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Ecotoxicological investigation of CeO_2 and TiO_2 nanoparticles on the soil nematode *Caenorhabditis elegans* using gene expression, growth, fertility, and survival as endpoints

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ABSTRACT

In this study, the potential harmful effect of cerium dioxide (CeO_2), and titanium dioxide (TiO_2) nanoparticles on the environment was investigated using *Caenorhabditis elegans* ecotoxicity tests. Multiple toxic endpoints, such as stress-response gene expression, growth, fertility, and survival, were analyzed in *C. elegans*, in response to the CeO_2 and TiO_2 exposure. To investigate relationship between sizes of nanoparticles and toxicity, *C. elegans* were exposed to nanoparticles to the different sizes of nanoparticles (15, 45 nm for CeO_2 and 7, 20 nm for TiO_2). An increase in the expression of the *cyp35a2* gene, decrease in fertility and survival parameters were observed in the 15 and 45 nm of CeO_2 and in the 7 nm of TiO_2 nanoparticles exposed to *C. elegans*. Gene knock-down experiment using RNA interference (RNAi) suggested that physiological level disturbances may be related with the *cyp35a2* gene expression. Smaller sized nanoparticles (7 nm of TiO_2 and 15 nm of CeO_2) seemed to be more toxic than larger sized ones (20 nm of TiO_2 and 45 nm of CeO_2) on the observed toxicity. The size-dependent effect in CeO_2 and TiO_2 nanoparticles-induced toxicity needs to be investigated under more detailed experimental settings with the various sizes of nanoparticles. Further studies on the mechanism by which CeO_2 and TiO_2 nanoparticles affect *cyp35a2* gene expression, fertility, and survival are warranted to better understand the CeO_2 and TiO_2 nanoparticles-induced ecotoxicity in *C. elegans*, as are studies with the causal relationships between these parameters. Overall results suggest that CeO_2 and TiO_2 nanoparticles have a potential for provoking ecotoxicity on *C. elegans* and the data obtained from this study can comprise a contribution to knowledge of the ecotoxicology of nanoparticles in *C. elegans*, about which little data are available.

1. Introduction

Nanoparticles toxicity studies are among the fastest growing areas of environmental toxicology research, because of the fast applications of the nanotechnology in a wide variety of fields (Zheng et al., 2005; Nohynek et al., 2007; Rogueda and Traini, 2007). The potential health and environmental risks related to the widespread production and use of the nanomaterials created by these technologies need to be investigated in terms of their toxic mechanism as well as their long term consequences (Hoet et al., 2004; Oberdorster et al., 2005; Nel et al., 2006; Handy et al., 2008). Nanoparticles exposure in the environment may cause a physiological response in certain organisms, which alter their fitness, and ultimately lead to population/community level changes. Most of the current literature on the toxicity of nanoparticles comes from mammalian studies on respiratory exposure, or from *in vitro* assays with mammalian cells (Xiao-Feng et al., 2005; Donaldson, 2006; Cha and Myung, 2007; Handy and Shaw, 2007; Warheit et al., 2007). Ecotoxicological studies with nanoparticles are currently increasing, with most focusing on aquatic organisms (Hund-Rinke and Simon, 2006; Lovern and Klaper, 2006; Federici et al., 2007; Lovern et al., 2007; Smith et al., 2007; Fujiwara et al., 2008; Blaise et al., 2008; Velzeboer et al., 2008). Few studies have been performed on terrestrial organisms (Jemec et al., 2008; Wang et al., 2009). In the current study, ecotoxicological assessments of nanoparticles were conducted on the soil nematodes, particularly *Caenorhabditis elegans*, using multiple toxic endpoints.

