The in vivo underlying mechanism for recovery response formation in nano-titanium dioxide exposed Caenorhabditis elegans after transfer to the normal condition

Yunli Zhao, Qiuli Wu, Meng Tang, Dayong Wang

Abstract

So far, we still know little about mechanism for recovery response of engineered nanomaterials (ENMs). Here we used Caenorhabditis elegans to investigate recovery responses of titanium dioxide nanoparticles (TiO2-NPs) exposed animals and the underlying mechanism. After acute exposure to TiO2-NPs (100 mg/L), endpoints including defecation and permeable state of intestinal barrier of exposed nematodes returned to control levels; however, after prolonged exposure to TiO2-NPs (100 μg/L), endpoints of exposed nematodes could not be recovered to control levels under the normal condition. After prolonged exposure to TiO2-NPs, nematodes exhibited severe deficits in development of intestinal barrier and AVL and DVB neurons controlling defecation; however, after acute exposure to TiO2-NPs, nematodes had normal developmental state of intestinal barrier and AVL and DVB neurons. Our results imply that developmental states of intestinal barrier and AVL and DVB neurons may serve as a pivotal determinant for recovery response in TiO2-NPs exposed nematodes.

From the Clinical Editor: This basic science study investigates the recovery response to TiO2 nanoparticles in a nematode model, and concludes that developmental states of the intestinal barrier and AVL and DVB neurons likely serve as determinants for recovery following TiO2-NP exposure.

Key words: TiO2-NPs; Recovery response; Intestinal barrier; Defecation; Caenorhabditis elegans

Engineered nanomaterials (ENMs) have been considered to confer enormous potential for human exposure and environmental release due to their increasing production and application in a number of industrial manufacturing processes and consumer products.1–3 Titanium dioxide nanoparticles (TiO2-NPs), one of the important products of nanotechnology, are widely used in cosmetics, pharmaceutical, paint, and paper industries, and approximately 5 million tons of pigmented TiO2-NPs are used annually worldwide.4 Previous studies have revealed the multiple adverse effects of TiO2-NPs on animals and human cell lines, such as induction of histopathological changes of organs, pulmonary toxicity, kidney injury, and neuronal toxicity.5–12 Especially, it was suggested that long-term or chronic exposure may be necessary for severe toxicity formation from TiO2-NPs on human and environmental organisms.13,14

Recently, it was further observed that the adverse effects of some ENMs including TiO2-NPs could be recovered in animals under the normal conditions.15–18 For example, there was a return of intravenously administered TiO2-NPs to control level in the lung and kidney of rats.17 Some endpoints returned to control levels by day 21 in TiO2-NPs exposed rats after short-term inhalation.18 Nevertheless, we know little about the mechanism explaining the formation of recovery response in ENMs exposed animals.

Nematode Caenorhabditis elegans, one of the most thoroughly studied model animals, offers a system best suited for asking in vivo toxicological questions with relevance at the organism level.19,20 Recently, C. elegans has been used for toxicological study of a series of metal-NPs or metal oxide-NPs.21–28 So far, C. elegans has been widely used in the study of environmental safety evaluation, toxicology, and translocation and transfer of ENMs.29 In C. elegans, acute exposure to TiO2-NPs at high concentrations caused increase in mortality, reduction in growth, and decrease in reproductive capacity.30,31

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*Corresponding author: Medical School of Southeast University, Nanjing 210009, China.
E-mail address: dayongw@seu.edu.cn (D. Wang).

1 They contributed equally to this work.

It was further found that the prolonged or chronic assay system can be used to assess the adverse effects of TiO$_2$-NPs at predicted environmental relevant concentrations on C. elegans. However, we still do not know whether endpoints of TiO$_2$-NPs exposed nematodes can return to control levels under the normal condition and the underlying mechanism. Thus, in the current study, we investigated the underlying mechanism for possible recovery responses of TiO$_2$-NPs exposed animals under the normal condition using the C. elegans in vivo assay system. Our data demonstrate here the important contributions of intestinal barrier and defecation to recovery response formation of TiO$_2$-NPs exposed animals after transfer to the normal condition.

