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Original Article

Effects of carbon nanotubes (CNTs) nano-materials on the giant freshwater prawn (*Macrobrachium rosenbergii*, de Man, 1879) in laboratory conditions

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Abstract: Carbon nanotubes (CNTs) are the most widely studied class of engineered nanoparticles due to carbon's unique hybridization properties and they are extensively used in several fields depending on their morphology, particle size, exposure time, and concentration. These nanoparticles are released into the aquatic ecosystems through domestic and industrial wastewater and induce adverse effects on the aquatic organisms. The present study evaluated the toxicity effects of CNTs nano-particles on crustacean hyperglycemic hormone (CHH) hormone release, hematology factors, and anti-oxidative enzymes' activity of *Macrobrachium rosenbergii*. This research was conducted in five treatments, including 0 (control), 5, 10, 20, and 30 mg/L CNT nanoparticles in triplicate for 28 days. The experimental units consisted of a 300-l recirculating system, stocked with, 10 prawns. The results indicated that *M. rosenbergii* reproductive performance, anti-oxidant enzyme activities, hematology parameters and CHH hormone release, survival rate, and growth performance were strongly affected by CNT NMs toxicity. The findings showed that SOD and CAT antioxidant enzymes activities have positive responses to the CNTS NMs in the experimental treatments and these NMs showed dose-dependent effects on the enzyme's activities. Also, CHH hormone in the experimental treatments showed significantly higher than the control treatment. The results of this work illustrate that because of the settling behavior of NMs, *M. rosenbergii* as a freshwater benthic decapod crustacean is an appropriate biological model to study NMs toxicity and also a suitable bio-monitor for NMs contaminations in freshwater aquatic environments.

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Introduction

Nanomaterials (NMs) have been categorized as those materials that have structured components with at least one dimension less than 100 nm and some of them have relatively high surface-to-volume ratios which are known as nano-absorbents (Qu et al., 2013). These NPs are widely used for various purposes in different applications and industries. Carbon nanotubes (CNTs) are tubes of graphite sheets with diameter in the nano-scale, including single-wall carbon nanotubes and multiwall carbon nanotubes; carbon nanotubes are named as the king of nano-materials. Nanotube drugs have been discovered to kill bacteria and are more effective than traditional antibiotics (Subramanian and Mehta, 2018). CNTs are the most widely studied class of engineered NPs due to carbon's unique hybridization properties and the sensitivity to variations in the synthesis conditions allowing tailoring of these nanostructures for specific applications (Aqel et al., 2012). The different synthesis, purification, and post-processing methods produce CNTs with different physical characteristics, which can be applied in different fields ranging from composite materials, medical applications, and electronics to energy storage (Helland et al., 2007). These nanoparticles are allotropes of carbon, made of graphite and constructed in cylindrical tubes that have been applied in pharmacy and medicine due to their high surface area that is capable of adsorbing or conjugating with a wide variety of therapeutic and diagnostic agents (He et al., 2013) and also for wastewaters treatment (Qu et al., 2013; Nezhadheydari et al., 2019) to remove impurities. Aquatic environments are the ultimate sink for these

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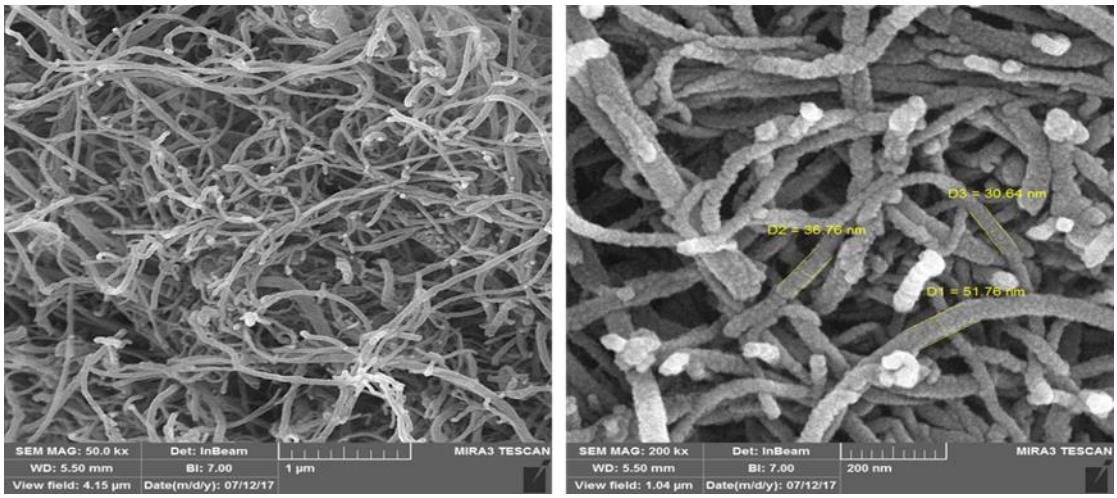