C. elegans, a free-living nematode that lives mainly in the liquid phase of soils, is the first multicellular organism to have its genome completely sequenced. The genome showed an unexpectedly high level of conservation with the vertebrate genome, which makes *C. elegans* an ideal system for biological studies, such as those in genetics, molecular biology, and development biology (Bettinger et al., 2004; Leacock and Reinke, 2006; Schafer, 2006; Schroeder, 2006). *C. elegans* is also a good animal model for the study of ecotoxicology. Due to its abundance in soil ecosystems, its convenient

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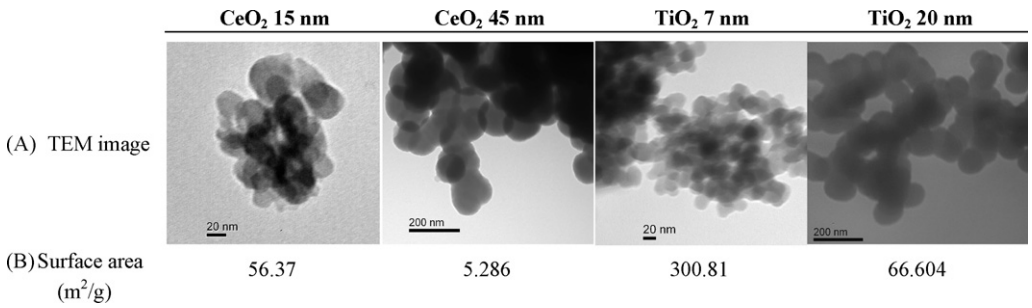

Fig. 1. Characterization of different sized CeO_2 and TiO_2 nanoparticles using TEM (A) and BET (B) methods. Structures and shapes of CeO_2 and TiO_2 nanoparticles used in this study were examined using transmission electron microscope. Two different sizes of CeO_2 (15 and 45 nm) and TiO_2 nanoparticles (7 and 10 nm) show different morphologies. Surface areas of nanoparticles were measured using the BET method.

handling in the laboratory, and its sensitivity to different kinds of stresses, *C. elegans* is frequently used in ecotoxicological studies utilizing various exposure media, including soil and water (Williams and Dusenbery, 1990; Peredney and Williams, 2000; Boyd and Williams, 2003; Roh et al., 2006, 2007).

Cerium dioxide (CeO_2) and titanium dioxide (TiO_2) were studied, as they are widely used manufactured nanomaterials. CeO_2 is one of the most important rare-earth oxides that have been widely investigated due to its unique properties and multiple applications, such as acting as a three-way catalyst in vehicle emission-control systems, electrolyte materials of solid oxide fuel cells, and ultraviolet blocking materials (Yu et al., 2001; Corma et al., 2004). A potent photocatalyst, TiO_2 has a potential for wide application in self-cleaning fabrics, paint, building materials, as well as, in pharmaceutical and cosmetic products (Gelis et al., 2003; Aitken et al., 2006).

The ecotoxicological assessment of CeO_2 and TiO_2 nanoparticles in *C. elegans* was conducted by investigating stress-response gene expressions, growth, fertility, and survival as toxic endpoints. To investigate relationship between the sizes of nanoparticles and toxicity, nanoparticles were exposed to *C. elegans* at a constant concentration (1 mg/L) with different sizes (15, 45 nm for CeO_2 and 7, 20 nm for TiO_2). Moreover, to test the ecotoxicological relevance of CeO_2 and TiO_2 nanoparticles-induced gene expression, integration of gene expression with the organism/population level endpoints was attempted using *C. elegans* functional genomics tool, RNAi.

2. Materials and methods

2.1. CeO_2 and TiO_2 nanoparticles

CeO_2 nanoparticles were synthesized by the supercritical synthesis method, as described previously (Park et al., 2008). The schematic diagram for the synthesis apparatus is shown in Supplementary Fig. 1. TiO_2 nanoparticles were purchased form Sigma–Aldrich Chemical (St. Louis, MO, USA). To measure the surface area of the nanoparticles, the Branauer, Emmett and Teller (BET) method was used, employing a volumetric adsorption apparatus, BELSORP-mini II (BEL Japan Inc., Osaka, Japan). To investigate the size and shape of the nanoparticles, 20 μL of a particle suspension from the test medium was dried on a 400 mesh carbon-coated copper grid and imaged using a JEM 1010 TEM (JEOL, Tokyo, Japan) at 40–100 kV (Fig. 1).