Results

Recovery response of development in TiO$_2$-NPs exposed nematodes after transfer to the normal condition

The nanosized TiO$_2$-NPs powder (core size, 10 nm) was obtained from commercial source. Structure of TiO$_2$-NPs is anatase (Figure 1, A). Surface area of TiO$_2$-NPs was 160 ± 12 m$^2$/g (Figure 1, A). Zeta potential of TiO$_2$-NPs (100 mg/L) was $-24.3 \pm 1.5$ mV (Figure 1, A). Hydrodynamic mean diameter of TiO$_2$-NPs (10 mg/L) was 97 ± 21 nm (Figure 1, A), which reflected the particle aggregation of TiO$_2$-NPs as also suggested by our previous study. To examine the possible effects of particle concentration on stability of TiO$_2$-NPs, we further investigated the zeta potential and the hydrodynamic mean diameter of different concentrations (100 mg/L and 100 μg/L) of TiO$_2$-NPs. Our data indicated that, under our experimental conditions, 100 μg/L of TiO$_2$-NPs had the similar zeta potential ($-23.5 \pm 2.1$ mV) and hydrodynamic mean diameter (90 ± 34 nm) to those of 100 mg/L of TiO$_2$-NPs.

Our previous studies demonstrated that acute exposure to 50 mg/L of TiO$_2$-NPs (10 nm) suppressed development, and acute exposure to 10-50 mg/L of TiO$_2$-NPs (10 nm) decreased locomotion behavior and increased reactive oxygen species (ROS) production. Prolonged exposure to 1-10 μg/L of TiO$_2$-NPs (10 nm) inhibited development and locomotion behavior, and increased intestinal autofluorescence and ROS production. In this study, we selected the concentrations of 10-100 μg/L for acute exposure to TiO$_2$-NPs (10 nm) and the concentrations of 1-100 μg/L for prolonged exposure to TiO$_2$-NPs (10 nm). TiO$_2$-NPs were given to C. elegans orally by mixing the particles into the food. Prolonged exposure included treatment of L1 larvae to adult, 4 days after hatching, while acute exposure included 24 h treatment of young adult.

Acute exposure to TiO$_2$-NPs at concentrations of 50-100 mg/L significantly ($p < 0.01$) suppressed body length of nematodes (Figure S1). After transfer to the normal condition for 24 h, body length of 50 mg/L of TiO$_2$-NPs exposed nematodes returned to control level; however, body length of 100 mg/L of TiO$_2$-NPs exposed nematodes was only moderately recovered (Figure S1). After transfer to the normal condition for 48 h, body length of 100 mg/L of TiO$_2$-NPs exposed nematodes also returned to control level (Figure S1). Prolonged exposure to TiO$_2$-NPs at concentrations of 10-100 μg/L significantly ($p < 0.01$) decreased body length of nematodes (Figure S1). In contrast, after transfer to the normal condition for 24 h, body lengths of both 10 μg/L of TiO$_2$-NPs exposed nematodes and 100 μg/L of TiO$_2$-NPs exposed nematodes did not return to control levels (Figure S1). After transfer to the normal condition for 48 h, only body length of 10 μg/L of TiO$_2$-NPs exposed nematodes was completely recovered to control level (Figure S1). These data imply the difference of recovery response in TiO$_2$-NPs exposed nematodes with different exposure periods after transfer to the normal condition.

