicators of the metabolic conditions (Rueda-Jasso *et al.,* 2004). Cellular energy allocation (CEA) that is calculated by dividing Ea by Ec, is a fast test for the energy budget measurement of organisms

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(Widdows and Donkin, 1992; De Coen and Janssen, 1997; Verslycke *et al.,* 2004; Bagheri *et al.,* 2010). Memarizadeh *et al.* (2014a) using CEA test surveyd the pollution potential of TiO_2-NPs on ecological health of *G. pyloalis*. Changes in the energy budget indicated the TiO_2-NPs as a toxic stress agent on the mentioned insect (Memarizadeh *et al.,* 2014). Świątek and Bednarska (2019) used CEA as an early biomarker to survey the effects and costs of different forms and concentrations of Zn on the earthworm *Eisenia andrei* Bouché (Świątek and Bednarska, 2019).

The aim of this study is to present an early biochemical indicator using CEA test in order to make a risk assessment of bare CNTs and also CNTs in combination with TiO_2-NPs on the newly-ecdysed fifth instar larvae of *G. pyloalis*.

Materials and Methods

Insects

As mentioned in our previous works (Memarizadeh *et al*., 2014a, b), mass rearing of *G. pyloalis* in the vicinity of Rasht, Iran was carried out in the laboratory under controlled conditions. Newly-ecdysed fifth instar larvae of *G. pyloalis* were used for sublethal experiments afterrearing at least two generations of *G. pyloalis* under laboratory conditions.

Synthesis of CNTs/TiO_2-NPs

According to previous studies (Memarizadeh *et al*., 2014a, b), TiO_2 nanoparticles were prepared by hydrolyzing titanium isopropoxide (Trung *et al.,* 2003). CNTs/TiO_2 nanocomposites were produced according to method of Li *et al.* (2011) utilizing sodium dodecylbenzesulfonate (NaDDBS) as the CNTs surface functionalizing agent.

Treatments

The newly-ecdysed fifth instar larvae of *G. pyloalis* were treated with different concentrations of CNTs and CNTs/TiO_2-NPs suspensions (0, 100, 200, 300, 400 and 500 mg/L). Over 72 h after treatments, representative samples were taken from surviving larvae. The collected samples were placed in a freezer at -20 °C until biochemical assays were performed.

Preparation of samples

Preparation of treated larvae, in order to assess the amount of energy reserves (i.e. total lipid and glycogen), was performed according to Yuval *et al.* (1998) method. Homogenization and preparation of samples for electron transport system (ETS) assay was performed through method of Verslycke *et al.* (2004).

As mentioned in our previous work (Memarizadeh *et al*., 2014a), measurement of the energy budget, cellular energy allocation (CEA), was assayed using the method described by Van Handel and Day (1988) with minor modifications.

CEA was calculated by determination of total energy reserves as energy available (Ea) in an insect body and the activity of electron transport system (ETS) as energy consumption (Ec) according to the following formula:

Ea = Σ (total lipid, carbohydrate, glycogen and total protein) (joule/insect);

Ec = ETS activity (joule/insect);

CEA = Ea/Ec.

Determination of the amount of total lipid

Measurement of total lipid of treated larvae compared to the control using Vanillin reagent was carried out as described in our previous work (Memarizadeh *et al*., 2014a). Standard curve for lipid assay was plotted using cholesterol as the standard (Yuval *et al.,* 1998).

Determination of the amount of total carbohydrate

Total carbohydrate in each individual larvae, using anthrone reagent as explained in detail in the previous work (Memarizadeh *et al*., 2014a), was calculated from standard curve using maltose as standard (Yuval *et al.,* 1998).

Determination of the protein content

Protein concentration was estimated according to the Bradford (1976) method, using bovine serum albumin as the standard (Bradford, 1976).

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Calculation of energy reserves

To calculate energy reserves, different components of energy reserves should be transformed to energetic equivalents which consisted of 17.5 j mg^{-1} glycogen and carbohydrate, 24 j mg^{-1}protein and 39.5 j mg^{-1} lipid (Gnaiger, 1983).

Determination and calculation of energy consumed

Energy consumed (Ec) of samples was calculated by amount of formazan formed based on an extinction coefficient of 15,900$M^{-1}cm^{-1}$. From a theoretical point of view, formation of 2 µl formazan will use 1 µmol of O_2 and the quantity of consumed oxygen was transformed into energetic equivalents (484 kJ/mol O_2) (Gnaiger, 1983).

Statistical analyses

Three replicates were conducted for all the biochemical assays and data were subjected to analysis of variance (ANOVA) and mean comparison performed in a split-plot in time design (combined analysis). Statistical analyses were performed at P = 0.05 by Tukey's test using the SAS software (SAS Institute, 2011).

Results

In this study effect of bare CNTs in addition to synthesized CNTs/TiO_2-NPs was surveyed on the *G. pyloalis*'s energy budget. Transmission electron microscope (TEM) image of CNTs/TiO_2-NPs is shown in the Fig. 1.