2.2. Organisms and sample preparation

The wildtype *C. elegans* Bristol strain N2 was used. *C. elegans* was maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50, at 20 °C, using the standard method previously described by Brenner (1974). Young adults (3 days old) from an age-synchronized culture were used in all the experiments. Worms were incubated at 20 °C for 24 h without a food source, and were then subjected to the analysis.

Nematodes were exposed to CeO_2 and TiO_2 nanoparticles prepared in a K-medium (0.032 M KCl, 0.051 M NaCl, Williams and Dusenbery, 1990) for assessment of 4 types of endpoints (stress-response gene expression, growth, fertility, and mortality). Test solutions of CeO_2 and TiO_2 nanoparticles were prepared in the K-media and dispersed for 20 min using a sonicator (Branson Inc., Danbury, CT, USA), to prevent aggregation. During the testing periods, the suspension of nanoparticles was stable and uniform throughout the K-media. The worms were treated with two different sizes of both nanoparticles for 24 h for ecotoxicological studies. Three replicates for each treatment and a control were conducted for all the test types.

2.3. Semi-quantitative reverse transcription-polymerase chain reaction

Following the 24 h incubation with exposure to two sizes of 1 mL of CeO_2 and TiO_2 nanoparticles, nematodes were harvested for the analysis of stress-response gene expression using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), as described previously (Roh et al., 2006). Briefly, the two-step RT-PCR method was used with RT Premix (Bioneer, Seoul, Korea) and PCR Premix kits (Bioneer), using a PTC-100 thermal cycler (MJ Research, Lincoln, MA, USA). The primers were designed based on the sequences retrieved from the *C. elegans* database (www.wormbase.org; supplementary Table 1).

2.4. Growth, fertility and survival

Following the 24 h incubation with exposure to two sizes of 1 mL of CeO_2 and TiO_2 nanoparticles, growth, fertility, and survival were assessed, as described previously (Roh et al., 2006). Growth was assessed by measuring the length of the worms that had been killed by the heat through microscopy, with a scaled lens in each metal concentration and a control. Fertility was assessed by counting the eggs of each worm through the microscopic inspection of the transparent *C. elegans* body in each sample. Survival was assessed by counting the number of live and dead worms, which were determined through visual inspection by probing the worms with a platinum wire under a dissecting microscope.

2.5. RNA interference by feeding

The RNAi feeding was performed, as previously described by Kamath et al. (2001). Bacteria containing plasmid constructs engineered to make double-stranded RNA were grown overnight in LB ampicillin (100 μg) at 37 °C and then seeded dropwise onto NGM agar plates. The RNAi bacteria were induced for 48 h at room temperature for dsRNA expression. We added about 10, L3–L4-stage animals, onto the plate and incubated at 20 °C. After 36–40 h, worms were transferred to another large-scale plate seeded with the same RNAi bacteria and then the worms were allowed to grow to adults and to lay eggs. When worms grown in full, adults were removed by the age-synchronized culture method. Each egg was seeded to new, freshly prepared RNAi feeding plates. To evaluate the efficiency of the dsRNA feeding, more than 1000 worms were assessed by semi-quantitative PCR.

2.6. Data analysis

Statistical differences between the control and treated cells were examined with the aid of one-way ANOVA test followed by Tukey's test, using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA).

