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Genotoxicity of CeO_2, SiO_2 and TiO_2 Nanoparticles in the Freshwater Crustacean *Daphnia magna*

Sung-Man Kim and Jinhee Choi※

Faculty of Environmental Engineering, College of Urban Study, University of Seoul, Seoul 130-743, Korea

*Daphnia magna*를 이용한 세리아, 실리카, 티타늄 나노물질의 유전독성 평가

김 성 만, 최 진 희※

서울시립대학교 환경공학부 독성학연구실

요 약

본 연구에서는, 세리아(CeO_2), 실리카(SiO_2) 및 티타늄(TiO_2) 나노입자의 유전독성과 생태독성 평가를 위하여 바이오 모니터링에 널리 이용되는 수생생태 감시종인 *Daphnia magna*를 사용하였다. 합성한 나노입자 세리아와 공업적으로 상용되는 실리카 및 티타늄을 유전독성 및 생태독성평가에 이용하였다. 세리아의 경우, *D. magna*의 DNA의 파괴가 증가함을 통해 세리아의 유전독성 가능성을 확인할 수 있었으나, 실리카 및 티타늄의 경우에는 두 물질 모두 유전독성 영향이 나타나지 않았다. 실리카는 DNA에는 영향을 미치지 않는 것으로 보이나, 실리카에 노출된 *D. magna*의 사멸은 증가하는 결과를 보였다. 그러나, 티타늄에 노출된 *D. magna*에서는 유전독성 및 생태독성 인자의 유의적인 변화를 관찰할 수 없었다. 이상의 전체 결과를 통하여 예상할 수 있는 것은 세리아 나노입자가 *D. magna*에 유전독성을 일으킬 수 있다는 점이다. 이 결과는 나노입자가 광범위하게 이용되고 있으나 독성 관련 자료가 미약한 현재에 수생태 관련 독성 연구 결과로서 이바지 할 수 있을 것으로 여겨진다.

Key words : nanoparticles, *Daphnia magna*, genotoxicity, ecotoxicity

INTRODUCTION

Numerous new industrial nanomaterials have been synthesized for commercial and industrial purposes. Environmental concerns, however, have arisen, due to their high production and widespread use. Despite the dramatic increase in the use of nano-sized materials, little information is available on their potential toxic effects on the environment. Their potential deleterious effects on ecological health should be identified to allow their safe use. Most current literature on the toxicity of nanoparticles come from mammalian studies that focus on respiratory exposure or from *in vitro* assays with mammalian cells (Lam *et al*., 2004; Braydich-Stolle *et al*., 2005; Hussain *et al*., 2005; Monteiro-Riviere *et al*., 2005; Limbach *et al*., 2007). Ecotoxicological studies of nanoparticles are much more limited, with only a few

※To whom correspondence should be addressed.
Tel: +82-2-2210-5622, Fax: +82-2-2244-2245
E-mail: jinhchoi@uos.ac.kr

reports focusing on the acute toxic effects of nanoparticles on aquatic organisms (Kerstin and Markus, 2006; Lovern and Klaper, 2006; Handy and Shaw, 2007; Lovern *et al*., 2007; Sarah *et al*., 2007). Few ecotoxicity studies on aquatic organisms have been performed that include genotoxic endpoints.

The continued presence of genotoxic and potentially carcinogenic compounds in the aquatic environment is a major concern with respect to the health of aquatic biotas (Houk and Waters, 1996; Ohe *et al*., 2004; Nehl and Segner, 2005). To effectively assess the presence of mutagens in water, genotoxicity assays should be used aside from conventional physio-chemical analysis as additional parameters in water quality monitoring programs. Many studies link DNA damage to subsequent molecular-, cellular- and tissue-level alteration of aquatic organisms (Jha, 2004; Ohe *et al*., 2004). Thus, genotoxic parameters have been proved to be sensitive and reliable tools in the detection of mutagenic activity in aquatic environments, and thus, currently the most valuable biomarkers for ecological risk assessment. Among the available genotoxicity indicator tests, Comet assay has recently attracted much attention. Comet assay, also called single-cell gel electrophoresis (SCGE) assay, primarily measures DNA strand breakage in single cells. Since Singh *et al*. published the protocol in (1988), it has been increasingly used in different fields of study. It is considered a sensitive and rapid technique for the detection of DNA strand breaks, and is an ideal non-specific biomarker of genotoxicity for environmental monitoring (Collins *et al*., 1997; Cotelle and Ferard, 1999; Tice *et al*., 2000; Brendler-Schwaab *et al*., 2005; Møller, 2006).