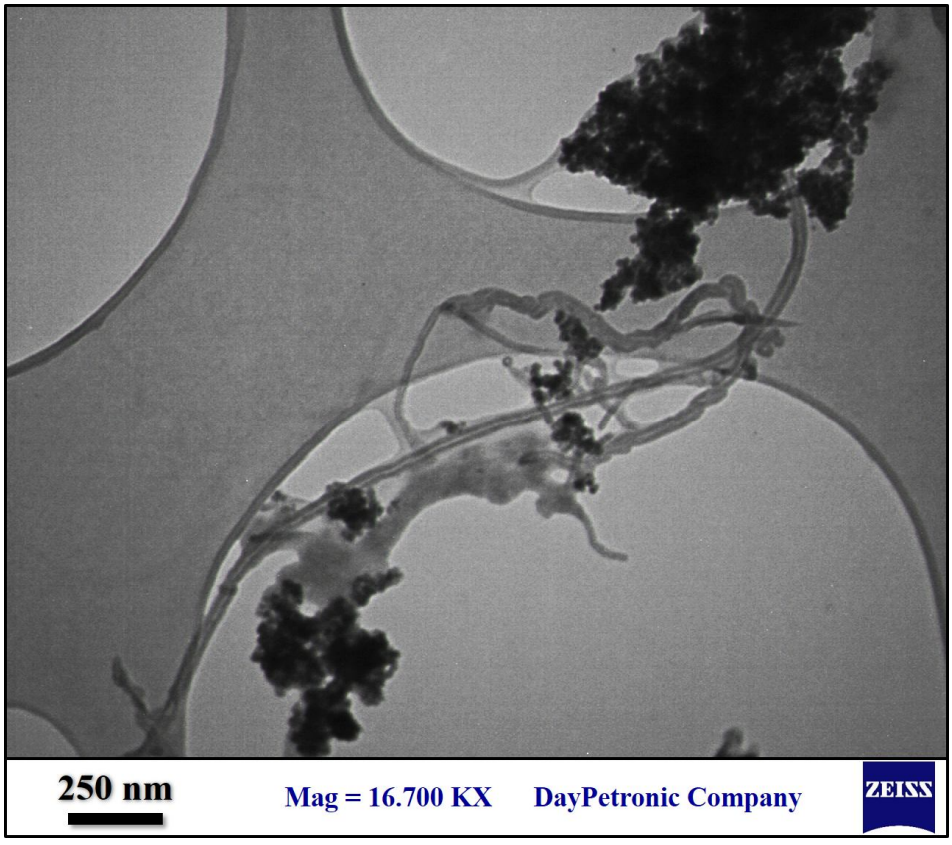

Figure 1 TEM image of synthesized CNTs/TiO_2-NPs. The structure of synthetic nanoparticles is resulting the bond of carbon nanotubes (CNTs) to TiO_2 nanoparticles (TiO_2-NPs). The hybrid structure, CNTs provide the essential surface needed to bind TiO_2-NPs in a specific way that can be seen in the TEM image. This bond enhances the special properties of TiO_2 nanoparticles such as photocatalytic activities. The structure of bare CNTs can be seen in the TEM image.

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Comparison of the amount of lipid in treated larvae by specified value of CNTs showed that unlike the control, the amount of lipid significantly decreased in the case of all concentrations of CNTs by increasing treatment time (Fig. 2). Although the highest amount of lipid was observed for control (36 joule/larvae) after 72 h, the lowest amount (4 joule/larvae) was calculated in the larvae treated with 500 ppm of CNTs for the same period of time (Fig. 2). As shown in the Fig. 2 lipid levels of treated larvae by specified value of CNTs/TiO_2-NPs had a similar trend to CNTs. This means that reduction of lipid in treated larvae occurs by increasing concentration level and treatment time. The only difference that can be noted is a greater reduction of lipid in the larvae treated with CNTs/TiO_2-NPs than in those treated with CNTs (Fig. 2).

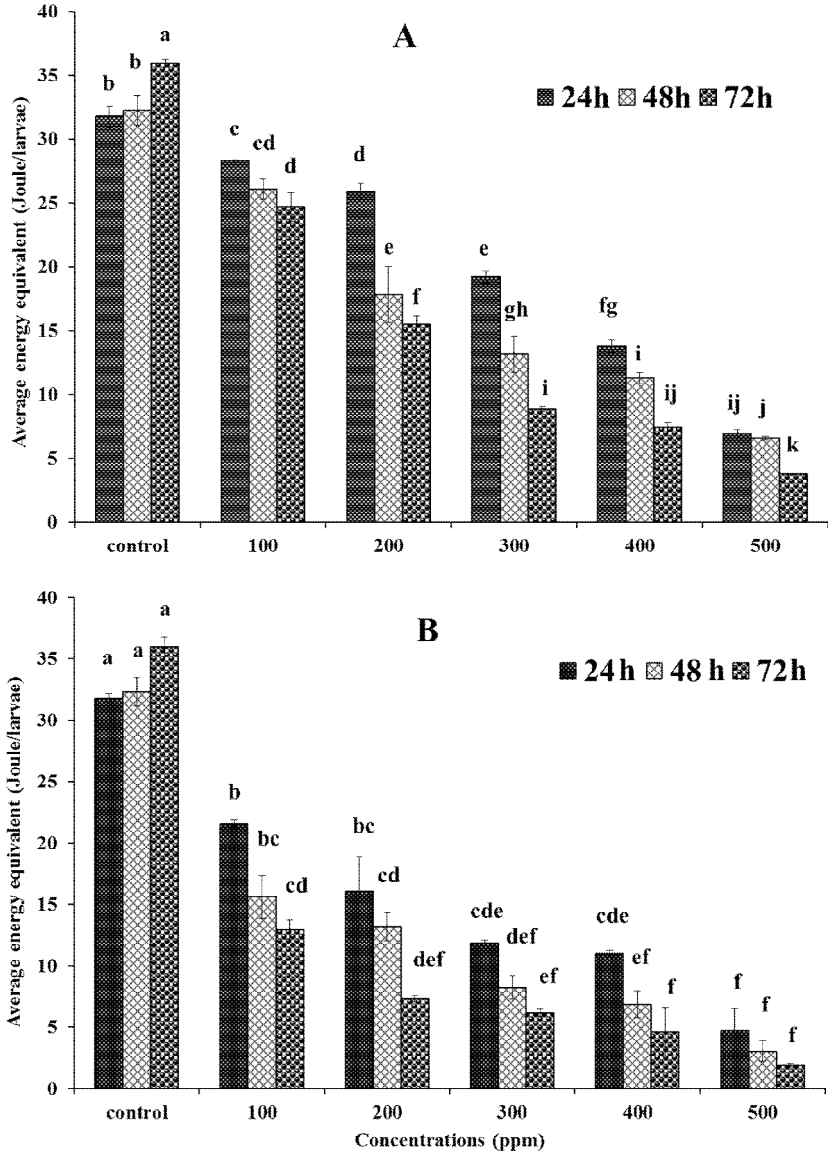

Figure 2 Average energy equivalents of lipids in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of (A) CNTs, and (B) CNTs/TiO_2-NPs.
Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