3. Results and discussions

The special properties of nanoparticles upon dispersion in aqueous media, such as, tendency to form large aggregates and poor solubility, hamper ecotoxicological studies. It has been recognized that results obtained with unstable nanoparticles dispersion cannot be fully representative of a monodispersed, non-aggregated nanoparticles system (Borm et al., 2006; Van Hoecke et al., 2008). As it is difficult to prepare stable, monodispersed, aqueous nanoparticles suspensions, special dispersion processes are needed, prior to and during toxicity assessments. In this study, sonication process was conducted for uniform dispersion, during the testing periods,

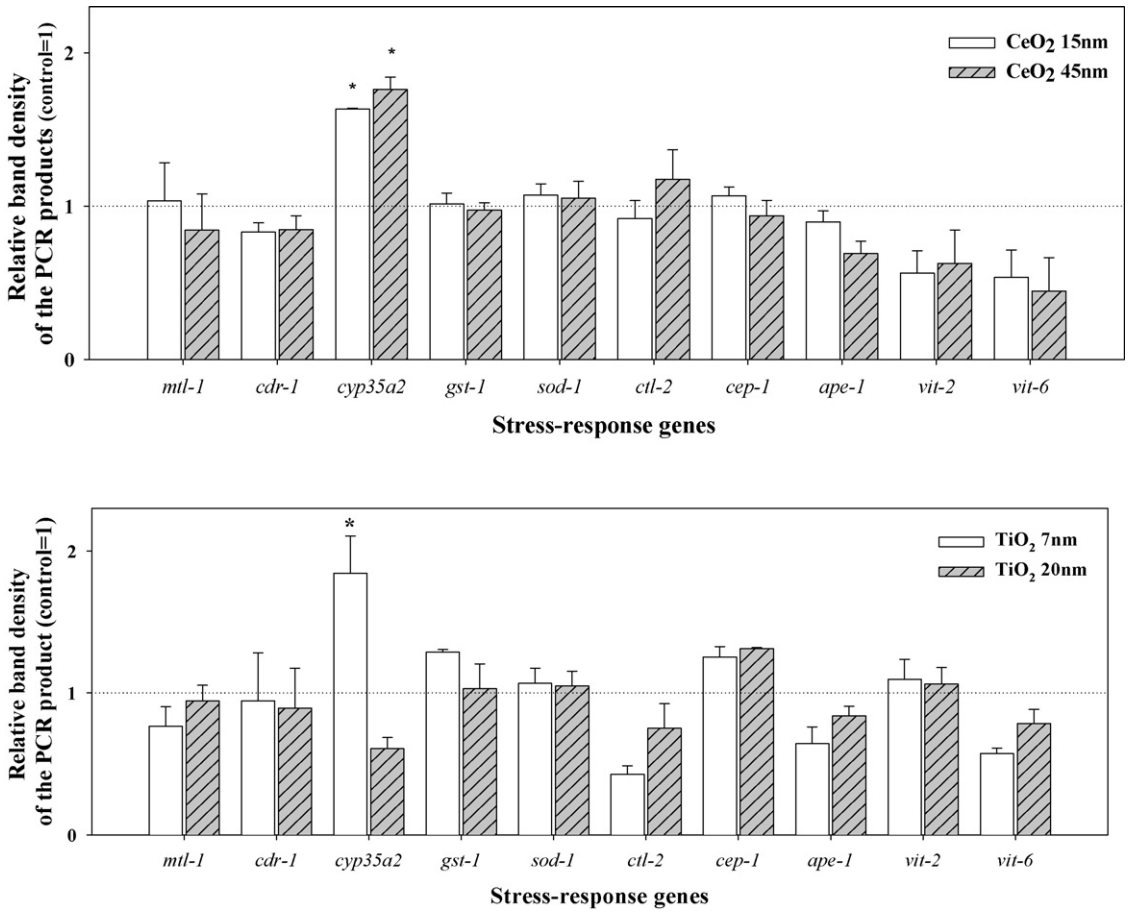

Fig. 2. Stress-response gene expression profiling in the young adult of *C. elegans* exposed to CeO_2 and TiO_2 nanoparticles for 24 h. Densitometric values normalized using actin mRNA are presented as relative units compared to control (control = 1; number = 3; mean ± standard error of the mean; $^*p < 0.05$).