Figure 1. SEM images of the prepared CNTs nanoparticle (Tescan mira 3, CZE).

nano-contaminants, via either direct discharge or hydrological processes (Rezaei Tavabe et al., 2010; Lee et al., 2015; Turan et al., 2019).

Yearly, a large quantity of carbon nanotubes is released into aquatic ecosystems through domestic and industrial wastewater and induce adverse effects on fish and other aquatic organisms (Wu et al., 2019), because animal cells and tissues can easily absorb these materials due to their tiny sizes. In aqueous environments, CNTs clump together to form aggregates in the micrometer range and cause harmful and toxic effects on aquatic organisms; on the other hand, the large surface area of CNTs may cause other molecules to adhere and potentially pick up pollutants and transport these throughout the environment (Subramanian and Mehta, 2018). So, CNTs NPs are hazardous to aquatic organisms and especially invertebrates as bio-indicators. Mwangi et al. (2012) confirmed CNTs toxicity on *Hyalella azteca*, *Chironomus dilutus*, *Lumbriculus variegatus*, and *Villosa iris* species of invertebrates in freshwater ecosystems. Also, Lee et al. (2015) demonstrated that CNTs exposure could cause antioxidant depletion and apoptosis in a manner influenced by tissue and gender in the *Oryzias latipes*. CNTs induce oxidative stress and neurotoxicity in fish models such as *Danio rerio* and *Astyanax altiparanae* after acute and sub-chronic exposure (Cimbaluk et al., 2018). Therefore, it is important to study the biological effects of NMs contaminants on aquatic animals in freshwater ecosystems. Because of the settling behavior of NMs, benthic organisms are likely to be exposed to a higher degree than pelagic organisms (Selck et al., 2016).

Decapod crustaceans because of their ecological and physiological characteristics are excellent bio-indicators and bio-monitors of different contaminants in freshwater ecosystems (Rezaei Tavabe et al., 2019; Zhang et al., 2020). The giant freshwater prawn, *Macrobrachium rosenbergii*, is one of the most important decapods crustacean aquaculture species, originally from Asia's southeastern region, but during past decades it has been moved throughout many other countries globally, for aquaculture activities (Tidwell et al., 2005). Since, this species readily breeds in captivity condition sequentially with high fecundity and fertilized eggs clutch attach to the abdomen section of the ovigerous females (Tavabe et al., 2013; Rafiee et al., 2015; Rezaei Tavabe et al., 2015a, b, 2017), it is an appropriate benthic biological model for toxicological studies in laboratory condition. The main objective of the present study was the assessment of CNT-NPs toxicity effects on hyperglycemic hormone (CHH) release, hematology factors, and anti-oxidative enzymes' activities of the *M. rosenbergii* in laboratory conditions.