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Amount of carbohydrate content in the treated larvae by each tested concentrations of CNTs, like the control, was not affected by increasing time of exposure (Fig. 3A). Results showed that amount of carbohydrate contents of larvae treated with all concentrations of CNTs significantly decreased compared to the control. However, the effect of different treatments of CNTs (i.e. 100-500 ppm) was similar in the reduction of carbohydrate contents (Fig. 3A). Results also showed that in the case of CNTs/TiO_2-NPs treatments, carbohydrate contents were affected by both concentration and time of exposure to treatments, except for 500 ppm (Fig. 3 B). Unlike CNTs, carbohydrate content significantly increased with increase in time of exposure to different concentrations of CNTs/TiO_2-NPs, except for 500 ppm (Fig. 3 B).

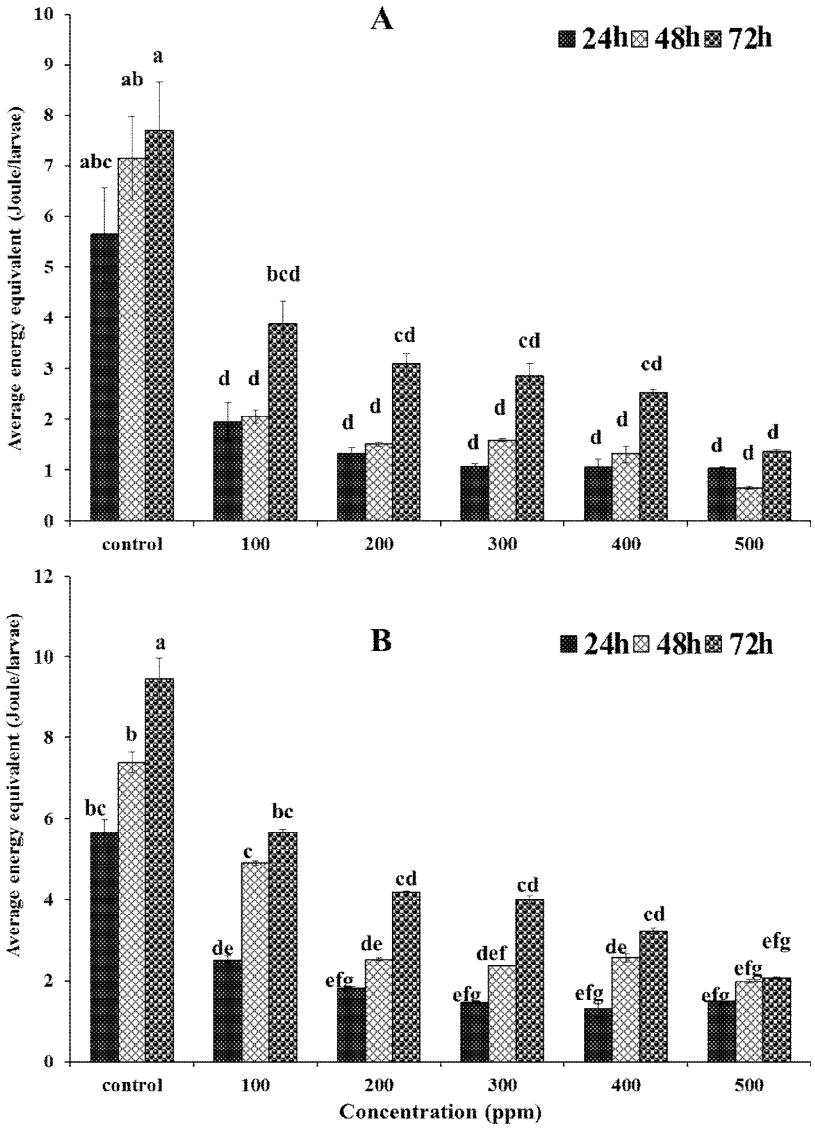

Figure 3 Average energy equivalents of carbohydrate in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of (A) CNTs, and (B) CNTs/TiO_2-NPs.
Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

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Glycogen content of the treated larvae with 100, 200 and 500 ppm of CNTs, like the control, showed significant increase after 48 h. However, in the case of 300 and 400 ppm concentrations, this significant increase was shown after 72 h (Fig. 4 A). It should be noted that glycogen content after 24 h treatment with 500 ppm of CNTs was significantly lower than those of 100, 200 and 300 ppm (Fig. 4A). Results of treatments by CNTs/TiO_2-NPs showed that glycogen content significantly increased after 72 h except for 300 ppm. Generally, results showed that effect of CNTs which was coupled to TiO_2-NPs appeared later than that of bare CNTs (Fig. 4). This result may indicate a faster effect of bare CNTs on the glycogen content of treated larvae than that of CNTs/TiO_2-NPs.