Recovery response of functions for the secondary targeted organs in TiO$_2$-NPs exposed nematodes after transfer to the normal condition

In C. elegans, neuron is one of the important secondary targeted organs for ENMs, and neurons function in the control of behaviors in animals. We first investigated the recovery response of locomotion behavior for TiO$_2$-NPs exposed nematodes after transfer to normal condition. Acute exposure to 10-100 mg/L of TiO$_2$-NPs significantly ($p < 0.01$) reduced both head thrashes and body bends of nematodes (Figure 1, B). After transfer to the normal condition for 24 h, only head thrashes and body bends of 10-50 mg/L of TiO$_2$-NPs exposed nematodes returned to control levels (Figure 1, B). After transfer to the normal condition for 48 h, head thrashes and body bends of 100 mg/L of TiO$_2$-NPs exposed nematodes returned to control levels (Figure 1, B). Prolonged exposure to 1-100 μg/L of TiO$_2$-NPs significantly ($p < 0.01$) decreased both head thrashes and body bends in nematodes (Figure 1, B). In contrast, after transfer to the normal condition for 24 h, head thrashes and body bends of 1-100 μg/L of TiO$_2$-NPs exposed nematodes were all not recovered to control levels (Figure 1, B). After transfer to the normal condition for 48 h, only head thrashes and body bends of 1 μg/L of TiO$_2$-NPs exposed nematodes returned to control levels (Figure 1, B).

In C. elegans, pharyngeal pumping is controlled by several neurons such as motor neurons MC and M3. We further examined the recovery response of pharyngeal pumping for TiO$_2$-NPs exposed nematodes after transfer to normal condition. Acute exposure to 50-100 mg/L of TiO$_2$-NPs significantly ($p < 0.01$) suppressed pumping rates of nematodes (Figure S2). Pumping rate of 50 mg/L of TiO$_2$-NPs exposed nematodes could be recovered to control level after transfer to the normal condition for 24 h, and pumping rate of 100 mg/L of TiO$_2$-NPs exposed nematodes could return to control level after transfer to the normal condition for 48 h (Figure S2). Prolonged exposure to 10-100 μg/L of TiO$_2$-NPs significantly ($p < 0.01$) suppressed pumping rates of nematodes (Figure S2). In contrast, after transfer to the normal condition for 24 h, pumping rates of 10-100 μg/L of TiO$_2$-NPs exposed nematodes could not be recovered to control levels (Figure S2). After transfer to the normal condition for 48 h, only the pumping rate of 10 μg/L of TiO$_2$-NPs exposed nematodes could return to control level (Figure S2). Therefore, different assay systems may cause different recovery response for altered functions of the secondary targeted organs in TiO$_2$-NPs exposed nematodes after transfer to the normal condition.
Recovery response of the primary targeted organs in TiO2-NPs exposed nematodes under the normal condition

Intestine is the key primary targeted organ for ENMs in C. elegans. Intestinal autofluorescence is caused by lysosomal deposits of lipofuscin, which can accumulate over time in aging nematodes. We next investigated the recovery response of intestinal autofluorescence for TiO2-NPs exposed nematodes after transfer to normal condition. Acute exposure to 100 mg/L of TiO2-NPs significantly enhanced intestinal autofluorescence of nematodes, and intestinal autofluorescence of 100 mg/L of TiO2-NPs exposed nematodes could be recovered to control levels after transfer to the normal condition for 24 h and 48 h (Figure 2, A and B). Prolonged exposure to 100 μg/L of TiO2-NPs also significantly increased intestinal autofluorescence of nematodes (Figure 2, C and D). In contrast, after transfer to the normal condition for 24 h and 48 h, the intestinal autofluorescence of 100 μg/L of TiO2-NPs exposed nematodes could not return to control levels.

The connection between nanotoxicity and excessive oxidative stress has been widely accepted. That is, oxidative stress has been considered as one of the important reasons to induce the toxicity of TiO2-NPs in animals. We further examined the recovery response of intestinal ROS production for TiO2-NPs exposed nematodes after transfer to normal condition. Acute exposure to 100 mg/L of TiO2-NPs significantly increased intestinal ROS production of nematodes (Figure 2, E and F). Intestinal ROS production of 100 mg/L of TiO2-NPs exposed nematodes could not be recovered to control levels after transfer to the normal condition for 24 h, and intestinal ROS production of 100 mg/L of TiO2-NPs exposed nematodes returned to control levels until after transfer to the normal condition for 48 h (Figure 2, E and F). Prolonged exposure to 100 μg/L of TiO2-NPs also significantly enhanced intestinal ROS production.
of nematodes (Figure 2, G and H). In contrast, after transfer to the normal condition for 24 h and 48 h, intestinal ROS production of 100 μg/L of TiO₂-NPs exposed nematodes were all not recovered to control levels (Figure 2, G and H). Therefore, different exposures may result in different recovery response for both the primary targeted and the secondary
targeted organs in TiO\textsubscript{2}-NPs exposed nematodes after transfer to the normal condition.