Materials and Methods

Preparation of CNT NPs: The required CNTs nanomaterials were purchased from Sigma-Aldrich Company (USA). After obtaining the nanomaterial,

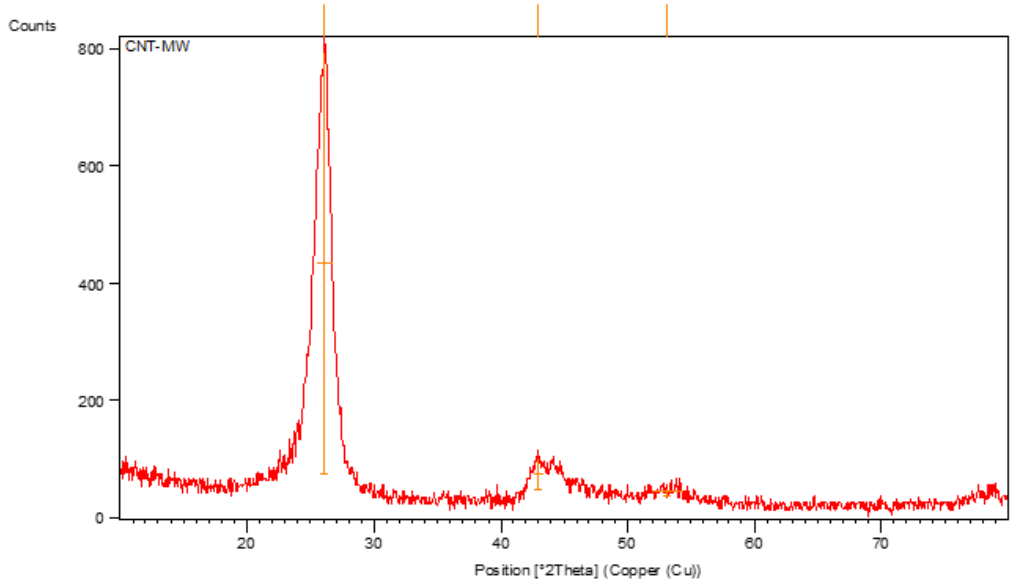

Figure 2. The XRD spectra of the obtained CNTS-Nanomaterials (Philips PW1730, NED).

SEM (Tescan Mira 3, CZE) images were taken for morph-structure of the surface, shape, and size of the NPs (Fig. 1). To assess the quality of NPs, the CNTs NPs X-ray diffraction pattern was recorded by a Philips PW1730, NED in the range of 10° to 80° (2θ). Figure 2 shows its XRD spectra and the peak points of 26.2, 43.1, and 54.5, and the recorded XRD was completely consistent with the standard patterns.

Experimental animals: Studied prawns (n = 350) were obtained from the Ghasreshirin freshwater prawn aquaculture center in Kermanshah Province in western Iran. Experiments were conducted at the aquatic animal laboratory of the Fisheries Sciences Department, University of Tehran. The prawn's average weight was 14±2 g and moved to the laboratory and stocked in a 1000 l tank for acclimation approximately 10 days before the experiment. In the acclimation tank, water quality parameters, photoperiod, and feedings were recorded and adjusted by the recommendations of Javanmardi et al. (2018), Javanmardi et al. (2020), Rezaei Tavabe and Rafiee (2015) and Rafiee et al. (2014).

Experimental setup and design: Experiments were conducted in five treatments including 0 (control), 5, 10, 20, and 30 mg/L of CNTs-NMs. Treatments were tested in triplicate and each of the experimental tanks (300-l recirculating systems) was stocked with, 10 prawns for 28 days research period. During the study, the photoperiod and temperature were 12 h light and 28±2°C, respectively, based on Rezaei Tavabe and Rafiee (2016).

Evaluation parameters:

Prawn broodstock reproductive parameters: Inter-spawn period, egg fertilization percentage, egg dry weight, total fecundity, egg-clutch somatic index (ESI), weight gain (WG), and survival of the females were recorded at the end of the experiment. Egg clusters were removed after spawning from berried females (n=3) to estimate fecundity, egg fertilization percentage, egg weight, and ESI. WG, ESI, egg fertilization percentage, and fecundity were calculated based on Rezaei Tavabe et al. (2015b).

CHH hormone release measurement: Measurement of CHH in the hemolymph was carried out according to the methodology described by Levenson et al. (1999). The sampled hemolymph was mixed 1:1 (v/v) with coating buffer (0.2 M/l sodium bicarbonate buffer, pH 9.4) and 100 µl was loaded in each well. After washing with buffer (10 mM/l PBS, pH 7.4 and 0.1% Tween 20) the plate was blocked with 100 µl of blocking buffer (10 mM/l PBS, 0.1% Tween 20, 2% BSA) for 2 hours and then incubated with anti-CHH solution (dilution 1:10 000 in blocking buffer) for 2 hours at room temperature. The plate was then washed

Table 1. Growth and reproductive parameters (mean±SD) of the prawns at different CNT-NMs treatments during the experiment period.

