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# Chronic $Al_2O_3$-nanoparticle exposure causes neurotoxic effects on locomotion behaviors by inducing severe ROS production and disruption of ROS defense mechanisms in nematode *Caenorhabditis elegans*

Yinxia Li[a,1], Shunhui Yu[b,1], Quili Wu[a,1], Meng Tang[c], Yuepu Pu[c], Dayong Wang[a,*]

[a] *Key Laboratory of Environmental Medicine Engineering in Ministry of Education, Department of Biochemistry and Molecular Biology, Medical School of Southeast University, Nanjing 210009, China*
[b] *College of Chemistry and Environmental Engineering, Chongqing Three Gorges University, Wanzhou 404000, China*
[c] *School of Public Health, Southeast University, Nanjing 210009, China*

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ABSTRACT

To date, knowledge on mechanisms regarding the chronic nanotoxicity is still largely minimal. In the present study, the effect of chronic (10-day) $Al_2O_3$-nanoparticles (NPs) toxicity on locomotion behavior was investigated in the nematode *Caenorhabditis elegans*. Exposure to 0.01–23.1 mg/L of $Al_2O_3$-NPs induced a decrease in locomotion behavior, a severe stress response, and a severe oxidative stress; however, these effects were only detected in nematodes exposed to 23.1 mg/L of bulk $Al_2O_3$. Formation of significant oxidative stress in nematodes exposed to $Al_2O_3$-NPs was due to both the increase in ROS production and the suppression of ROS defense mechanisms. More pronounced increases in ROS, decreases in SOD activity, and decrease in expression of genes encoding Mn-SODs (*sod-2* and *sod-3*) were detected in nematodes exposed to $Al_2O_3$-NPs compared with bulk $Al_2O_3$. Moreover, treatment with antioxidants or SOD-3 overexpression not only suppressed oxidative stress but also prevented adverse effects on locomotion behaviors from $Al_2O_3$-NPs exposure. Thus, chronic exposure to $Al_2O_3$-NPs may have adverse effects on locomotion behaviors by both induction of ROS production and disruption of ROS defense mechanisms. Furthermore, *sod-2* and *sod-3* mutants were more susceptible than the wild-type to chronic $Al_2O_3$-NPs-induced neurotoxicity inhibition.



## 1. Introduction

With the explosive growth of nanotechnology, a large number of novel nano-materials (1–100 nm dimensions) have been generated for industry and biomedical applications. In addition to analysis of acute toxicity from nanoparticles (NPs), toxicity from chronic NPs exposure has recently been investigated [1–3]. Nevertheless, only a few studies have been performed to evaluate the chronic toxicity from NPs exposure [1–4]. In particular, knowledge on mechanisms regarding their chronic toxicity is limited.

Research directed towards invertebrate tests employing long-term and low-concentration exposure with chronic endpoints has been already recommended [4]. *Caenorhabditis elegans*, a typical invertebrate model animal, has been successfully chosen as a useful bioindicator and toxicity test organism because of its short life cycle, ease of generating mass cultures, low cost, and ability to change reproductive speed, life cycle, locomotion behavior, and other properties while exposed to toxicants or stresses [5]. Among endpoints used to evaluate toxicity, locomotion behavior can be easily monitored under the microscope, and such an endpoint can allow us rapidly monitor the toxicity from toxicants or effects of stressors [6]. Recently, toxicity evaluation after chronic metal exposure has been further performed in nematodes at day 10, and metals can affect the behavior of *C. elegans* after chronic exposure [3,7,8].

Among the NPs, $Al_2O_3$-NPs have been used in industry and biomedical applications [9]; however studies on $Al_2O_3$-NPs ecotoxicology are mainly limited to reports on acute exposure [9,10]. More recently, acute toxicity of $Al_2O_3$-NPs has been examined in nematodes with endpoints of lethality, growth, reproduction, stress response, and intestinal autofluorescence [11,12]. Aluminum (Al) is a vital etiopathogenic agent responsible for the incidence of neurodegenerative diseases [13]. In the present study, the chronic toxicity (10-day exposure) of $Al_2O_3$-NPs, compared with that of bulk $Al_2O_3$, was evaluated in adult *C. elegans* using locomotion behaviors as endpoints. Moreover, we examined the regulation of oxidative stress on the formation of severe locomotion behavior decreases after chronic exposure to $Al_2O_3$-NPs. The main aim of this study was to investigate the mechanisms of oxidative stress

* Corresponding author.
*E-mail address:* dayongw@seu.edu.cn (D. Wang).
[1] They contributed to this work equally.

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in regulating the formation of locomotion behavior alterations in nematodes after chronic $Al_2O_3$-NPs exposure.