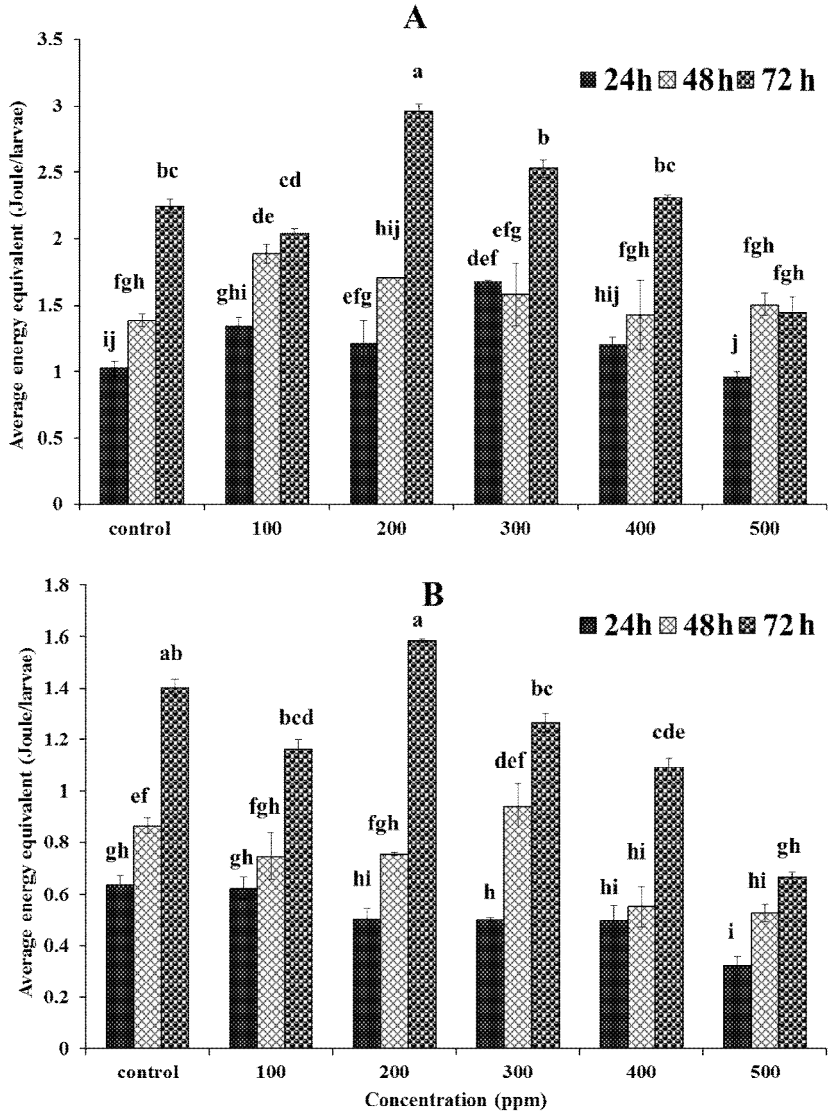

Figure 4 Average energy equivalent of Glycogen in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of (A) CNTs, and (B) CNTs/TiO_2-NPs.
Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

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Effect of different concentrations of CNTs appears in the form of a decrease in the amount of protein content of fifth instar larvae of *G. pyloalis*. So, after 24 h of exposure to each concentration, protein content significantly decreased (Fig. 5). Furthermore, all tested concentrations of CNTs, except for 100 ppm, prevented normal increase in protein content up to 72 h of exposure (Fig. 5). The amount of protein content in the fifth instar larvae of *G. pyloalis* when exposed to CNTs/TiO_2-NPs showed different trend to that of CNTs (Fig. 5). After 72 h of treatment with all concentrations of CNTs/TiO_2-NPs, the protein content significantly decreased. Furthermore, it should be noted that the level of total protein in the exposure to pure CNTs was less than that in the exposure to CNTs in combination with TiO_2-NPs (Fig. 5). This result showed that the effect of bare CNTs on the protein contents is faster and greater than that of CNTs/TiO_2-NPs (Fig. 5).

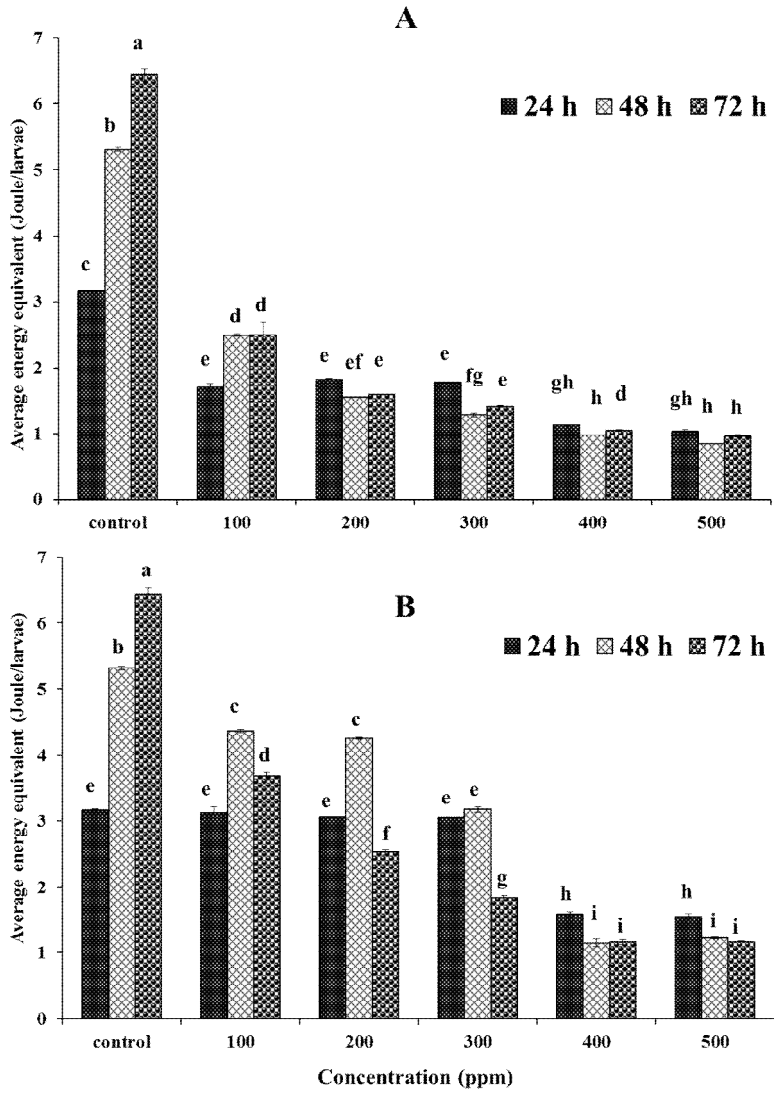

Figure 5 Average energy equivalent of protein in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of (A) CNTs, and (B) CNTs/TiO_2-NPs.
Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