CNT Treatments (mg/L)	Initial weight (g)	WG%	Survival (%)	Inter-spawn period (days)	Egg dry weight (µg)	Fecundity (eggs/ female)	Eggs fertilization (%)	ESI (%)
Control	14±2	84.5±5.2^{a}	92 %	26±4	25±2^{a}	3350±641	95±4^{a}	9±1^{a}
5	14±2	60.7±3.9^{b}	83 %	25±3	22±3^{ab}	3045±325	81±6^{b}	8±2^{ab}
10	14±2	58.6±5.5^{b}	75 %	25±5	19±2^{b}	3184±501	48±11^{c}	6±1^{b}
20	14±2	31.8±6.7^{c}	42 %	At this treatment the brood stock spawned, but detached immediately the egg clutches from the swimming legs after spawning.				
30	14±2	27.9±8.5^{c}	40 %	At this treatment the brood stock did not spawn.				

The comparison is intergroup and means with different superscript letters in same columns are significantly different (P<0.05).

and incubated with the secondary antibody, anti-rabbit IgG peroxidase for 2 hours at room temperature. Again, the plate was washed and 100 µl of tetramethylbenzidine (TMB) ELISA substrate was added to each well to initiate the enzymatic reaction. The plate was incubated in the dark for 10-30 min at 37°C. The reaction was stopped by adding 2 M/l H_2SO_4. After that multi-well plates were read at 450 nm in ELISA-reader (Cyberlab Inc., USA).

Anti-Oxidative enzymes activity assay: At the end of the research period, three prawns for each tank were sampled and anaesthetized and the hepatopancreas tissue of an individual prawn was removed then Superoxide dismutase (SOD) enzymes activities were assayed. SOD enzyme activity in hepatopancreas was assayed according to Du et al. (2019), based on the oxidation of epinephrine to adreno-chrome by the enzyme. According to this method, 0.1 ml volume of hepatopancreatic homogenate was added to a tube containing 0.75 ml ethanol and 0.15 ml chloroform and centrifuged. The supernatant obtained (0.5 ml) was treated with 0.5 ml EDTA solution and 1 ml buffer. The enzyme reaction, increase in absorbance, and activity were expressed as 50% inhibition of epinephrine auto-oxidation/min/mg protein.

Hematology assay: SGH (Small granular haemocyte) and LGH (large granular haemocyte) were determined at the end of the experiment period by monitored light microscope as hematological assay parameters. Hemolymph sampling and hemocyte counting were carried out based on Leigh and Antoinette (1997).

Data analysis: The data were normalized by the Shapiro-Wilk test then the parameters were analyzed by one-way ANOVA and significant differences among the means were calculated (P<0.05) by Duncan's test by SPSS version 24 (IBM, USA).

Results and

During the experiment period, water physic-chemical factors including temperature, pH, dissolved oxygen (DO), ammonia-N, and nitrite-N were recorded 28±2°C, 7-7.6, 6±1 mg/l, < 0.2 mg/l, and < 0.1 mg/l respectively.

Growth and reproductive performances: Growth and reproductive results were sharp; so, by increasing CNT NMs concentrations on the treatments WG and survival rate were strongly decreased. Although, WG and survival rate in the control treatment were 84.5 and 92%, respectively, but these parameters for the 30 mg/L CNT treatment were 27.9 and 40%. On the other hand, high concentrations of CNT NMs disrupted the reproduction process in the experimental tanks. At the 20 mg/L treatment, the broodstock spawned, but detached immediately the egg clutches from the swimming legs after spawning while at the 30 mg/L, the broodstock did not spawn at all. In other treatments, while inter-spawn period and fecundity were not different among the treatments but egg dry weight, egg fertilization rate, and ESI parameters were significantly different and showed a decreasing trend (Table 1).