In this study, genotoxic assessments of nanoparticles were conducted on the aquatic sentinel species that are widely used in biomonitoring the freshwater crustacean *Daphnia magna*. The small-sized freshwater crustacean *D. magna* holds an important position in the aquatic food chain and are sensitive to many pollutants, aside from being easy to culture and having a short life cycle. Thus, it is considered suitable species for aquatic biomonitoring (Giesy *et al*., 1988; Cranston, 1995; Choi *et al*., 2000; Atienzar *et al*., 2001; Lee and Choi, 2006). Given the importance of *D. magna* in the aquatic ecosystem, information concerning genotoxicity on this species can be valuable for freshwater monitoring.

Cerium dioxide (CeO_2), silicon dioxide (SiO_2) and titanium dioxide (TiO_2) nanoparticles were studied as they are widely used. As lanthanide element oxides, CeO_2 nanoparticles are among the most important nanomaterials used in a wide range of applications, including in catalysis, solar, fuel cells, phosphor/luminescence, abrasives for chemical/mechanical planarizations gas sensors, oxygen pumps, and metallurgical and glass/ceramic applications (Murray *et al*., 1999; Corma *et al*., 2004; Izu *et al*., 2004; Zheng *et al*., 2005). As a non-metal oxide, the SiO_2 nanoparticle has found extensive applications in chemical /mechanical polishing and as additives to drugs, cosmetics, printer toners, varnishes, and food. In recent years, the use of SiO_2 nanoparticles has been extended to biomedical and biotechnological fields, such as biosensors for simultaneous assays of glucose, lactate, L-glutamate, and hypoxanthine levels in the rat striatum as biomarkers for leukemia cell identification using optical microscopy imaging and for cancer therapy, DNA delivery, drug delivery, and enzyme immobilization (Hirsch *et al*., 2003; Zhang *et al*., 2004; Gemeinhart *et al*., 2005; Venkatesan *et al*., 2005). TiO_2 is a potent photocatalyst that can break down almost any organic compound when exposed to sunlight, and has a potential for wide application in self-cleaning fabrics, auto body finishes, and ceramic tiles (Gratzel *et al*., 1999; Fujishima *et al*., 2000; Caruso *et al*., 2001).

MATERIALS AND METHODS

1. *Daphnia* culture

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), adult test organisms (*D. magna*) reared in our laboratory were obtained. *D. magna* were individually placed in glass

beakers containing culture media (M4) for 2 days. Cultured daphnids were fed the green alga *Chlorella* sp. at concentrations of $1 \times 10^6 \sim 10^9$ cells/mL everydays. Culture of *D. magna* was maintained at 20± 1°C, with 16 hr light and 8 hr dark cycle photoperiod regime.

2. CeO_2, SiO_2, and TiO_2 nanoparticles

SiO_2 and TiO_2 nanoparticles were purchased from Sigma Corp. (St. Louis, MO, USA), whereas CeO_2 nanoparticles were synthesized as described previously (Park *et al*., 2008).

3. Exposure conditions

Test solutions of CeO_2, SiO_2 and TiO_2 nanoparticles were prepared in culture media and dispersed for 15 minutes using a sonicator (Branson Inc., Danbury, CT, USA) to prevent aggregation. During the tests, the suspended nanoparticles were stable and uniform in the culture media. The concentration used in this study was 1 mg/L to prevent the aggregation and/or precipitation of the particles.

4. Comet assay

For the preparation of *Daphnia*, a total of 150 neonates aged less than 24 hr were collected from the control and experimental tanks 24 hr after treatment and were pooled for a Comet assay. Treated organisms were placed in 1 mL of phosphate-buffered saline (PBS) containing 20 mM ethylene diamine tetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and disintegrated mechanically by mincing. An alkaline comet assay was performed, as described by Singh *et al*. (1988). Briefly, 100 µL of 1% low melting point (LMP) agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4°C for 5 min to allow solidification. The cells were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 NaCl, 10% DMSO (only organisms), 10% Triton ×100, pH 10), and sub-sequently exposed to alkaline conditions (300 mM NaOH, 1 mM EDTA, pH > 13) at 4°C for 20 min to allow the DNA to unwind and the alkaline-liable sites to be expressed. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min, after which, the slides were neutralized and dehydrated with 70% ethanol. The slides were stored in a dry place prior to image analysis. Before their analyses, the slides were stained with 50 µL ethidium bromide (5 µg/mL), and then analyzed using a fluorescence microscope (Nikon, Kanagawa, Japan) equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm at 400× magnification. Approximately, 50 cells per slide (3 slides per treatment) were examined. DNA damage was expressed as the tail moment using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

5. Mortality test

Mortality test for *D. magna* was performed by counting the number of dead individuals. For *Daphnia*, 10 neonates aged less than 24 hr were individually transferred into 100 mL glass beakers filled with 50 mL of test solutions and incubated at 20±1°C for 24 hr.