## 2. Materials and methods

### 2.1. Reagents and preparation of $Al_2O_3$-NPs suspensions

$Al_2O_3$-NPs (purity, >99.99%) were from Hongchen Material Sci. & Tech. Co., Zhoushan, China. Bulk $Al_2O_3$ (purity, >98.5%) was from Baker Chemical Co., Phillipsburg, NJ, U.S.A. Diameters of $Al_2O_3$-NPs and $Al_2O_3$ are 60 nm and 429 nm, respectively. Surface areas of $Al_2O_3$-NPs and $Al_2O_3$ were re-measured by $N_2$ sorption at 77 K using a NOVA 1000e instrument. The surface areas of the $Al_2O_3$-NPs and $Al_2O_3$ were 186 $m^2$/g and 11.4 $m^2$/g, respectively. All the other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Stock suspensions of $Al_2O_3$-NPs and bulk $Al_2O_3$ (0.005–203.9 mg/L) were prepared partially as described [9]. Series of stock suspensions of $Al_2O_3$-NPs and bulk $Al_2O_3$ were dispersed in ultrapure water by probe sonication at 100 W and 40 kHz for 30-min to form homogeneous suspensions. Size of bulk $Al_2O_3$ in stock suspensions was visualized by transmission electron microscopy to ensure that sonication did not break up the material of bulk $Al_2O_3$. To avoid precipitation of $Al(OH)_3$, the pH values of control, $Al_2O_3$-NPs and bulk $Al_2O_3$ were adjusted to 5.6, which had little effects on development of *C. elegans* because they have a wide pH tolerance range from 3 to 12 [14]. The prepared stock suspensions could be sustained at least 24 h. The zeta potentials for $Al_2O_3$-NPs and $Al_2O_3$ were 34.2 and 5.9 mV, respectively, as analyzed with a Nano Zetasizer (Malvern Instrument Ltd., Malvern, UK).

### 2.2. Strain preparation and exposure conditions

Nematodes used were wild-type N2, VC433 [*sod-3*(*gk235*)], RB1072 [*sod-2*(*ok1030*)], KC136 [P*hsp-16.2::gfp*], and *Ex*(P*sod-3-sod-3*) (the genetically modified nematodes were come from strain N2). They were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C [15]. Gravid nematodes were washed off the plates and lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl). Age synchronous populations of young adults were obtained as described [16]. The collected nematodes were washed with a modified K medium (50 mM NaCl, 30 mM KCl, 10 mM NaOAc, pH 5.5) [17]. Acute exposures were performed on young adults in 12-well sterile tissue culture plates for 6 h or 48 h at 20 °C in the presence of food. To perform chronic exposure (10 days), each prepared solution with the volume of 500 μL was added onto the NGM plate. The bacterial (OP50) lawn was ringed with a line of 10 mg/mL palmitic acid in ethanol to keep animals from crawling off the lawn. To prevent production of offspring, 5′-fluoro-2′-deoxyuridine (FUdR), a DNA synthesis inhibitor used to prevent a synchronous population from reproducing without otherwise interfering with post-maturational development [18], was added into the NGM plates at a final concentration of 25 μM [7]. The exposed nematodes were transferred to new NGM plates with new prepared suspension solutions every 12 h with injection needle to ensure the homogeneous state of used suspensions. The tip of the injection needle was wide enough to allow pick up nematodes. The transfer with injection needle did not influence the physiology of nematodes

### 2.3. Locomotion behavior

The endpoints of head thrash and body bend reflect locomotion behaviors of head and body of the nematodes. To assay head thrash, every nematode was transferred into a microtiter well containing 60 μL of modified K medium on the top of agar. After a 1 min recovery period, head thrashes were counted for 1 min. A thrash was defined as a change in the direction of bending at the mid body. To assay body bend, nematodes were picked onto a second plate and body bends were counted for 20 s. A body bend was counted as a change in the direction of the part of nematodes corresponding to posterior bulb of pharynx along the *y* axis, assuming that nematode was moving along the *x* axis. The endpoints of head thrash and body bend were used for both the acute (6 h and 48 h) and chronic (10 days) toxicity assay. Fifty nematodes were examined per replicate. Five replicates were performed.

### 2.4. Analysis of transgenic strain

Stress response reflects the physiological response of animals to a specific stress, and can be characterized by the induced synthesis of a unique set of polypeptides, such as heat-shock proteins [19]. The KC136 strain, carrying a transgene of *gfp* reporter driven by *hsp-16.2* heat-shock promoter (P*hsp-16.2::gfp*), can reveal the presence of toxic stress [19]. To analyze *hsp-16.2* expression patterns, treated KC136 nematodes were allowed to settle for 10 min, and pipetted onto an agar pad on a glass slide, mounted and observed for the fluorescent signals. Observations of fluorescence in the second pharyngeal bulb were recorded and images were taken for documentation of results with Magnafire® software (Olympus, Irving, TX, USA). Fluorescence of more than 50 nematodes was examined per replicate. Five replicates were performed.

### 2.5. Oxidative damage

The collected nematodes from at least ten large NGM plates (9 cm) were washed free of bacteria, pelleted and frozen for carbonylated protein quantification. To avoid the possible influence of food in water, the collected nematodes in water were placed on ice first, and then discard the supernatant with most of food in it. Oxidative damage was analyzed using an Oxyblot assay kit (Millipore, Billerica, MA, USA) to detect the carbonylated proteins. Quantification of carbonylated proteins was obtained by taking the ratio of DNP staining to tubulin staining. Five replicates were performed.

To further quantify whether treatment increases reactive oxygen species (ROS) levels, ROS production was assayed. Nematodes were transferred to M9 buffer containing 1 μM CM-H2DCFDA to pre-incubate for 3 h at 20 °C, and then mounted on agar pads for examination with a laser scanning confocal microscope (Leica, TCS SP2, Bensheim, Germany) at 488 nm of excitation wavelength and 510 nm of emission filter. Relative fluorescence intensities of the second pharyngeal bulbs were semi-quantified. The semiquantified ROS was expressed as relative fluorescent units (RFU). More than 50 nematodes were examined per replicate. Five replicates were performed.

### 2.6. Superoxide dismutase (SOD) activity

SOD activity was measured at 500 nm with a commercially available kit (kit Ransod superoxide dismutase) from Randox Laboratories. To avoid the possible effect of food in solutions, the collected nematodes in solutions were placed on ice first, and then discard the supernatant with most of food in it. Nematodes of at least ten large NGM plates (9 cm) were collected. The data were summary of three trials.