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Reserve energy was calculated by summation of total carbohydrate, glycogen, lipids and protein contents (Fig. 6). Ea for untreated larvae (control) and 100 and 200 ppm concentrations significantly increased as time of exposure increased (Fig. 6). However, using concentrations of 300 and 400ppm of CNTs, Ea significantly decreased after 48 h rather than 24 h and then increased after 72 h of treatment (Fig. 6). Generally, results showed that, except for 500 ppm, time of exposure to other tested concentrations of CNTs could affect the reserve energy of the fifth instar larvae of *G. pyloalis*. The lowest amount of Ea was recorded for larvae treated with 500 ppm of CNTs (Fig. 6). In general, CNTs/TiO_2-NPs treatments significantly decreased Ea compared to the control in all three time points of exposure (Fig. 6). At 100 ppm of CNTs/TiO_2-NPs Ea significantly increased from 24 h to 72 h showing the same trend as the control. On the contrary at 200, 300, 400 ppm of CNTs/TiO_2-NPs, Ea significantly decreased from 24 h to 72 h of treatment (Fig. 6). The lowest amount of Ea was calculated in the exposure to 400 and 500 ppm of CNTs/TiO_2-NPs after 48 h and 24 h, respectively (Fig. 6).

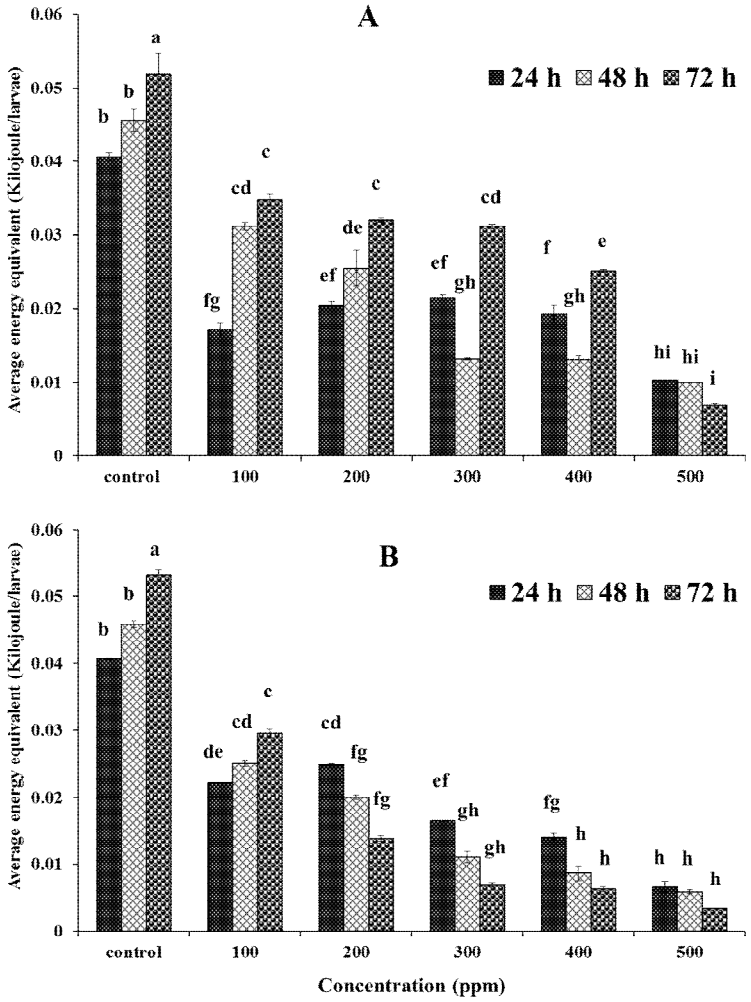

Figure 6 Average energy equivalent of energy reserves (Ea) in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of (A) CNTs, and (B) CNTs/TiO_2-NPs.
Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

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Energy consumption (Ec) during the exposure times was calculated based on the mitochondrial ETS activity and the results pertaining to the CNTs and CNTs/TiO_2-NPs treatments are presented in the Fig. 7. Results showed that by increasing time of exposure to 100 ppm of CNTs, from 24 to 72 h, Ec of the fifth instar larvae of *G. pyloalis* significantly decreased compared to the control (Fig. 7). There was no significant difference between Ec of larvae larvae treated with 200 and 300 ppm of CNTs and Ec of the control in all three time points of treatments. However, using 400 ppm of CNTs, Ec was significantly decreased after 72 h compared to the control. Significant reduction of Ec compared to the control was also recorded after 24, 48 and 72 h of treatment with 500 ppm of CNTs (Fig. 7). Comparison of CNTs/TiO_2-NPs treatments with the control at each time point showed that Ec significantly increased at all three time points of the exposure to the concentrations of 100 and 200 ppm. Furthermore, after 24 h of exposure to 200 and 300 ppm of CNTs/TiO_2-NPs Ec increased more than that of the control. However, 24 h of exposure, to 500 ppm of CNTs/TiO_2-NPs significantly decreased Ec compared to the control (Fig. 7). The highest amount of Ec was recorded after 24 h of treatment with 100 and 200 ppm of CNTs/TiO_2-NPs (Fig. 7).