**Contribution of defecation control to recovery response formation in TiO\textsubscript{2}-NPs exposed nematodes under the normal condition**

To examine the possible mechanisms explaining the recovery response formation for TiO\textsubscript{2}-NPs exposed nematodes after transfer to the normal condition, we first investigated whether there are differences of defecation behavior in TiO\textsubscript{2}-NPs exposed nematodes using different assay systems. Acute exposure to 100 mg/L of TiO\textsubscript{2}-NPs significantly (\(p < 0.01\)) increased mean defecation cycle time of nematodes (Figure 3, A). After transfer to the normal condition for 24 h and 48 h, mean defecation cycle time of 100 mg/L of TiO\textsubscript{2}-NPs exposed nematodes returned to control levels (Figure 3, A). Prolonged exposure to 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs also significantly (\(p < 0.01\)) increased mean defecation cycle time of nematodes (Figure 3, A). In contrast, after transfer to the normal condition for 24 h and 48 h, mean defecation cycle time of 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs could not be recovered to control levels (Figure 3, A).

In *C. elegans*, defecation behavior is controlled by neurons such as AVL and DVB.\textsuperscript{36} Again, we explored a transgenic strain of *oxIs12* labeling AVL and DVB neurons in GABAergic nervous system\textsuperscript{37} to investigate the possible alterations of AVL and DVB neurons during recovery response in TiO\textsubscript{2}-NPs exposed nematodes. Interestingly, we found that acute exposure to 100 mg/L of TiO\textsubscript{2}-NPs did not obviously affect development of AVL neuron in head region and DVB neuron in tail region (Figure 3, B and C). During the recovery response, the development of both AVL neuron and DVB neuron was also normal compared with control (Figure 3, B and C). However, prolonged exposure to 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs influenced the development of AVL and DVB neurons, because relative sizes of fluorescent puncta for AVL and DVB neurons were significantly (\(p < 0.01\)) reduced compared with control (Figure 3, B and C). Moreover, under the normal condition, the deficit in development of AVL and DVB neurons in 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs exposed nematodes could not be obviously recovered (Figure 3, B and C). Therefore, the defecation state may serve as an important contributor for recovery response formation in TiO\textsubscript{2}-NPs exposed nematodes after transfer to the normal condition.

**Contribution of intestinal barrier to recovery response formation in TiO\textsubscript{2}-NPs exposed nematodes after transfer to the normal condition**