### 2.7. Reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) from nematodes of at least ten large NGM

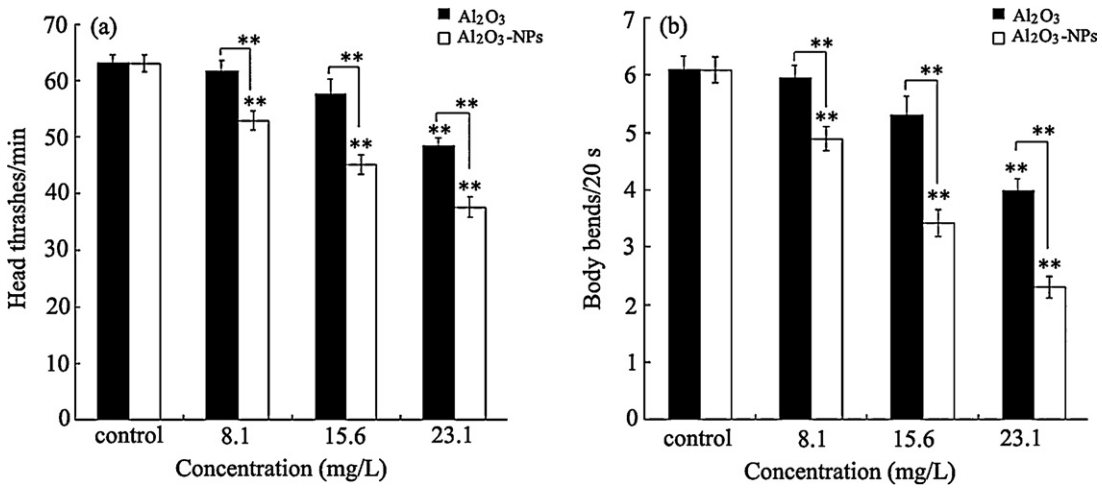


**Fig. 1.** Comparison of locomotion behaviors in nematodes exposed to $Al_2O_3$-NPs and bulk $Al_2O_3$ for 10-day. $n = 5$. Bars represent mean ± S.E.M. $^{**}P < 0.01$ *vs* control (if not otherwise indicated).

plates (9 cm). Total RNA (~1 μg) was reverse-transcribed using cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), and real-time PCR was performed using primers for target genes of *sod-2* (forward primer, 5′-AGCACTCGCTGCCAGATTTA-3′; reverse primer, 5′-AGGACGGAGGAGAACCATCG-3′) and *sod-3* (forward primer, 5′-CTTGGCTAAGGATGGTGGAG-3′; reverse primer, 5′-ATCACTATTGCGGTTCAAGG-3′), and reference gene of *ubp-1* (forward primer, 5′-CACTTGGTTCTTCGTCTTAG-3′; reverse primer, 5′-CCTCCTTGTCTTGAATCTTG-3′). In nematodes, *ubq-1* encodes an ubiquitin protein, a highly conserved protein in eukaryotic organisms. Relative quantification of *sod-2* or *sod-3* gene in comparison to reference *ubq-1* gene was determined, and the final results were expressed as relative expression ratio (between target gene and reference gene) in the treatments as compared to the ratio in the control.

### 2.8. Pharmacological assay

The day-9 $Al_2O_3$-NPs exposed (23.1 mg/L) adults were treated with 0.1% DMSO, an effective free radical scavenger, for 24-h [20], 10 mM ascorbate for 24 h [21] or 5 mM *N*-acetyl-L-cysteine (NAC) for 24 h [21]. Ascorbate and NAC are two antioxidants and used to treat mitochondrial dysfunction, and treatment with 10 mM ascorbate or 5 mM NAC did not influence survival of nematodes [21]. Graphs are representative of at least ten trials.

### 2.9. DNA construct and germline transformation

The *sod-3* (1010 bp, XbaI/KpnI) promoter fragments was subcloned into the pPD95_75 vector, and the *sod-3* full length cDNA were inserted into sites of KpnI/XhoI of the pPD95_75 vector behind P*sod-3*fragments to obtain plasmid of P*sod-3-sod-3*. Transgenic worms were generated as described [22]. The plasmids were injected as a mix at 20 ng/μl using P*dop-1::rfp* as a transgenic marker.

### 2.10. Statistical analysis

All data were expressed as means ± standard error of the mean (S.E.M.). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to determine the significance of differences between the groups. Probability levels of 0.05 and 0.01 were considered statistically significant.

## 3. Results and discussion

In this study, we first investigated the effects of acute exposure to $Al_2O_3$-NPs and bulk $Al_2O_3$ on locomotion behaviors. After exposure for 6-h, the significant decreases in head thrashes ($P < 0.01$) and body bends ($P < 0.01$) were observed in nematodes exposed to 51–203.9 mg/L of $Al_2O_3$-NPs (data not shown). Significant decreases in head thrashes (51 mg/L, $P < 0.05$; 102–203.9 mg/L, $P < 0.01$) and body bends ($P < 0.01$) were observed in nematodes exposed to 51–203.9 mg/L of bulk $Al_2O_3$ (data not shown). Nevertheless, both head thrashes and body bends in $Al_2O_3$-NPs exposed nematodes were lower than those in bulk $Al_2O_3$ exposed nematodes at concentrations of 51–203.9 mg/L (data not shown).