Anti-Oxidative enzymes activities assay: As expected, antioxidant enzymes' activities showed a positive response to the CNTS NMs in the experimental treatments. SOD enzyme activity in the hepatopancreas tissue for the 50 mg/L treatment was

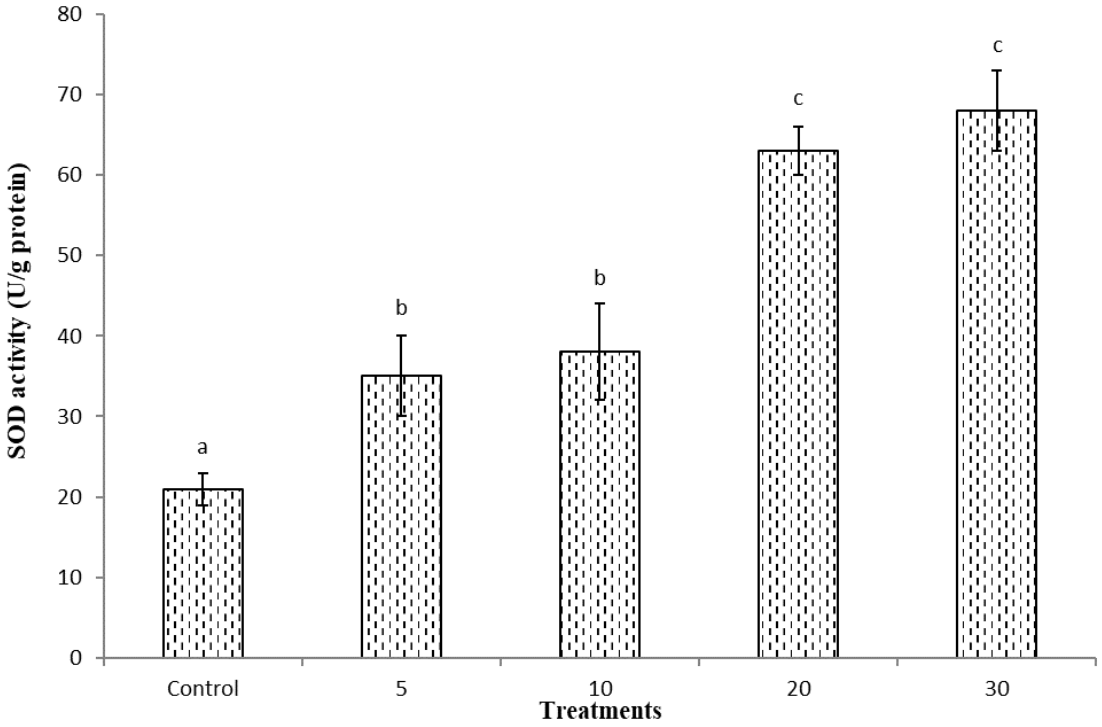

Figure 3. SOD enzyme activity in hepatopancreas tissue of *Macrobrachium rosenbergii* (mean±SD) at different CNT-NMs treatments (mg/L). Different letters denote significant differences (P<0.05) among the treatments.