the suspension of nanoparticles was stable and uniform throughout the test media (data not shown). It was reported that aggregates form more easily when particle concentration is increased (Crane et al., 2008). The concentration we used in this study was 1 mg/L, to prevent formation of aggregates and/or the precipitation of particle. Particle precipitation was visible in the test media at the higher concentrations (data not shown). However, there are ecologically based criticisms of artificial dispersion and stabilization methods. Nanoparticles discharged to the environment are not likely to occur in the presence of milligram quantities of dispersant chemicals or sonication. It could be argued that the non-dispersed material is likely to be more relevant to what will happen in the real environment, and toxicity test design should reflect that reality (Crane et al., 2008).

Recently, gene expression as an environmental stress-response has been increasingly used in ecotoxicology, as it offers high sensitivity and mechanistic values to diagnose environmental contamination (Snell et al., 2003; Lee and Choi, 2006; Roh et al., 2006, 2007; Poynton et al., 2007). In this study, in order to investigate whether nanoparticles lead to alteration of gene expressions in *C. elegans*, stress-response gene expression profiling analysis was conducted on the selected genes, such as, metal response proteins (*mtl-1*, *cdr-1*), xenobiotic metabolism enzymes (*cyp35a2*, *gst-1*), antioxidant enzymes (*sod-1*, *ctl-2*), tumor suppressor and apoptosis proteins (*cep-1*, *ape-1*) and yolk proteins (*vit-2*, *vit-6*) (Fig. 2). Although some minor fluctuations were observed, the expression of most of the tested genes was not significantly changed, either by CeO_2 or by TiO_2 nanoparticles exposure. An increase in the expression of *cyp35a2* gene was the most pronounced by both 15 and 45 nm of CeO_2 and by 7 nm of TiO_2 nanoparticles exposure. Cytochrome P450 is a well known phase I enzyme involved in xenobiotic biotransformation in vertebrate systems (Usmani et al., 2004). In *C. elegans*, *cyp35a2* was reported to be involved in fat storage pathway (Ashrafi et al., 2003; Menzel et al., 2007); however, the exact biological function of *cyp35a2* is still unknown. Therefore, our data can only suggest that *cyp35a2* may be involved in CeO_2 and TiO_2 metabolism and/or toxicity in *C. elegans*, without providing any evidence on the biological role of this gene in nanoparticles toxicity.

Even though gene expression as environmental stress-response provides high sensitivity and thus can act as early warning signal, relating such a molecular level response to ecological effects represents a substantial challenge. A link or correlation between a validated toxicity endpoint (e.g., growth fertility and survival) and upstream-induced gene expression is interesting, particularly for ecotoxicological purposes. There have been few direct experimental demonstrations for the relations between molecular/biochemical effects and the consequences at higher levels of biological organization (Choi et al., 2002; Lee and Choi, 2006, 2007; Kim et al., 2008). Recent ecotoxicity study of TiO_2 nanoparticles on terrestrial isopods, *Porcellio scaber,* using biochemical and organism level endpoints showed that activities of antioxidant enzymes, such as catalase and glutathione-*S* transferase, were affected, but higher level endpoints, including weight change and survival, were not affected (Jemec et al., 2008). To identify whether increased *cyp35a2* gene expression by nanoparticles exposure is a compensatory homeostatic response or not, the organism level response was subsequently investigated, in the presence of nanoparticles.