Moreover, after exposure for 48 h, significant decreases in head thrashes ($P < 0.01$) and body bends ($P < 0.01$) were observed in nematodes exposed to 30.6–203.9 mg/L of $Al_2O_3$-NPs. In contrast, significant decreases in head thrashes ($P < 0.01$) and body bends ($P < 0.01$) were observed in nematodes exposed to 51–203.9 mg/L of bulk $Al_2O_3$ (data not shown). In addition, both head thrashes and body bends in $Al_2O_3$-NPs exposed nematodes were lower than those in bulk $Al_2O_3$ exposed nematodes at concentrations of 30.6–203.9 mg/L (data not shown). These data suggest that locomotion behavior may be a useful endpoint for evaluating the chronic toxicity of $Al_2O_3$-NPs, and that exposure to 8.1–30.6 mg/L of $Al_2O_3$-NPs for 6 h and 8.1–23.1 mg/L of $Al_2O_3$-NPs for 48-h does not influence locomotion behaviors.

We further examined the 10-day chronic toxicity from 8.1–23.1 mg/L of $Al_2O_3$-NPs and bulk $Al_2O_3$ exposure on locomotion behavior of nematodes. The lethality of adult nematodes exposed to $Al_2O_3$-NPs (<15.6 mg/L) or $Al_2O_3$ (<23.1 mg/L) was not more than 20% at day 10 (Supplementary Fig. 1). As shown in Fig. 1, significant decreases in head thrashes ($P < 0.01$) and body bends ($P < 0.01$) were observed in nematodes exposed to 8.1–23.1 mg/L of $Al_2O_3$-NPs. In contrast, significant decreases in head thrashes ($P < 0.01$) and body bends ($P < 0.01$) were detected in nematodes exposed to 23.1 mg/L of bulk $Al_2O_3$. Furthermore, both head thrashes and body bends in $Al_2O_3$-NPs exposed nematodes were lower than those in bulk $Al_2O_3$ exposed nematodes. Therefore, chronic exposure to 8.1–23.1 mg/L of $Al_2O_3$-NPs induces toxic effects on locomotion behavior in nematodes. One of the important reasons for selection of *C. elegans* as a bioindicator to evaluate the chronic toxicity of NPs is its short life cycle (approximately 24-day) of nematodes, and the use of *C. elegans* may largely reduce the evaluation period of the chronic toxicity from NPs exposure [5].

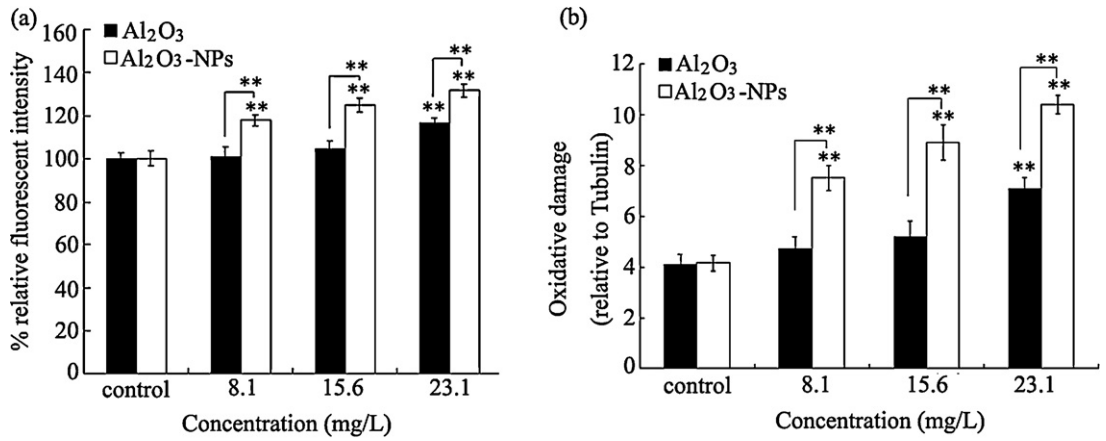


**Fig. 2.** Stress responses (a) and oxidative damage (b) of nematodes exposed to $Al_2O_3$-NPs and bulk $Al_2O_3$ for 10-day. Increased relative fluorescent intensities for P*hsp-16.2::gfp* expression were observed to reflect stress response. Examination of carbonylated proteins reveals increased oxidative damage. $n = 5$. Bars represent mean ± S.E.M. $^{**}P < 0.01$ *vs* control (if not otherwise indicated).

We next examined the toxicities from chronic (10-d) $Al_2O_3$-NPs and bulk $Al_2O_3$ exposure on the stress response in a transgenic KC136 strain. Chronic exposure to $Al_2O_3$-NPs at the examined concentrations induced a significant ($P < 0.01$) increase in the relative fluorescent intensities of P*hsp-16.2::gfp* expression, whereas only chronic exposure to 23.1 mg/L of bulk $Al_2O_3$ activated a significant ($P < 0.01$) increase in the relative fluorescent intensities of P*hsp-16.2::gfp* expression (Fig. 2a). Noticeable differences in the relative fluorescent intensities of P*hsp-16.2::gfp* expression in $Al_2O_3$-NPs exposed nematodes from those in bulk $Al_2O_3$ exposed nematodes were detected at the examined concentrations (Fig. 2a), suggesting that chronic exposure to $Al_2O_3$-NPs at the examined concentrations causes induction of the stress response.