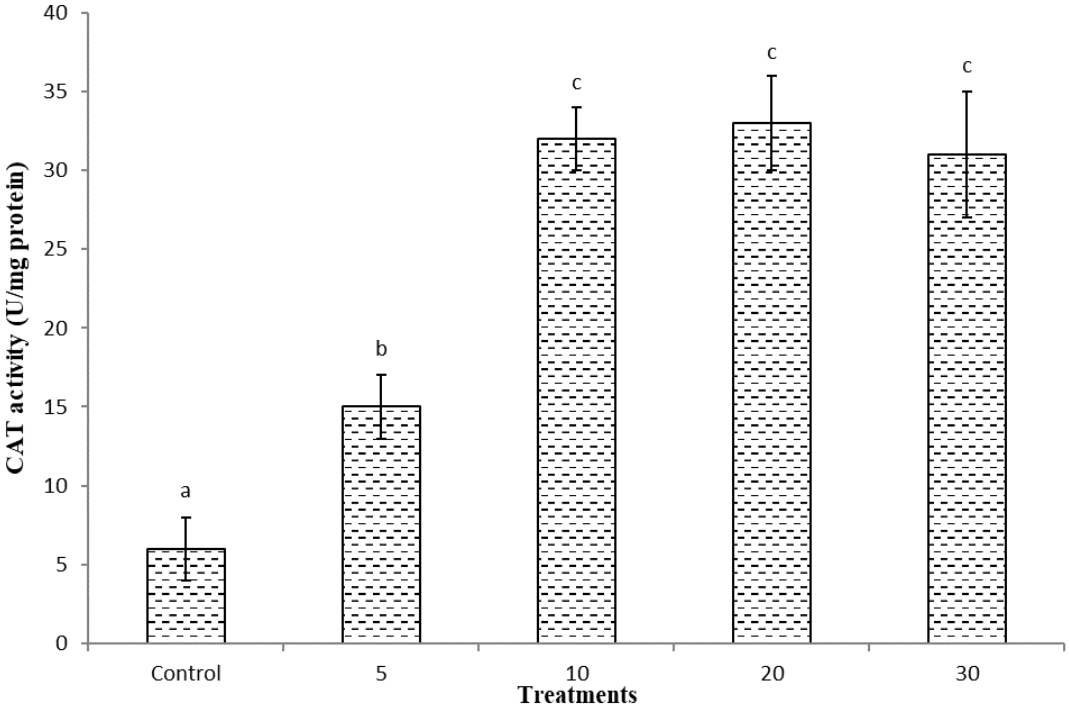

Figure 4. Catalase enzyme activity in hepatopancreas tissue of *Macrobrachium rosenbergii* (mean±SD) at different CNT-NPs treatments (mg/L). Different letters denote significant differences (P<0.05) among the treatments. Different letters denote significant differences ($P < 0.05$) among the treatments.

67.5 U/g protein while at the control treatment, this enzyme activity was 20.9 U/g protein (Fig. 3). Based on the results, catalase activity in the same tissue in 10, 20 and 30 mg/L treatments were same but these treatments were significantly different from the control one and 5 mg/L treatments (Fig. 4).

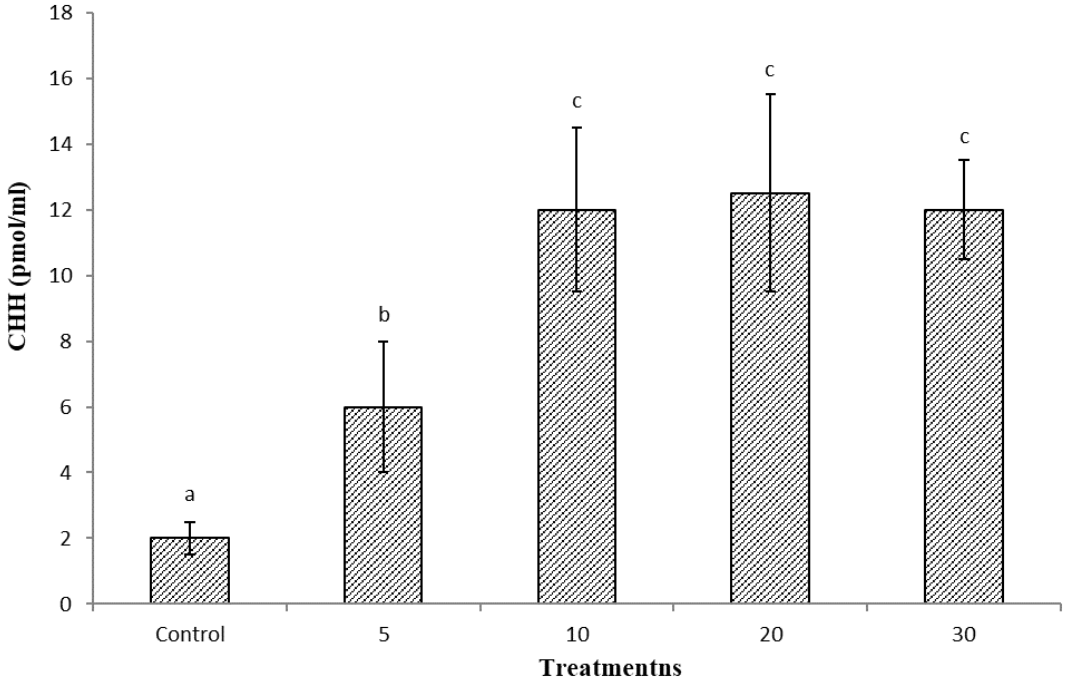

Figure 5. CHH of *Macrobrachium rosenbergii* (mean±SD) at different CNT-NPs treatments (mg/L). Different letters denote significant differences (P<0.05) among the treatments. Different letters denote significant differences (P<0.05) among the treatments.