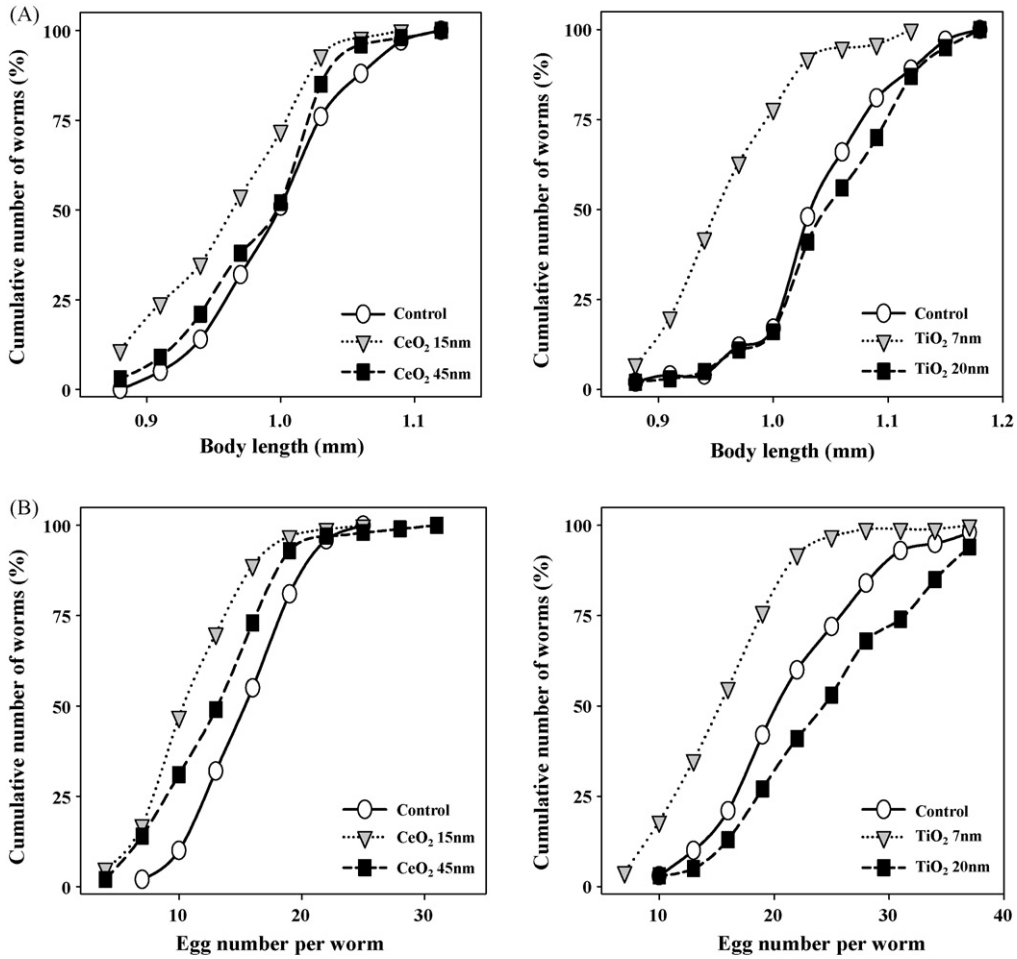

Fig. 3. Growth (A) and fertility indicators (B) examined in the young adults of *C. elegans* exposed to CeO_2 and TiO_2 nanoparticles for 24 h. Results are expressed as cumulative number of worms for each response. Growth was assessed by measuring the length of the worms and fertility was assessed by counting the number of eggs per worms. Fertility was investigated by counting the number of offspring per individual.

In this study, ecotoxicological parameters, such as, growth/fertility (Fig. 3) and survival (Table 1), were investigated in the CeO_2 and TiO_2 exposed *C. elegans*. CeO_2 nanoparticles exposure did not provoke significant effect on growth, whereas, it seemed to affect the worm's fertility, as a decrease of 28 and 11% in the number of egg per worms was observed, compared to control by 15 and 45 nm of CeO_2 exposure, respectively. Seven nanometers of TiO_2 nanoparticles exposure led to decrease in growth and fertility potentials about 9 and 21%, compared to the control, respectively. An decrease in the survival rate was also observed by 15 nm of CeO_2 and 7 nm of TiO_2 nanoparticles exposure (about 20 and 30% compared to control, respectively).