Considering the important role of the primary targeted organs for translocation and distribution of ENMs, we further examined the permeability of the primary targeted organs for TiO\textsubscript{2}-NPs exposed nematodes under the normal condition. We first explored the lipophilic fluorescent dye, Nile Red to stain TiO\textsubscript{2}-NPs exposed nematodes before and after transfer to the normal condition. Acute exposure to 100 mg/L of TiO\textsubscript{2}-NPs significantly (\(p < 0.01\)) enhanced the relative fluorescent intensity of Nile Red in intestine compared with control (Figure 4, A and B). After transfer to the normal condition for 24 h, the relative fluorescent intensity of Nile Red in intestine of 100 mg/L of TiO\textsubscript{2}-NPs exposed nematodes did not return to control level (Figure 4, A and B). The relative fluorescent intensity of Nile Red in intestine of 100 mg/L of TiO\textsubscript{2}-NPs exposed nematodes was recovered to control level until transfer to the normal condition for 48 h (Figure 4, A and B). Prolonged exposure to 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs also significantly (\(p < 0.01\)) increased the relative fluorescent intensity of Nile Red in intestine (Figure 4, A and B). However, even after transfer to the normal condition for 48 h, the relative fluorescent intensity of Nile Red in intestine of 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs exposed nematodes could not be recovered to control level (Figure 4, A and B). Because Nile Red can be used to label fat storage in nematodes, we also investigated triglyceride content in TiO\textsubscript{2}-NPs exposed nematodes. Exposure to 100 mg/L or 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs did not significantly influence the triglyceride content of nematodes (Figure 4, C). Under the normal condition, the triglyceride contents in 100 mg/L or 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs exposed nematodes were also not obviously altered compared with control (Figure 4, C). Based on these data, we obtain the conclusion that acute exposure to 100 mg/L of TiO\textsubscript{2}-NPs or prolonged exposure to 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs may result in a hyper-permeable intestinal barrier rather than increased lipid accumulation. Under the normal condition, the permeable state of intestinal barrier for 100 mg/L of TiO\textsubscript{2}-NPs exposed nematodes can return to the normal state; however, the permeable state of intestinal barrier for 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs exposed nematodes may not be able to be recovered.

To reveal the corresponding mechanism explaining the altered permeable state of intestinal barrier, we further examined the structural changes of intestinal barrier using a transmission electron microscope (TEM). We found that acute exposure to 100 mg/L of TiO\textsubscript{2}-NPs did not obviously alter ultrastructure of intestine in nematodes compared with control, although some TiO\textsubscript{2}-NPs already gradually entered into intestinal cells (Figure 5, A). In contrast, ultrastructure of intestine in 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs exposed nematodes was severely disrupted, and some microvilli were lost, which was accompanied with more deposition of TiO\textsubscript{2}-NPs into intestinal cells of nematodes (Figure 5, A). Moreover, the disrupted ultrastructure of microvilli in 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs exposed nematodes was not noticeably recovered under the normal condition (Figure 5, A). In 100 \(\mu\)g/L of TiO\textsubscript{2}-NPs exposed nematodes before and after transfer to the normal condition, some TiO\textsubscript{2}-NPs were observed to be located into the mitochondria (Figure 5, A). Therefore, the different permeable state of intestinal barrier during the recovery response may be closely associated with the altered intestinal structure in nematodes.
Figure 3. Alterations of defecation behavior and neurons controlling defecation in TiO$_2$-NPs exposed nematodes after transfer to the normal condition. (A) Alterations of defecation in TiO$_2$-NPs exposed nematodes after transfer to the normal condition. (B) Alterations of AVL neuron in TiO$_2$-NPs exposed nematodes after transfer to the normal condition. (C) Alterations of DVB neuron in TiO$_2$-NPs exposed nematodes after transfer to the normal condition. Acute exposure concentration for TiO$_2$-NPs was 100 mg/L, and prolonged exposure concentration for TiO$_2$-NPs was 100 μg/L. Asterisks indicate the position of AVL or DVB neuron. Bars represent means ± S.E.M. **p < 0.01.
accumulation in body than acute exposure to 100 mg/L of TiO₂-NPs. After transfer to the normal condition for 48 h, only a small amount of Ti was detected in TiO₂-NPs (100 mg/L) acutely exposed nematodes; however, still a large amount of Ti could be detected in nematodes undergoing prolonged exposure to TiO₂-NPs (100 μg/L). These data suggested that TiO₂-NPs can be ingested by nematodes after acute or prolonged exposure, and the normal condition cannot successfully help nematodes undergoing prolonged exposure to TiO₂-NPs (100 μg/L) to excrete the ingested TiO₂-NPs from the body during the recovery response duration.