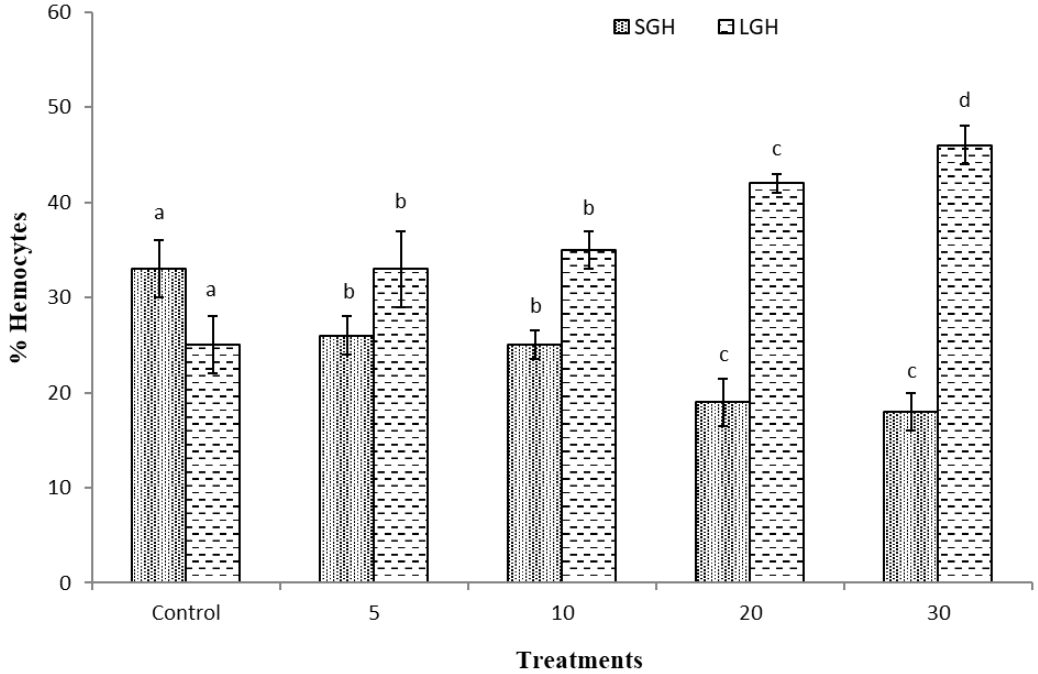

Figure 6. Hematocytes changes of *Macrobrachium rosenbergii* (mean±SD) at different CNT-NPs treatments (mg/L). The comparison is intergroup and different letters denote significant differences (P<0.05) among the treatments.

CHH hormone assay: CHH hormone in crustaceans is known as a metabolizing stress hormone. In the present study, the experimental treatments showed significant differences from the control treatment. While in the 50 mg/L CNT treatment CHH release was 11.5 pmol/ml, but this value for the control treatment was 1.7 pmol/ml (Fig. 5).

Hematology parameters: Hemocyte changes are common responses in crustacean hemolymph in challenge with stressors. According to the hematology

data, by increasing CNTs NMs concentrations in the experimental treatments, SGH and LGH hemocyte values were decreased and increased, respectively (Fig. 6).