Previous studies have suggested that acute exposure to metals such as cadmium will induce the formation of oxidative stress in nematodes [23]. As shown in Fig. 2b, chronic exposure to 8.1–23.1 mg/L of $Al_2O_3$-NPs caused the formation of oxidative damage; however, only chronic exposure to 23.1 mg/L of bulk $Al_2O_3$ induced the formation of oxidative damage. Therefore, chronic

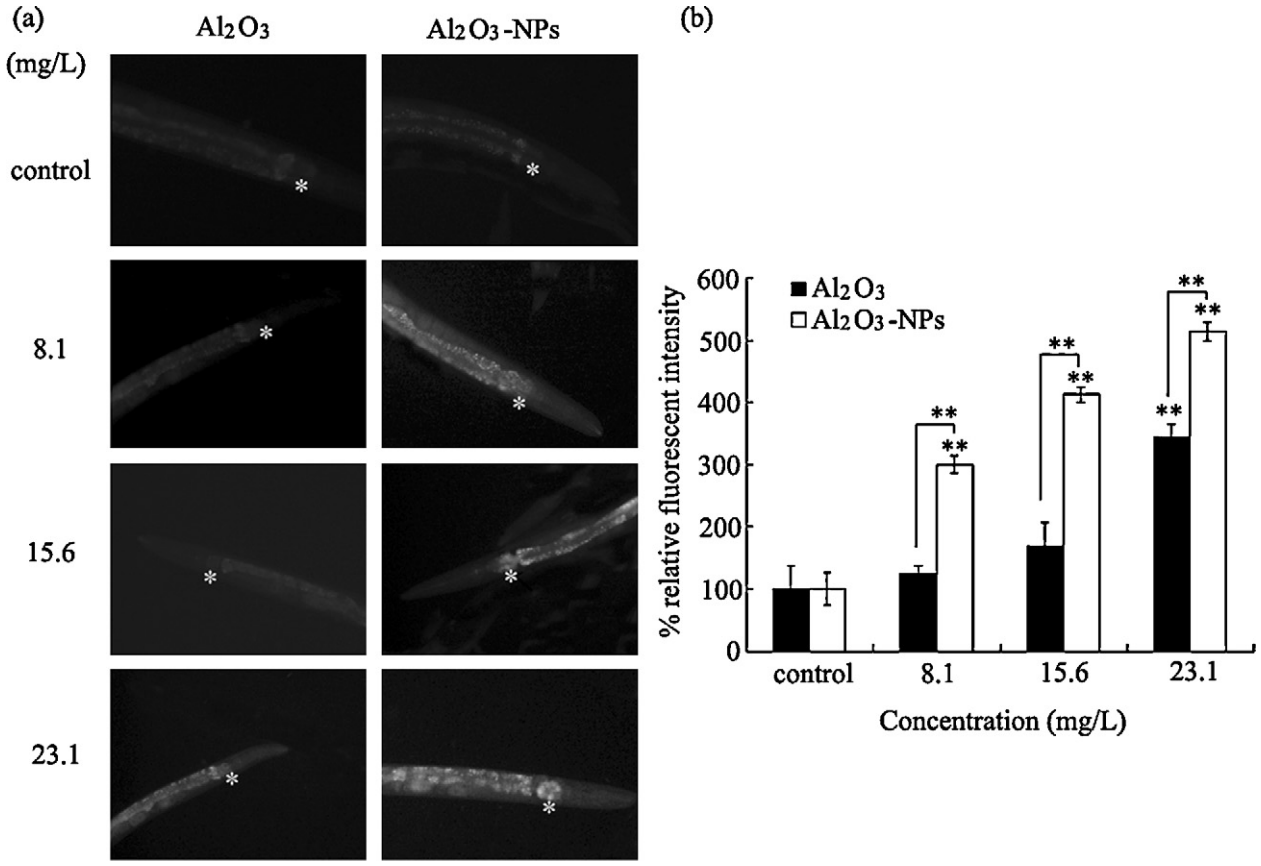


**Fig. 3.** ROS production of nematodes exposed to $Al_2O_3$-NPs and bulk $Al_2O_3$ for 10-day. (a) Pictures showing the ROS production. Asterisks indicate position of second pharyngeal bulb. (b) Comparison of ROS production in nematodes exposed to $Al_2O_3$-NPs and bulk $Al_2O_3$. CM-$H_2$DCFDA was used to detect ROS production, and semiquantified ROS was expressed as relative fluorescent units (RFU). $n = 5$. Bars represent mean ± S.E.M. $^{**}P < 0.01$ *vs* control (if not otherwise indicated).

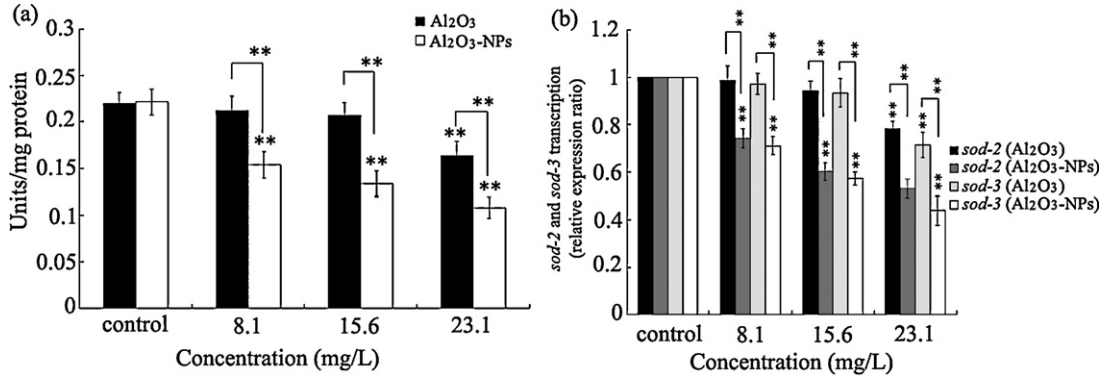


**Fig. 4.** Superoxide dismutase (SOD) activities (a) and of *sod-2* and *sod-3* gene expressions (b) in nematodes exposed to $Al_2O_3$-NPs and bulk $Al_2O_3$ for 10-day. Relative expression ratios (between *sod-2* or *sod-3* target gene and *ubq-1* reference gene) in treatments are normalized to the control. $n = 5$. Bars represent mean ± S.E.M. $^{**}P < 0.01$ *vs* control (if not otherwise indicated).

exposure to 8.1–23.1 mg/L of $Al_2O_3$-NPs further results in the formation of oxidative stress.