Discussion

In the present study, we first confirm the existence of recovery response in TiO₂-NPs exposed nematodes after transfer to the normal condition. Moreover, we found that nematodes after acute exposure to TiO₂-NPs more tend to perform recovery response under the normal condition compared with nematodes undergoing prolonged exposure to TiO₂-NPs. The data presented here further support the notion that L1-larvae may be more sensitive to toxicants than L4-larvae or adult nematodes. The predicted environmental relevant concentration of TiO₂-NPs in...
Figure 5. Ultrastructural changes of intestine and uptake of Ti in TiO$_2$-NPs exposed nematodes after transfer to the normal condition. (A) Ultrastructure of intestine in TiO$_2$-NPs exposed nematodes after transfer to the normal condition. Asterisks indicate the position without microvilli. Arrowheads indicate the location of TiO$_2$-NPs. mt, mitochondria. (B) Uptake of Ti in TiO$_2$-NPs exposed nematodes after transfer to the normal condition. The Ti content was expressed as concentrations of titanium element, ng Ti mg$^{-1}$ total protein. Bars represent means ± S.E.M. Acute exposure concentration for TiO$_2$-NPs was 100 mg/L, and prolonged exposure concentration for TiO$_2$-NPs was 100 μg/L.
sewage treatment effluents is 4 μg/L. Here we show that the altered body length and pharyngeal pumping rate of nematodes undergoing prolonged exposure to 1-10 μg/L of TiO2-NPs could return to control levels under the normal condition. Locomotion behavior and intestinal ROS production of nematodes undergoing prolonged exposure to 10 μg/L of TiO2-NPs were also recovered to control levels under the normal condition (data not shown). Together with the recent observation that the gum TiO2-NPs are non-toxic for gastrointestinal cells even at a concentration of 200 mg/L after acute exposure, we hypothesize that at least short-term exposure to environmental concentrations of TiO2-NPs should be relatively safe to human and environmental organisms.

Nevertheless, we found that animals undergoing prolonged exposure to high concentrations of TiO2-NPs will be difficult to exhibit the recovery response under the normal condition. One of the possible mechanisms explaining the recovery response formation raised here is that nematodes undergoing prolonged exposure to high concentrations of TiO2-NPs had the disrupted structure for neurons controlling defecation behavior, which might in turn cause nematodes to use a prolonged defecation cycle time for ENMs excretion through the intestine. In contrast, although nematodes undergoing acute exposure to TiO2-NPs also had a prolonged defecation cycle time, their structure of neurons controlling defecation behavior was unaffected compared with control. These may be partially due to the fact that the nervous system of nematodes at adult stage is already completely established, whereas the L1-larvae are still undergoing the establishment of entire nervous system. Previous study demonstrated that design of ENMs by enhancing the excretion should be a viable strategy to reduce their toxicity.

TiO2-NPs can be translocated into several targeted organs of body in animals. Another possible mechanism explaining the recovery response formation raised here is that the nematodes undergoing prolonged exposure to high concentrations of TiO2-NPs exhibited the deficit in intestinal structure with the loss of many microvilli, implying that their intestinal barrier was severely disrupted and such a disruption might be irreversible. In contrast, the nematodes undergoing acute exposure to TiO2-NPs still had the normal intestinal structure, although permeability of the intestinal barrier was also influenced. The still relatively entire intestinal structure may ensure the nematodes acutely exposed to TiO2-NPs can successfully perform the recovery response under the normal condition.

In conclusion, using model animal of C. elegans as the assay system, we provided the evidence to indicate the possibility that, after acute exposure to high concentrations of TiO2-NPs, animals may undergo the recovery response under the normal condition. In contrast, after prolonged exposure to high concentrations of TiO2-NPs, animals may meet difficulties to exhibit the recovery response under the normal condition. Moreover, we raised a hypothesis that both defecation behavior and intestinal barrier contribute greatly to the recovery response formation for TiO2-NPs exposed animals. Especially, structural state of both neurons controlling defecation behavior and intestinal barrier may play a pivotal role in regulating the recovery response formation in TiO2-NPs exposed animals under the normal condition. The findings here will be useful for explaining the recovery response in animals and even human exposed to other ENMs. Furthermore, our data suggest that design for both prevention strategy and new safe ENMs should pay attention to the maintenance or control of both the defecation behavior and the primary biological barrier.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2013.07.004.

References


