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Adverse effects of MWCNTs on life parameters, antioxidant systems, and activation of MAPK signaling pathways in the copepod *Paracyclopina nana*

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ABSTRACT

Engineered multi-walled carbon nanotubes (MWCNTs) have received widespread applications in a broad variety of commercial products due to low production cost. Despite their significant commercial applications, CNTs are being discharged to aquatic ecosystem, leading a threat to aquatic life. Thus, we investigated the adverse effect of CNTs on the marine copepod *Paracyclopina nana*. Additional to the study on the uptake of CNTs and acute toxicity, adverse effects on life parameters (*e.g.* growth, fecundity, and size) were analyzed in response to various concentrations of CNTs. Also, as a measurement of cellular damage, oxidative stress-related markers were examined in a time-dependent manner. Moreover, activation of redox-sensitive mitogen-activated protein kinase (MAPK) signaling pathways along with the phosphorylation pattern of extracellular signal-regulated kinase (ERK), p38, and c-Jun-N-terminal kinases (JNK) were analyzed to obtain a better understanding of molecular mechanism of oxidative stress-induced toxicity in the copepod *P. nana*. As a result, significant inhibition on life parameters and evoked antioxidant systems were observed without ROS induction. In addition, CNTs activated MAPK signaling pathway *via* ERK, suggesting that phosphorylated ERK (*p*-ERK)-mediated adverse effects are the primary cause of *in vitro* and *in vivo* endpoints in response to CNTs exposure. Moreover, ROS-independent activation of MAPK signaling pathway was observed. These findings will provide a better understanding of the mode of action of CNTs on the copepod *P. nana* at cellular and molecular level and insight on possible ecotoxicological implications in the marine environment.

1. Introduction

Carbon nanotubes (CNTs) are low-dimensional allotropes of carbon, and are classified into single-walled (SWNT), double-walled (DWNT), and multi-walled (MWNT) carbon nanotubes (Saleh et al., 2008) based on the number of concentrically rolled-up graphene sheets. Owing to the unusual physicochemical, mechanical, and electrical properties (Kang et al., 2008), engineered MWCNTs have received considerable attention as subjects of intense research with envisage on the widespread application in a broad variety of commercial products (Liu et al., 2009). The lower production cost of MWCNTs than SWCNTs favoured broadening the field of application including environmental management (De Volder et al., 2013; Long and Yang 2001). As MWCNTs playing a major role in the industry, it is likely to be released in the aquatic environment, posing a serious threat (Stegeman and Hahn, 1994; Sabba and Thomas, 2004; Scott-Fordsmand et al., 2008). Therefore, the consequences of possible dispersal of nanomaterials in environmental systems and its impact on human and the environment are a great concern (Wiesner et al., 2006). Even though nanotechnology is playing an increasing role in the human society, little is known about the environmental toxicity of nanotechnological products (Scott-Fordsmand et al., 2008). Nanoparticles in general and CNTs, in particular, have the capacity to interact with a biological molecule that leads to diverse impacts on living organisms (Guan et al., 2014; Lee et al., 2016a). However, little is known about the effects of CNTs on marine invertebrates (Lee et al., 2016a), which play a major role in maintaining the energy equilibrium of the marine ecosystem and is important to reflect profound implications on the food chain

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(Won et al., 2016). Thus, the evaluation of toxic impairments on marine invertebrates is critical to foreseen CNTs-induced consequences on marine ecosystems.

To date, ecotoxicological effects of CNTs have been investigated in diverse aquatic organisms including fish (Lee et al., 2015a), *Daphnia* (Kim et al., 2009), copepod (Lee et al., 2016a), rotifer (Lee et al., 2016a), amphibian (Mouchet et al., 2010), algae (Pereira et al., 2014), protozoa, and bacteria (Ghafari et al., 2008). Among marine invertebrates, copepods play a major role in the marine ecosystem as a link between producers and high trophic consumers (Raisuddin et al., 2007). Particularly, the cyclopoid copepod *Paracyclopina nana* has been used as an aquatic model organism, considering the planktonic nature, small size (<0.6 mm), short life cycle (~14 days), and ease of maintenance in the laboratory (Hwang et al., 2010; Lee et al., 2012; Won et al., 2014; Han et al., 2015; Dahms et al., 2016). As an extremely sensitive organism to the environmental conditions (*e.g.*, temperature, salinity and pollutants) and stressors (*e.g.*, heavy metal and radiation), this species has received considerable attention as suitable model for ecotoxicological and environmental genomics research; which provides a platform for molecular mechanistic study of marine pollutants (Hwang et al., 2010; Won and Lee, 2014; Han et al., 2015; Dahms et al., 2016). Moreover, in recent studies, various ecotoxicological biomarkers have been evaluated in *P. nana* (Won and Lee, 2014; Lee et al., 2015a; Dahms et al., 2016), which can be used for environmental risk assessment in aquatic organisms (Lee et al., 2015b). However, so far, no information is available on effects of carbon allotrope nanomaterials, particularly MWCNTs on *P. nana*. Thus, this species will be a suitable model species for increasing our understanding of the effects of emerging nanomaterials in marine environments.