6. Data analysis

The genotoxic- and ecotoxic assays results were tested for significance using the analysis of variance (ANOVA) test with Dunnett's multiple comparison test. All statistical tests were performed using SPSS® 12.0 KO (SPSS Incorporated, Chicago, IL, USA).

RESULTS AND DISCUSSION

In this study, the potential hazards of widely used nanoparticles (CeO_2, SiO_2 and TiO_2) on ecological health were evaluated using geno- and ecotoxicity tests on the aquatic sentinel species, *D. magna*. These aquatic toxicity tests may provide insights on the relative sensitivity of these species to the tested nano-

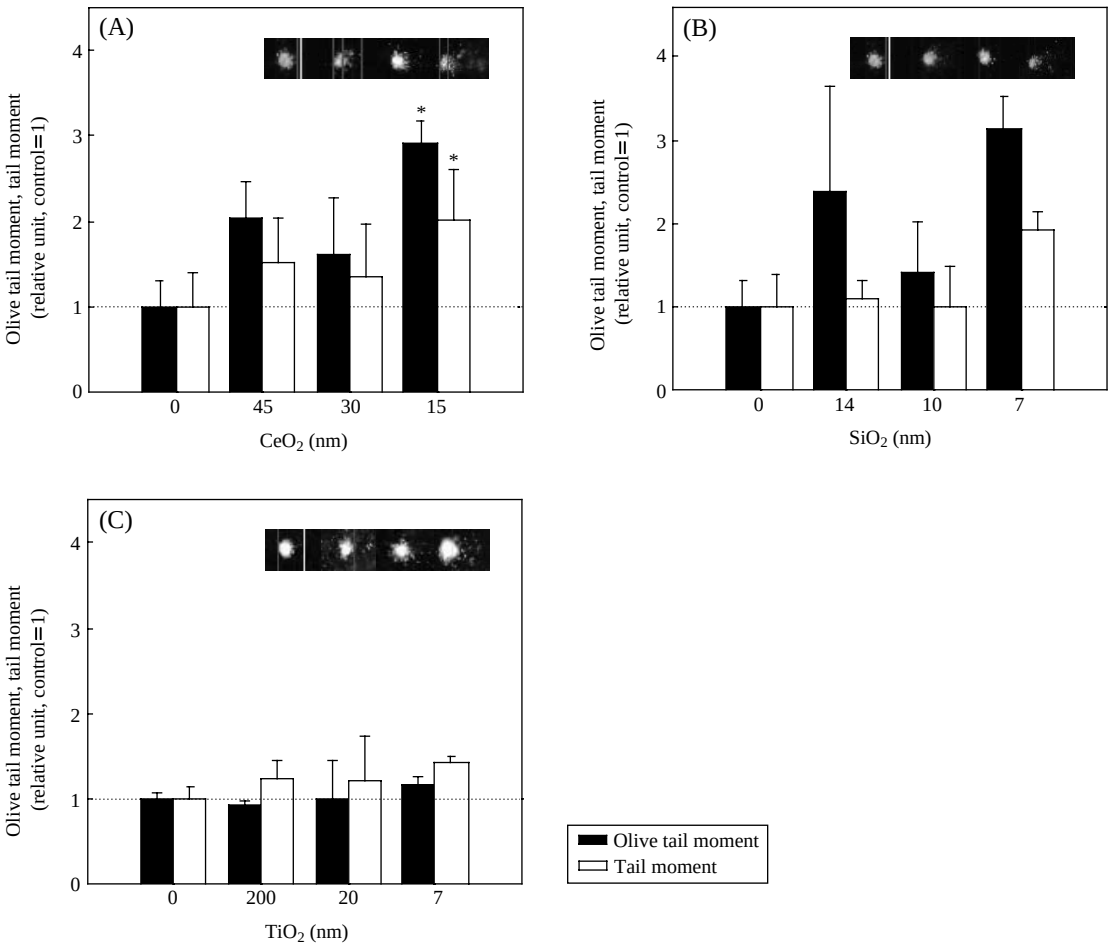

Fig. 1. DNA damage measured for *D. magna* exposed to CeO_2 (A), SiO_2 (B) and TiO_2 (C). The results were expressed as Olive tail moment and tail moment obtained by Comet assay (n=3, mean±standard error of mean, $*p < 0.05$).

particles, which may also provide information on the impact of nanoparticles on water systems, as these species hold an important position in aquatic ecosystems (OECD, 1984; Okamura *et al*., 1999; Kikuchi *et al*., 2000; Lee and Choi, 2006).