Physiological disturbances, such as, reduced fertility and survival parameters, may be considered as a progression of toxicity in consequence of induced *cyp35a2* gene expression by CeO_2 and TiO_2 nanoparticles, as an increased expression of this gene was concomitantly observed. This explanation, however, is based on the speculation of the responses at the different biological levels (from molecular to organism levels). The biological- and ecophysiological consequences of changes in expression of *cyp35a2* gene, and their roles in the defense against CeO_2 and TiO_2 nanoparticles toxicity are unknown. As functional genomic study can provide clearer experimental evidence on causal relationships between gene expressions and altered physiological indicators, we knocked down *cyp35a2* gene expression, using RNAi (Fig. 4) and compared the response of *cyp35a2* gene knocked down worms with that of wildtype worms in terms of growth, fertility, and survival (Fig. 5). In this regard, *C. elegans* is particularly an attractive animal model. *C. elegans* functional genomic tools, such as individual gene knock-downs via RNAi, can offer the possibility to assess the physiological meaning of up- or down-regulated gene expressions by chemical exposure and can provide indicators of the toxic modes of action from the level of a single gene to that of the whole organism (Menzel et al., 2007).

In wildtype experiment set, significant decrease in the number of eggs per worm was observed in the CeO_2 and TiO_2 nanoparticles exposed worms compared to control, whereas, the response pattern of worms, which had *cyp35a2* knock-down gene, was dif-

Table 1
Survival rates examined in the wildtype *C. elegans* exposed to CeO_2 and TiO_2 nanoparticles for 24 h (control = 100; number = 5; mean ± standard error of mean, $p < 0.05$).

Control	CeO_2		TiO_2	
(%)	15 nm	45 nm	7 nm	20 nm
100 ± 0	80 ± 14*	100 ± 0	70 ± 1.4*	90 ± 0

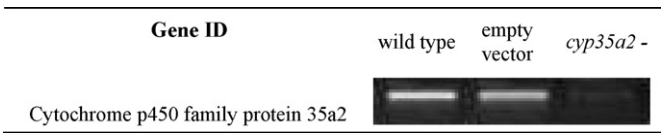

Fig. 4. Knock-down of *cyp35a2* by feeding RNAi.

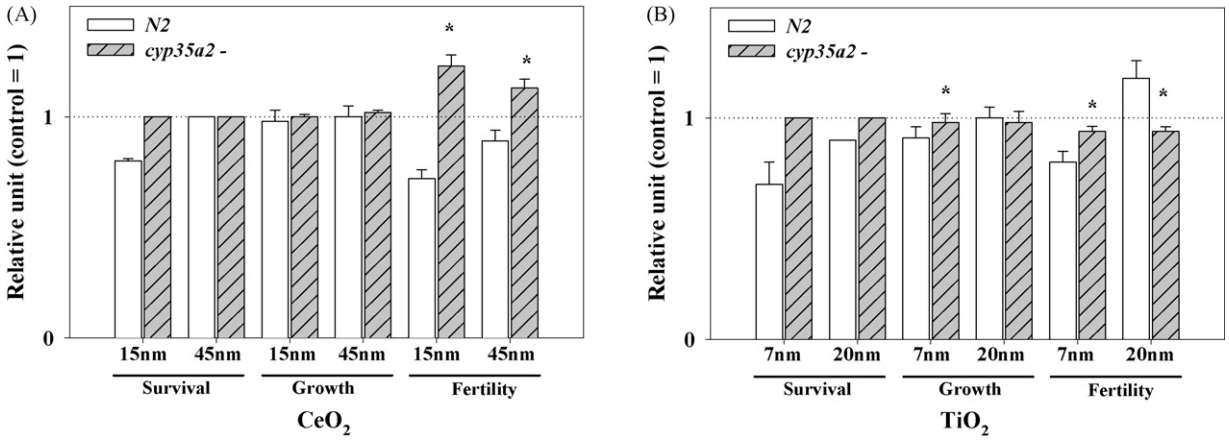

Fig. 5. Survival, growth and fertility indicators examined in the wildtype and cyp35a2 RNAi *C. elegans* exposed to CeO_2 (A) and TiO_2 (B) nanoparticles for 24 h. Results are presented as relative units compared to control (control = 1; number = 3; mean ± standard error of the mean; *significantly different from wildtype).