To date, CNT-induced cellular damages *viz*., oxidative stress (Pereira et al., 2014), modulation of antioxidant systems (Lee et al., 2016a,b), apoptosis (Smith et al., 2007; Lee et al., 2015a), abnormal activation of the mitogen-activated protein kinase (MAPK) signaling pathway (Lee et al., 2016a,b), and increased metal toxicity (Kim et al., 2009; Kim et al., 2010) have been reported from various organisms (*e.g.*, *Chlorella vulgaris*, *Tigriopus japonicus*, *D. magna*, *Brachionus koreanus*, *Oryzias latipes*). Also, the impairment on physiological parameters (*e.g.*, growth, behavior, fertilization, mobility, and molting) have been reported in response to CNTs (Templeton et al., 2006; Zhu et al., 2009; Kwok et al., 2010; Jackson et al., 2013; Lee et al., 2016b). Recently, MWCNTs induced modulation of antioxidant systems and extracellular signal-regulated kinase (ERK) activation without the generation of reactive oxygen species (ROS) was reported in the copepod *T. japonicus* (Lee et al., 2016a). However, in the rotifer *B. koreanus*, the activation of intracellular ROS was observed in response to MWCNTs, along with antioxidant depletion and ERK activation (Lee et al., 2016b). Therefore, it is apparent that CNTs have different modes of action according to the type of cells (Park et al., 2008) and the organisms which are exposed. Thus, more elaborative studies are required to attain better knowledge about the underlying mechanistic aspects of the toxicity of CNTs in marine invertebrates.

In this paper, we demonstrated internalized MWCNTs-induced significant alteration in the antioxidant mechanism, modulation in MAPK pathways and impairment on life parameters of the marine copepod *P. nana*.

2. Materials and methods

2.1. Carbon nanotubes and chemicals

The MWCNTs (>98% purity on carbon basis) with a diameter 6–13 nm, length (average) 10 μm, and a wall thickness (average) of 7–13 graphene layers were purchased from Sigma-Aldrich Co. (Catalog no. 698849; St. Louis, MO, USA). According to the datasheet, MWCNTs were produced by chemical vapour deposition (CVD) method followed by demineralization using hydrochloric acid (HCl). All other chemicals and reagents used for this study, unless specifically stated otherwise, were also purchased from Sigma-Aldrich Co.

2.2. Maintenance of P. nana

The copepod *P. nana* collected from Lake Songji (Gangneung, South Korea, 38°20′9.89″ N, 128°30′55.17″ E), isolated under a stereomicroscope (SZX-ILLK200, Olympus, Tokyo, Japan), and maintained over 10 years in the Department of Biological Science, Sungkyunkwan University, Suwon, South Korea, was used for this study (Han et al., 2015). The animals were fed with the green marine microalgae *Tetraselmis suecica* (~6×10^4 cells/ml) every 24 h, maintained in filtered artificial seawater (ASW) (TetraMarine Salt Pro, Tetra™, Cincinnati, OH, USA) under confined laboratory conditions of 15 psu salinity, 12:12 h (light: dark) photoperiod at 25 °C. The population density was monitored every 48 h under a stereomicroscope and was sub-cultured during the sigmoidal growth phase. The species identity of *P. nana* was confirmed by morphometric analysis followed by molecular characterization of cytochrome oxidase 1 (*CO1*) DNA of mitochondrial genome (Ki et al., 2009).

2.3. Preparation of seawater-dispersed MWCNTs

Preparation of seawater-dispersed MWCNTs was performed by our previous study (Lee et al., 2016a). Briefly, MWCNTs was dispersed in ASW by sonication; 5 s run and 1 s pause with 100% amplitude for 10 min, in glass bottles in duplicate.

2.4. Uptake of MWCNTs by P. nana *and acute toxicity test*

To confirm the uptake of MWCNTs by *P. nana*, 30 adult species were exposed to 20 mg/L MWCNT in ASW for 96 h. After exposure, animals were fixed in 1 mL formaldehyde (10%) on a clean glass slid and monitored using an Inverted fluorescent microscope (Ix71, lamp TH4-200, Olympus America Inc., Center Valley, PA, USA). Microscopic images of the control groups and test groups were taken to confirm the distribution of MWCNTs inside the body. All experiments were conducted in triplicate.

Acute toxicity tests for 48 h were carried out with various concentrations of MWCNTs 0 (ASW only), 25, 50, 100, 200, and 400 mg/L. Briefly, ten adults were placed into 10 mL of test solution in a glass beaker (100 mL) and kept undisturbed for 48 h without food in confined laboratory conditions of 12:12 h (light: dark) photoperiod at 25 °C. All tests were conducted in triplicate. Mortality was monitored every 24 h during the 48 h toxicity test under a stereomicroscope (M205A, Leica Microsystems Ltd., Wetzlar, Germany). A half lethality at 48 h (LC50–48 h) value was calculated by the Probit method (Finney, 1971) using the Finney computer program BioStat™ 2009 (AnalystSoft Inc., Vancouver, BC, Canada).

2.5. Effect of MWCNTs on life parameters of P. nana

Life parameters such as developmental rate, fecundity, and morphometric analysis in *P. nana* in response to MWCNTs exposure were performed as described in Lee et al. (2016b). For each experiment, ten individuals were exposed to different concentrations of MWCNTs (0, 2.5, 5, 10, and 20 mg/L) in a 4 mL glass beaker filled with 1 mL test solution. The highest concentration was varied for each experiment and was fixed based on the toxicity studies. Live feed diet *T. suecica* was supplied once daily, irrespective of the experiments. All experiments were conducted in triplicate.