One possible cause of induction of oxidative stress is an increase in ROS generation [24]. As shown in Fig. 3, after a 10-day exposure, significant ($P < 0.01$) ROS production was observed in nematodes exposed to 8.1–23.1 mg/L of $Al_2O_3$-NPs. In contrast, significant ($P < 0.01$) ROS production was only detected in nematodes exposed to 23.1 mg/L of bulk $Al_2O_3$. ROS generation in $Al_2O_3$-NPs exposed nematodes was higher than that in bulk $Al_2O_3$ exposed nematodes at the examined concentrations. Therefore, ROS production can occur in nematodes chronically exposed to $Al_2O_3$-NPs, implying that one of the causes of oxidative stress induction in nematodes chronically exposed to $Al_2O_3$-NPs is induction of ROS production.

Usually, when ROS generation increases or when ROS defense mechanisms are compromised, cells and animals are said to be under oxidative stress [21]. SOD is one of many antioxidant enzymes involved in protection against stress by converting superoxide to hydrogen peroxide in animals. As shown in Fig. 4a, after a 10-day exposure, significant ($P < 0.01$) decreases in SOD activities were detected in nematodes exposed to 8.1–23.1 mg/L of $Al_2O_3$-NPs. In contrast, significant ($P < 0.01$) decrease in SOD activity was only detected in nematodes exposed to 23.1 mg/L of bulk $Al_2O_3$. More interestingly, we found that after pretreatment with paraquat, a known oxidative stressor, exposure to $Al_2O_3$-NPs enhanced the adverse effects of paraquat on nematodes as indicated by SOD activity (Supplementary Fig. 2). In *C. elegans*, *sod-2* and *sod-3* encodes Mn-SODs that are localized in the mitochondrial matrix [25]. As shown in Fig. 4b, after a 10-day exposure, noticeable decreases in *sod-2* and *sod-3* expression were detected in nematodes exposed to 8.1–23.1 mg/L of $Al_2O_3$-NPs. In contrast, obvious decreases in *sod-2* and *sod-3* expression were only detected in nematodes exposed to 23.1 mg/L of bulk $Al_2O_3$. The expression levels of *sod-2* and *sod-3* genes in $Al_2O_3$-NPs exposed nematodes were lower than those in bulk $Al_2O_3$ exposed nematodes at the examined concentrations. Therefore, the formation of oxidative stress in nematodes chronically exposed to $Al_2O_3$-NPs may be due to alterations in both ROS production and ROS defense mechanisms.

To confirm the roles of oxidative stress in induction of locomotion behavior decreases in nematodes chronically exposed to $Al_2O_3$-NPs, we investigated the neurotoxic effects of antioxidants on locomotion behaviors in nematodes chronically exposed to $Al_2O_3$-NPs at the concentration of 23.1 mg/L. As indicated above, chronic exposure to 23.1 mg/L of $Al_2O_3$-NPs induced the most severe adverse effects on locomotion behavior in nematodes (Fig. 1). Treatment with 0.1% DMSO effectively suppressed induction of the stress response, formation of oxidative damage, increase in ROS generation, and decrease in SOD activities in nematodes chronically exposed to 23.1 mg/L of $Al_2O_3$-NPs (Fig. 5a–d). Moreover, treatment with 0.1% DMSO effectively retrieved the neurotoxic effects on head thrashes and body bends in nematodes chronically exposed to 23.1 mg/L of $Al_2O_3$-NPs (Fig. 5e and f).

Some antioxidants, such as ascorbate and NAC, can be used to treat mitochondrial dysfunction. Treatment with 10 mM of ascorbate or 5 mM of NAC effectively inhibited the induction of the stress response, formation of oxidative damage, increase in ROS generation, and decrease in SOD activities in nematodes chronically exposed to 23.1 mg/L of $Al_2O_3$-NPs (Fig. 5a–d). Furthermore, treatment with 10 mM of ascorbate or 5 mM of NAC effectively suppressed the neurotoxic effects on locomotion behaviors in nematodes chronically exposed to 23.1 mg/L of $Al_2O_3$-NPs (Fig. 5e and f). Therefore, treatment with antioxidants can suppress the formation of oxidative stress and retrieve the defects of locomotion behaviors in nematodes chronically exposed to $Al_2O_3$-NPs.

To further confirm the roles of oxidative stress in induction of locomotion behavior decreases in nematodes chronically exposed to $Al_2O_3$-NPs, we selected the *sod-3* gene to construct a SOD-3 overexpression transgenic strain. This allowed for investigation of the effects of SOD-3 overexpression on locomotion behaviors in nematodes chronically exposed to $Al_2O_3$-NPs. As shown in Fig. 6, SOD-3 overexpression effectively suppressed formation of the stress response and oxidative damage in nematodes chronically exposed to $Al_2O_3$-NPs (Fig. 6a and b). Moreover, SOD-3 overexpression effectively prevented the occurrence of ROS generation and the disruption of ROS defense mechanisms formed in nematodes chronically exposed to $Al_2O_3$-NPs (Fig. 6c and d). Furthermore, SOD-3 overexpression effectively inhibited the decreases in locomotion behaviors in nematodes chronically exposed to $Al_2O_3$-NPs (Fig. 6e and f), implying that SOD-3 overexpression can suppress the adverse effects of chronic exposure to $Al_2O_3$-NPs on locomotion behaviors by inhibiting the formation of oxidative stress. A similar conclusion was obtained in *clk* mutants; *clk* mutants with an extended lifespan show increased expression of SOD-2 and no decrease in oxidative damage [26].