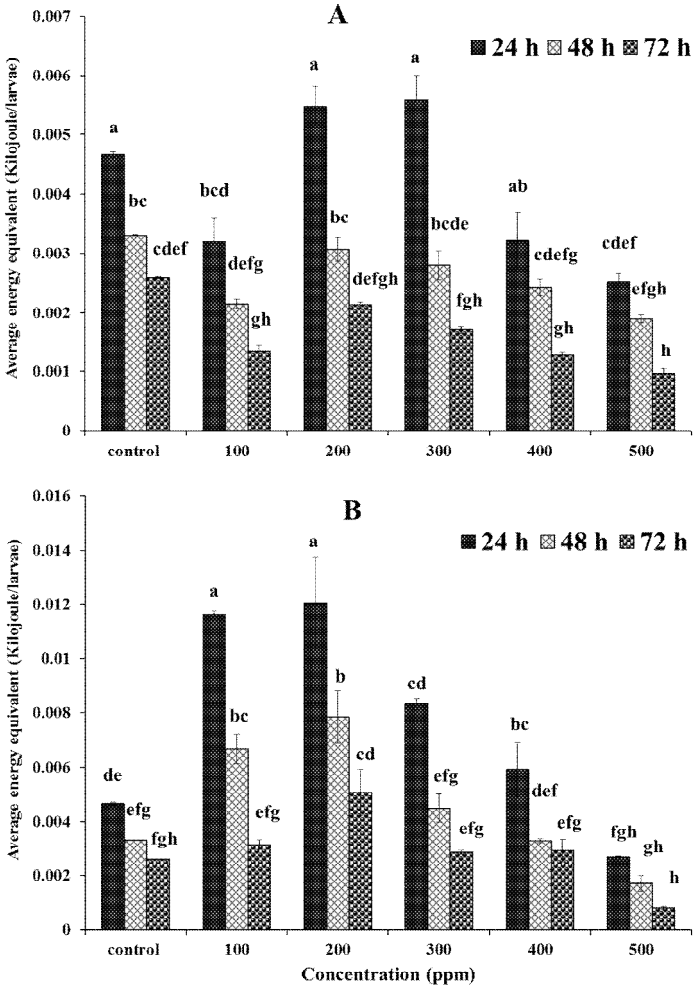

Figure 7 Average energy equivalent of energy consumed (Ec) in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of (A) CNTs, and (B) CNTs/TiO_2-NPs.
Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

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Results also showed that contrary to Ec, generally CEA was significantly decreased as the concentration of CNTs treatments increased (Fig. 8). CEA was not affected by 100 ppm of CNTs after 24 h. However, under treatment with other concentrations of CNTs (i.e. 200, 300, 400 and 500 ppm) CEA significantly decreased compared to the control in all three time points (Fig. 8). Interestingly results showed that the reduction of CEA amounts of treated larvae at each time point of exposure to CNTs is in a dose response related manner (Fig. 8). As shown in Fig. 8, there was very significant reduction in CEA amount of all treatments with CNTs/TiO_2-NPs compared to the control. Among different concentrations there was significant enhancement in CEA amount just after 72 h treatment with 100 and 500 ppm of CNTs/TiO_2-NPs (Fig. 8). Hence, there was no significant difference between CEA amount of treated larvae at concentrations of 200, 300 and 400 ppm of CNTs/TiO_2-NPs in all three time points (Fig. 8).

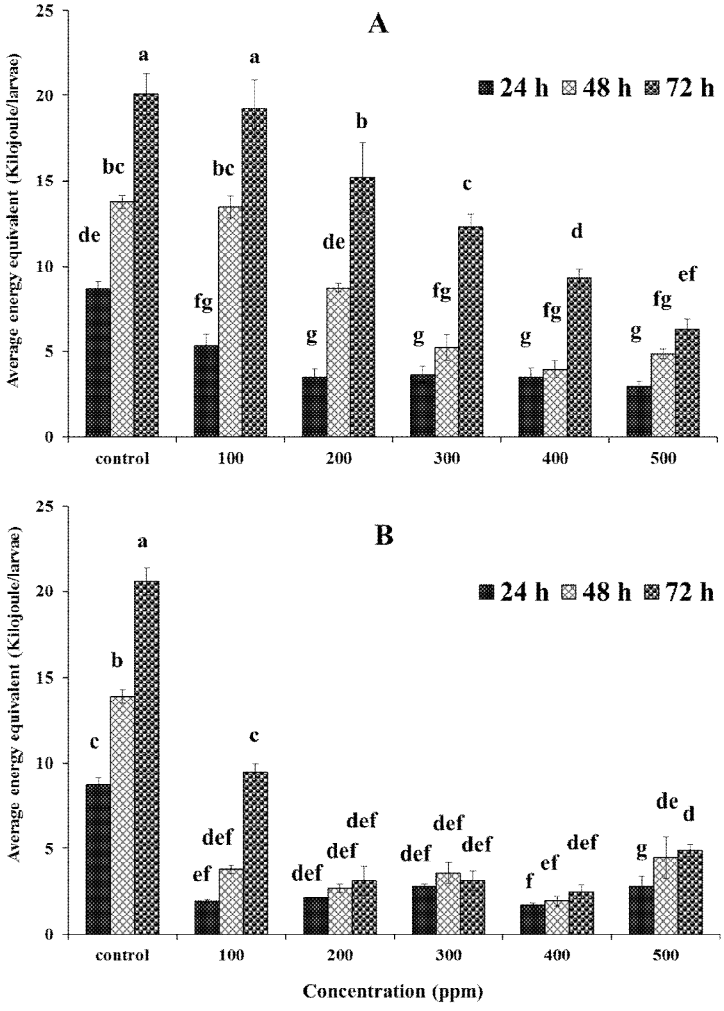

Figure 8 Average energy equivalent of cellular energy allocation (CEA) in *Glyphodes pyloalis*; 24, 48 and 72 hours after treatment with different concentrations of (A) CNTs, and (B) CNTs/TiO_2-NPs.
Means followed by similar letters showed no significant difference from each other by Tukey's test ($P < 0.05$).