Discussions

Nanoparticles have been widely used in different fields resulting in their intentional or unintentional release into the aquatic environment (Ashori et al., 2019; Turan et al., 2019). The fate of nanomaterials in the aquatic environment depends both on their physicochemical properties and the characteristics of the receiving environment and this environment because of the settling behavior of NMs, benthic organisms are likely to be exposed to a higher degree than pelagic organisms (Selck et al., 2016). Therefore, aquatic environments receive a large quantity of carbon nanotubes due to their increasing production and applications via direct discharge or hydrological processes, yearly (Zhang et al., 2020), and cause adverse effects on aquatic organisms (Vali et al., 2020). The present study showed that *M. rosenbergii* reproductive performance, anti-oxidant enzymes' activities, hematology parameters and CHH hormone release, survival rate, and growth performance were strongly affected by CNT NMs toxicity. Nano-materials enhance the formation of reactive oxygen species (ROS), which is one of the main toxic mechanisms observed in aquatic organisms (Lee et al., 2012; Rezaei Tavabe et al., 2018). These materials of varying chemical composition such as CNTs have been shown to induce oxidative stress (Bonner, 2007). Hence, knowledge of the fate and behavior of CNTs in different types of natural aquatic ecosystems and their potential eco-toxicity is essential for the quantitative assessment of the environmental risks of these NMs (Lukhele et al., 2015).

The findings of the current study showed that SOD and CAT antioxidant enzymes' activities have positive responses to the CNTS NMs in the experimental treatments and these NMs showed dose-dependent effects on the enzymes' activities. Oxidative stress is defined as a situation where the redox balance is shifted toward a pro-oxidant state as compared to an antioxidant state. Oxidative stress endpoints can be assessed in experimental models ranging from simple cellular conditions to molecular epidemiology using biomarkers of oxidative-generated biomolecules, antioxidant depletion, or other indicators (Møller et al., 2014; Rezaei Tavabe et al., 2020). ROS as pro-oxidants cause oxidative stress and oxidation of proteins, lipids, and DNA, which can lead to significant cellular and tissue damage (Tripathy, 2016). Liu et al. (2018) indicated that SOD and CAT enzymes can maintain steady-state levels of ROS in cells and protect cells against the adverse effects of them. Wang et al. (2015) showed that exposure of goldfish to different CNTs NPs causes obvious changes in antioxidant enzymes' activities such as SOD and catalase in the liver. Saria et al. (2014) confirmed the effects of MWCNT NPs toxicity on SOD and catalase enzymes' activities in the African frog (*Xenopus laevis*) tadpoles. Also, increased activities of these enzymes were shown in zebrafish (Souza et al., 2019), Rare minnow (*Gobiocypris rarus*) larvae (Zhu et al., 2015; Tavabe et al., 2020), and *Channa punctatus* fish (Amjad et al., 2018) in a dose-dependent manner. The results of the present study not only confirm the past reports but also depict that CAT enzyme activity in *M. rosenbergii* hepatopancreas is more intense than SOD in exposure to CNT NMs.

CHH hormone in crustaceans is known as a metabolizing stress hormone. In the present study, the experimental treatments showed significantly higher than the control treatment. Lorenzon et al. (2004) indicated that CHH hormone excretion in decapod crustaceans is directly related to their exposure to stressors. The stressors induce hyperglycemia and glucose release from the hepatopancreas storage cells by secretion of CHH hormone from the X-Organ (Santos and Keller, 1993) to provide energy to deal with stressors. Also, Sreenivasula Reddy et al. (2011) indicated that stressor factors cause glucose release from the hepatopancreas tissue in the developed crustaceans via excretion of CHH hormone. This hormone release in the crustacean's body has a direct relation to the severity of the stressor factor. Lukhele

et al. (2015) confirmed the toxicity effects of CNT NMs on three aquatic animals including *Pseudokirchneriella subcapitata*, *Daphnia pulex*, and *Poecilia reticulate*. Like most invertebrates, crustaceans facing stressful conditions switch to an alternative anaerobic energy metabolism via glycolysis and hyperglycemia, the process that is regulated by CHH hormone secretion (Chung et al., 2010). Reddy and Sainath (2009) indicated that the variations in the CHH values and hemolymph glucose level in relation to stressor factors could be used as an efficient tool to monitor a variety of stress responses in decapod crustaceans. Therefore, CHH hormone not only is a stress-metabolizing hormone but is also known as a reproductive regulation hormone (Chung et al., 2010).

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