ferent from that of wildtype worms. Increase in fertility potential was observed by CeO_2 nanoparticles exposures in *cyp35a2* RNAi worms. The differences of fertility potential between wildtype and *cyp35a2* RNAi worms were 50% in 15 nm of CeO_2 and 20% in 45 nm of CeO_2 exposure. This result suggests that *cyp35a2* gene may have negative effect on the worm's fertility. Reduced fertility capacity may be explained as a part of defense and/or compensatory mechanism to metabolize the toxicity induced by CeO_2 nanoparticles. Survival rate was decreased by CeO_2 and TiO_2 nanoparticles in wildtype worms, whereas, it was not affected in RNAi worms; which suggest that *cyp35a2* gene may be involved to some extent in nanoparticles-induced survival (Fig. 5). If more genes had been tested with more biochemical/physiological processes, involvement of observed CeO_2 and TiO_2 nanoparticles-induced altered gene expressions in physiological pathways could probably be better evaluated and explained. Moreover, the responses of a broad range of gene knock-down sets to various physiological process (i.e. development, behavior, etc.) may be needed to elucidate CeO_2 and TiO_2 toxicity.

Nanoparticles are often expected that the smaller the size, the stronger is the exerted toxicity (Oberdorster et al., 2005). Particle size and surface area are considered important factors for determining the toxicity of nanoparticles, because, as the particle size decreases its surface area increases (Eom and Choi, 2009). In the present study we tested the hypothesis that ecotoxicity of nanoparticles is related to their surface area by testing assessing the toxicity of nanoparticles of the same material of different sizes. We exposed the worms to the constant concentration (1 mg/L) with different sizes of nanoparticles (15, 45 nm for CeO_2 and 7, 20 nm for TiO_2), expecting to identify a relationship between sizes of nanoparticles and toxicity. In this study, smaller sized nanoparticles (7 nm of TiO_2 and 15 nm of CeO_2) seems to be more toxic than larger sized ones (20 nm of TiO_2 and 45 nm of CeO_2) on the observed toxicity. Nevertheless, to confirm the size-dependent effect in CeO_2 and TiO_2 nanoparticles-induced toxicity, further experiments on various sizes of nanoparticles with longer exposure periods may be needed, because, it is also believed that parameters other than size (i.e. shape, charge, concentration, etc.) may also influence the toxicity of these nanoparticles. Indeed, most studies could not show clear relationships between toxicity and size of nanoparticles (Hussain et al., 2005; Yin et al., 2005).

4. Conclusions

Overall results suggest that CeO_2 and TiO_2 nanoparticles provoke ecotoxicity on *C. elegans* fertility and survival, which may be related with *cyp35a2* gene expression. Further studies on the mechanism by which CeO_2 and TiO_2 nanoparticles affect *cyp35a2* gene expression, fertility, and survival are warranted to better understand the CeO_2 and TiO_2 nanoparticles-induced ecotoxicity in *C. elegans*, as are studies with the causal relationships between these parameters. The genome wide transcription responses, may also be needed to elucidate toxicity from CeO_2 and TiO_2 nanoparticles in *C. elegans*. The present study focused on the identification of the potential harmful effect of CeO_2 and TiO_2 nanoparticles in *C. elegans* under laboratory conditions. The calibration and validation of the identified toxicity using dose–response assessment and time course studies will be addressed in future studies under environmentally relevant exposure settings.

Conflict of interest statement

We have nothing to declare in conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.etap.2009.12.003.

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