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Analysis of variance (split plot in time) showed that generally the amount of protein, lipid, glycogen, Ec, Ea and CEA in treated larvae were significantly affected by: (1) concentrations of treatments, (2) kind of treatments (3) time of exposure to the treatments, (4) interplay effect of concentration and exposure time and (5) interplay effect of concentration, exposure time and kind of treatments. This means that the effect of CNTs concentrations on the amount of lipid, glycogen, carbohydrate, protein, Ec, Ea and CEA is correlated to the length of exposure time. Furthermore, analysis of variance showed that the effect of CNTs/TiO_2-NPs on the amount of lipid, glycogen, carbohydrate, protein, Ec, Ea in treated larvae is similar to those of CNTs treatments and affected by the length of exposure time.

Discussion

Nowadays with growing application of nanotechnology in various fields, mainly food and feed industries, risk assessment of possible side effects of applied nanotechnology is inevitable (Li *et al.,* 2019). Toxicity of CNTs is specified by its composition, geometry and surface functionalization. So, moderate dosages of CNTs without significant functionalization has shown significant toxicity to human or animal cell lines (Venkataraman *et al.,* 2019).

The reduction of total lipid in the exposure to CNTs in a dose response related manner can be due to increase in the levels of lipid peroxidation. This is one of the results of the oxidative stress which arises from generation of reactive oxygen species (ROS) and free radicals in the exposure to CNTs. On the other hand, the reduction of lipid can be justified by consumption of lipids as the first energy source during exposure to the environmental contaminations (De Coen and Janssen, 1997; Novais *et al.,* 2013; Amorim *et al.,* 2012). The same trend of reduction of total lipid in the exposure to CNTs/TiO_2-NPs is justified by the mentioned reasons. It is worth noting that our previous study on the toxicity of bare TiO_2-NPs showed the role of these nanoparticles in the reduction of lipid content (Memarizadeh *et al.,* 2014a). Thus, more inhibitory effect of CNTs/TiO_2-NPs on lipid production can be correlated to the effect of TiO_2 nanoparticles that are added to CNTs.

Once again by reduction of carbohydrate content in a dose response related manner in the exposure to both CNTs and CNTs/TiO_2-NPs; the possibility of consumption of the reserved energy sources proves to be as the cost of detoxification of these nanoparticles by organism.

Świątek and Bednarska (2019) pointed out a decrease in carbohydrate content in the earthworm *E. andrei*, exposed to Zn-NPs (Świątek and Bednarska, 2019). Khalil (2015), reported a decrease in carbohydrate content in the guts of *Pheretima hawayana* Rosa earthworms exposed to TiO_2-NPs (Khalil, 2015).

Lipids in particular along with carbohydrates are highly efficient and preferred storage components rather than protein substrates to be mobilized under toxic stress (Smolders *et al.,* 2003).

There was no specific trend in the case of glycogen content in the larvae exposed to CNTs and also to CNTs/TiO_2-NPs. Hence, increase of tested concentrations of nanoparticles, contrary to lipid and carbohydrate contents, couldn't effect to glycogen content in a dose response related manner. It should be noted that under the influence of CNTs/TiO_2-NPs, impact on the glycogen content occurred more intensely than that of CNTs and in less time of exposure. This result indicated the effect of TiO_2 nanoparticles on the physiology of treated insects when CNTs coupled to TiO_2 nanoparticles were used (Memarizadeh *et al*., 2014a). Holmstrup *et al*. (2011) reported decrease of glycogen content in *Dendrobaena octaedra* Savigny earthworms by increase in the concentrations of Ni, Al, and Zn treatments after specific time of exposure (Holmstrup *et al.,* 2011).

Comparison of protein content of larvae treated with CNTs and CNTs/TiO_2-NPs showed that protein content was affected by bare CNTs more than that by CNTs conjugated to TiO_2

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nanoparticles. Since in the case of treatments by bare CNTs, larvae were treated with higher concentrations of CNTs than that of CNTs/TiO_2-NPs treatments; it could be concluded that CNTs causes further disruption by protein content. Oxidation of proteins can be one of the toxicity mechanisms of nanoparticles. Furthermore, nanoparticles by penetrating through the exoskeleton of insects and entering the intracellular spaces bind to sulfur from proteins which leads to the rapid denaturation of organelles and enzymes (Rai *et al.,* 2014). Similarly, Fouad *et al.* (2018) observed significantly decreased levels of total protein in *Aedes albopictus* Skuse and *Culex pipiens* Pallens when exposed to Ag-NPs. Proteins as fundamental components can participate in the metabolism only during extreme energy deficiency. Therefore, metabolic compensation is performed by elevated protein turnover under stress conditions (Sokolova *et al.,* 2012).

Interruption of energy transduction is considered as one of the toxicity mechanisms of nanoparticles. The reduction of energy reserves could be due to decreased food consumption and/or increased metabolic activity (De Coen and Janssen, 2003; Novais *et al.,* 2013). Significant effects on the components of the energetic budg*et al*so could be caused by influence of nanoparticles (Novais *et al.,* 2013). The increase in the available energy reserves according to increase of exposure time which was shown in treatments with 100 and 200 ppm of CNTs, similar to the control, was mainly due to increased glycogen levels. Such an increase has been also observed for *Enchytraeus albidus* Henle exposed for 8 days to three different pesticides (dimethoate, atrazine, and carbendazim) and also *E. andrei* after exposure to zinc-NPs due to increased lipid and protein levels (Świątek and Bednarska, 2019; Novais *et al.,* 2013). In the present study, similar trend of changes in available energy reserves levels to the control was observed only for 100 and 200 ppm of CNTs treatments. And in the case of other treatments there wasn't specific manner over three time points of treatments. However, In the case of treatments by CNTs coupled with TiO_2 nanoparticles (CNTs/TiO_2-NPs), all concentrations except for 100 ppm showed completely different behavior compared to the control over three time points of treatments. These results once again showed the effect of TiO_2 nanoparticles on the total energy budget of organism (Memarizadeh *et al*., 2014a). Świątek and Bednarska (2019) showed that there were no differences in carbohydrate, protein, and lipid levels and Ea for any of the applied treatments and for any of time points of exposure to ZnO-NP except for 500 ppm, indicating that none of the toxicants had significant effects on the components of the energetic budget (Świątek and Bednarska, 2019).