In *C. elegans*, *sod-2* and *sod-3* mutants are very sensitive to oxidative stress [27]. We next investigated the possible susceptible properties of *sod-2* and *sod-3* mutants to chronic $Al_2O_3$-NPs neurotoxicity. For this purpose, we selected the concentration of 8.1 mg/L to examine the possible susceptible properties of *sod-2* and *sod-3* mutants after exposure to $Al_2O_3$-NPs because, as indicated above, chronic exposure to 8.1 mg/L of $Al_2O_3$-NPs induced relatively less severe adverse effects on locomotion behavior in nematodes (Fig. 1). As shown in Fig. 7, after chronic exposure to 8.1 mg/L of $Al_2O_3$-NPs, mutations of *sod-2* and *sod-3* induced

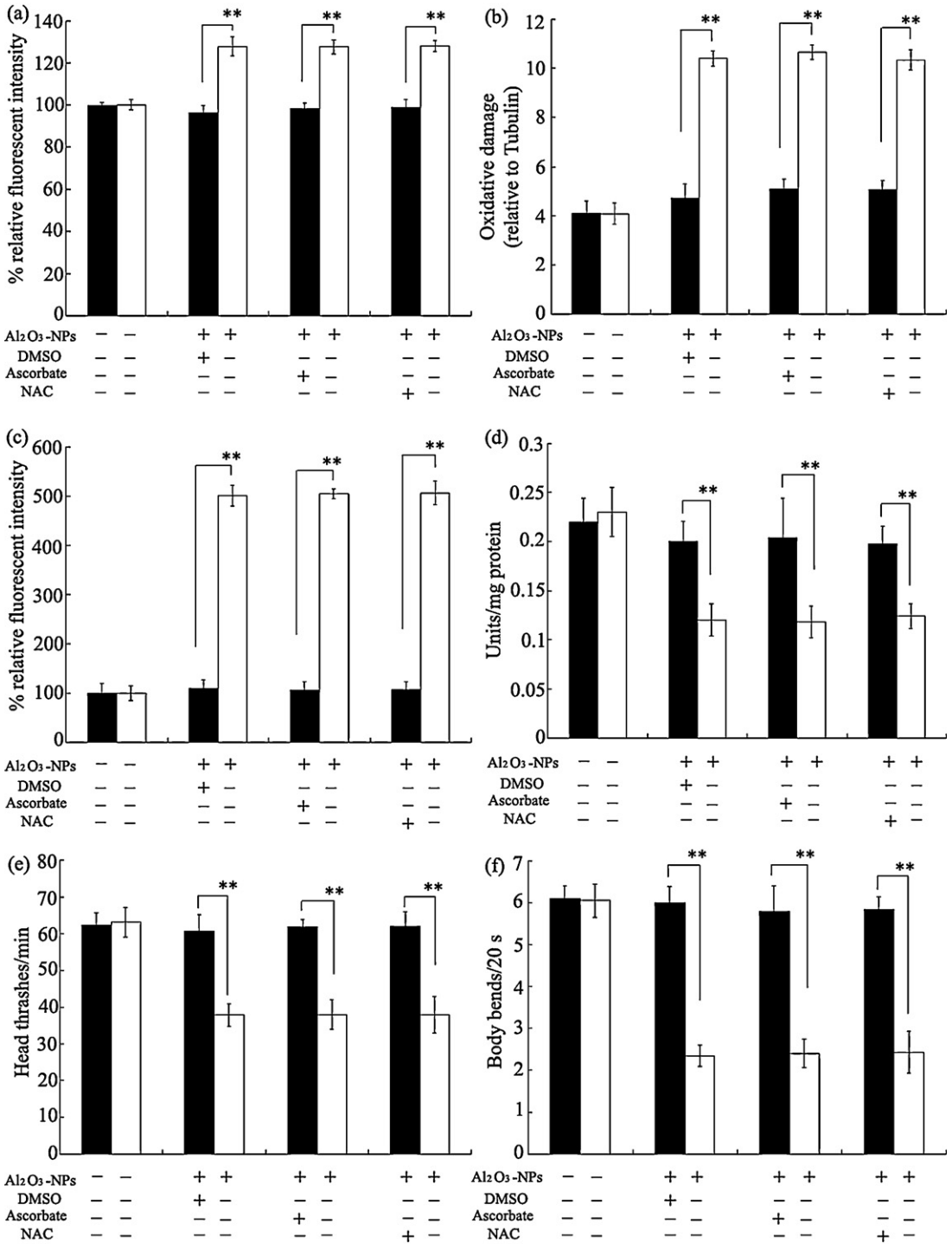


**Fig. 5.** Effects of antioxidant treatment on stress response (a), oxidative damage (b), ROS production (c), SOD activity (d), head thrash (e) and body bend (f) in nematodes chronically exposed to 23.1 mg/L $Al_2O_3$-NPs. Increased relative fluorescent intensities for P*hsp-16.2::gfp* expression were observed to reflect stress response. Examination of carbonylated proteins reveals increased oxidative damage. CM-$H_2$DCFDA was used to detect ROS production, and semiquantified ROS was expressed as relative fluorescent units (RFU). The day-9 $Al_2O_3$-NPs exposed nematodes were treated with 0.1% DMSO, 10 mM ascorbate, or 5 mM *N*-acetyl-L-cysteine (NAC) for 24 h. $n = 5$. Bars represent mean ± S.E.M. $^{**}P < 0.01$.

more marked decreases in head thrashes and body bends compared with those in wild-type nematodes. These data suggest that *sod-2* and *sod-3* mutants are more susceptible than wild-type to chronic $Al_2O_3$-NPs exposure-induced neurotoxicity.