DNA damage, particularly DNA strand breaks, was measured using Comet assay, to evaluate whether CeO_2, SiO_2 and TiO_2 nanoparticles exert genotoxicity on *D. magna* (Fig. 1). CeO_2 may have genotoxic effects on *D. magna*, given that DNA strand breaks (tail/Olive tail moments) increased in this species exposed to this nanoparticle. Among the three sizes tested, the greatest degree of DNA damage was observed in the *Daphnia* exposed to the smallest CeO_2 nanoparticle (15 nm). Neither the SiO_2 exposure nor the TiO_2 exposure showed a genotoxic effect on the species since no significant increase in the tail/Olive tail moments was observed in these species exposed to the nanoparticles.

To screen genotoxic activities in aquatic environments, most genotoxic tests using Comet assays have been performed in *in vitro* systems from aquatic species, using mostly fish-derived cell lines (Cotelle and Ferard, 1999; Nehl and Segner, 2005). In this study, however, the *D. magna* was exposed to various sizes of different nanoparticles *in vivo*, and DNA

damage was assessed in the cells that were subsequently isolated from them. The *in vivo* genotoxic biomarker obtained in the aquatic sentinel species in this study could be a powerful tool in aquatic environment monitoring.

The responses of genotoxic parameters could provide useful information and could be used as an 'early warning system' for ecotoxicity monitoring of the potential hazards of nanomaterials to aquatic organisms. Chemical-induced genotoxic effects may have consequences at higher levels of biological organization, such as changes in population dynamics and in biological diversity at both the intra- and inter-species levels. The genotoxic biomarker alone is not sufficient, however, in evaluating the ecotoxicological response of nanomaterials in aquatic organisms to diagnose the potential risks of nanomaterials in aquatic ecosystems. The multi-parametric approach, wherein different biological responses ranging from the molecular/cellular to the physiological/ecological are evaluated, is essential to perform a better prospective assessment of the risks engendered by the presence of nanoparticles in aquatic ecosystems. Simultaneous measurement of various toxicological/ecological parameters gives the opportunity to obtain data at different levels of biological organization and may help fully uncover the effects of chemicals on organisms. In addition, the determination of population-level parameters improves the interpretation of the data collected at lower biological levels (Lee and Choi, 2006; Lee *et al*., 2008). Therefore, in this study, conventional ecotoxicity tests were conducted using mortality growth and reproduction as endpoints, to investigate the physiological- and organism-level effects of the tested nanoparticles and to validate the ecotoxicological relevance of the response of DNA damage from nanoparticle exposure of *D. magna* (Table 1).

A slight increase in the mortality rate of *Daphnia* was observed after it was treated with 15 nm of CeO_2 nanoparticles. A narrow increase in mortality was observed in *D. magna* exposed to 7 and 10 nm of the SiO_2 nanoparticle. The TiO_2 nanoparticle did not significantly alter the mortality.

Table 1. Mortality investigated for *D. magna* exposed to CeO_2, SiO_2 and TiO_2. (number=3, mean±standard error of mean)

Nanoparticles (Size: nm)		Mortality (%)
CeO_2	0 (control)	5±4.08
	45	5±4.08
	30	0
	15	10±0
SiO_2	14	5±4.08
	10	15±4.08
	7	10±8.16
TiO_2	200	5±4.08
	20	0
	7	5±4.08

DNA damage in wildlife species measured with Comet assay could be used as a sensitive and rapid genotoxic biomarker in environmental monitoring (Chen and White, 2004; Jha, 2004; Ohe *et al*., 2004). Nanomaterials may influence the genetic constitution of populations by directly damaging DNA molecules within the individual cell nucleus, but the ecological relevance of changes in single cells within some tissues of some individual organisms is extremely difficult to assess (Depledge, 1998). Nonetheless, a sensitive detection of DNA damage in wildlife species is necessary, as chemical-induced DNA damage may influence the genetic constitution of populations. The relationships between the responses of genotoxic biomarkers and physiological/individual /population effects are complicated because of the compensatory mechanisms that regulate physiological/individual fitness and population dynamics in natural ecosystems. As the mere presence of genotoxic compounds, which are potentially carcinogenic, is a major concern in human and ecosystem health perspectives, however, sensitive and rapid detection of genotoxic properties of aquatic systems themselves is considered important, although it does not necessarily include alteration at a higher level of biological organization. Especially for the nanomaterials concerned, despite the dramatic increase in the use of

nanomaterials and hence, their ubiquitous distribution in aquatic environments, little information has been available on their potential genotoxic effects on aquatic organisms. Considering the potential of *D. magna* as bioindicator species and the importance of the genotoxicity of nanoparticles in ecotoxicity monitoring, the measurement of the DNA damage in this species after exposure to nanoparticles could provide useful information for freshwater monitoring.

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