An increase in Ec that was observed in all concentrations of CNTs/TiO_2-NPs and all three time points of exposure except for higher concentration (i.e. 500 ppm) in comparison to the control, showed that the uptake, distribution and excretion of excess CNTs conjugated to TiO_2 can increase the energetic cost. Comparison between CNTs and CNTs/TiO_2-NPs treatments showed that CNTs alone couldn't increase the energetic cost in the trend which was observed for CNTs/TiO_2-NPs treatments. Our last study on the impacts of TiO_2 nanoparticles on the fifth instar larvae of *G. pyloalis* confirmed the high effect of these nanoparticles on the increase of Ec as an energetic cost (Memarizadeh *et al*., 2014a). Although no differences in Ec were reported in the earthworm *E. andrei* after exposure to zinc in nanoparticle and ionic form in the contamination phase; but a significantly increased Ec just for 500 ppm of $ZnCl_2$, indicated a strong effect of this treatment on earthworm metabolism even after completion of exposure (Świątek and Bednarska, 2019).

Amount of CEA under influence of CNTs was reduced in a dose response related manner compared to the control in all three time points of treatment. Negative correlation between CNTs concentrations and the net energy budget indicated that energy was spent to overcome the toxicity of CNTs and thus there will be less energy available for other physiological

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functions (Novais *et al.,* 2013). However, under influence of CNTs/TiO_2-NPs, despite the sharp decline in CEA values for all concentrations, except for the lower concentration of treatments, there weren't significant reductions in CEA from 48 h to 72 h of treatment. This means that 48 h is a sufficient time for incidence significant effects of CNTs/TiO_2-NPs on the energy budget of organism. Reduced energy budget resulted in the extra energy requirements for detoxification (Amorim *et al.,* 2012).

Results of present study showed that the energy metabolism rate of *G. pyloalis* was affected by CNTs and also in particular by CNTs/TiO_2-NPs. Thus, energy reserves were reduced and mitochondrial electron transport system activity changed due to increased cellular respiration. Consequently, using CEA test as an early indicator and by indirect measurement of possibility of an organism's survival, the toxic effects of CNTs and CNTs when coupled to other nanoparticles such as TiO_2 could be foreseen could be detected.

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ارزیابی بیوشیمیایی جزئی ریسک نانولوله‌های کربنی و نانولوله‌های کربنی/نانوذرات دی‌اکسید تیتانیوم روی پروانه برگ‌خوار توت *Glyphodes pyloalis* (Lepidoptera: Pyralidae)

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چکیده: آزمون تخصیص انرژی سلولی (CEA) در راستای بررسی اثرات و هزینه‌های نانولوله‌های کربنی خالص (CNTs) و نانولوله‌های کربنی ترکیب با نانو ذرات دی‌اکسید تیتانیوم ($CNTs/TiO_2$-NPs) بر پروانه برگ‌خوار توت Walker *Glyphodes pyloalis*، در مورد افراد مواجه شده با تیمارهای ۱۰۰، ۲۰۰، ۳۰۰، ۴۰۰ و ۵۰۰ پی‌پی‌ام، ۲۴، ۴۸ و ۷۲ ساعت پس از تیمار انجام گرفت. نتایج هم‌بستگی منفی بین مقادیر چربی کل و غلظت‌های تیمارها (یعنی: CNTs و $CNTs/TiO_2$-NPs) و هم‌چنین مدت زمان مواجهه با آنها را نشان داد. محتوای کربوهیدرات، برخلاف تیمارهای CNTs، تحت تأثیر فاکتور غلظت و مدت زمان مواجهه با تیمارهای $CNTs/TiO_2$-NPs قرار گرفت. نتایج نشان داد که تأثیر CNTs خالص در افزایش محتوای گلیکوژن سریع‌تر از CNTs کوپل شده با نانوذرات TiO_2 ظاهر می‌شود. با افزایش زمان مواجهه با تمام غلظت‌های CNTs، به‌جز ۱۰۰ پی‌پی‌ام، از افزایش مقدار پروتئین جلوگیری شد. تأثیر CNTs خالص در کاهش میزان محتوای پروتئین سریع‌تر و بیش‌تر از $CNTs/TiO_2$-NPs بود. نتایج نشان داد که *G. pyloalis*، بدون تأثیر قابل توجه بر ذخیره انرژی (Ea)، قادر به تنظیم مؤثر ترکیبات CNTs و $CNTs/TiO_2$-NPs وارد شده به بدن نیست. مقایسه مقدار مصرف انرژی (Ec) لاروهای تیمار شده با CNTs خالص و CNTs ترکیب شده با نانوذرات TiO_2 نشان‌دهنده مصرف انرژی بیش‌تر در پاسخ به استرس $CNTs/TiO_2$-NPs نسبت به استرس CNTs بود. به‌طور کلی، با افزایش غلظت تیمارهای CNTs، CEA به‌طور قابل‌توجهی کاهش یافت. کاهش شدیدتر در مقدار CEA در مورد تیمارهای -CNTs/TiO2 NPs نسبت به شاهد نیز احتمالاً به‌دلیل بالارفتن هزینه لازم جهت سم‌زدایی با افزایش غلظت تیمار و هم‌چنین زمان مواجهه است. بنابراین، این امکان وجود دارد که آزمون CEA را به‌عنوان یک نشانگر بیوشیمیایی سریع در جهت ارزیابی پاسخ فوری موجودات زنده بعد از مواجهه با مقدار حاد عوامل استرس‌زا درنظر گرفت و آن را برای ارزیابی ریسک نانومواد مورد کاربرد قرار داد.

واژگان کلیدی: آزمون CEA، $CNTs/TiO_2$-NPs، زیست نشانگرهای بیوشیمیایی، ارزیابی ریسک

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