We also examined the effects of chronic exposure to $Al_2O_3$-NPs supernatant on locomotion behaviors of wild-type N2 nematodes. As shown in Fig. 8, chronic exposure to 8.1–15.6 mg/L of $Al_2O_3$-NPs supernatants did not obviously affect head thrashes or body bends; however, head thrashes and body bends in nematodes chronically exposed to 23.1 mg/L of $Al_2O_3$-NPs supernatant were moderately decreased. In contrast, head thrashes and body bends in nematodes chronically exposed to resuspensions of 8.1–23.1 mg/L of $Al_2O_3$-NPs pellets were significantly decreased. Therefore, although some metal may be dissolved to ionic Al in prepared suspensions, the toxicity of $Al_2O_3$-NPs can be mainly explained by NP-specific toxic mechanisms.

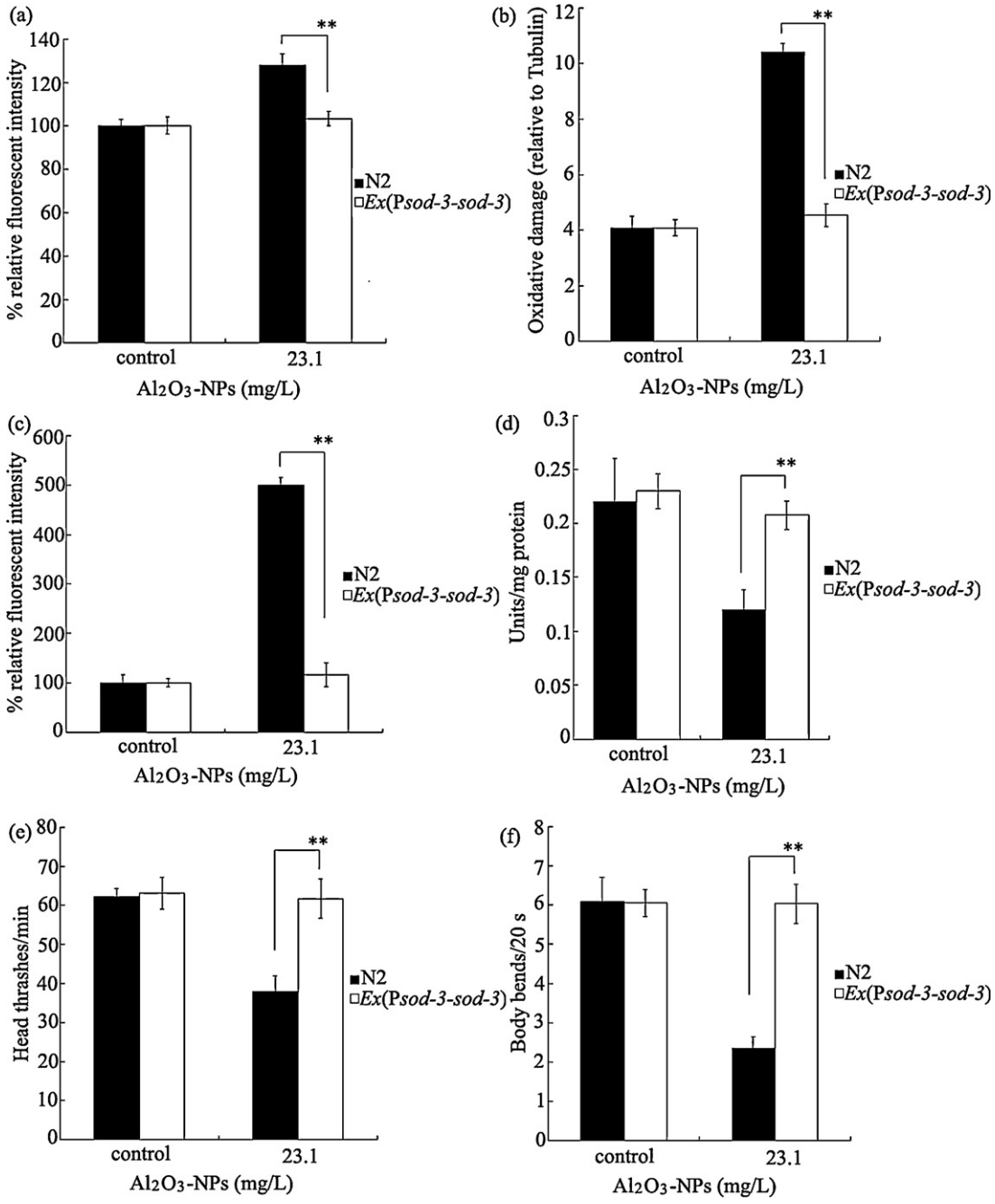


**Fig. 6.** Effects of SOD-3 over-expression on stress response (a), oxidative damage (b), ROS production (c), SOD activity (d), head thrash (e) and body bend (f) in nematodes chronically exposed to 23.1 mg/L $Al_2O_3$-NPs. Increased relative fluorescent intensities for P*hsp-16.2::gfp* expression were observed to reflect stress response. Examination of carbonylated proteins reveals increased oxidative damage. CM-$H_2$DCFDA was used to detect ROS production, and semiquantified ROS was expressed as relative fluorescent units (RFU). $n = 5$. Bars represent mean ± S.E.M. $^{**}P < 0.01$.

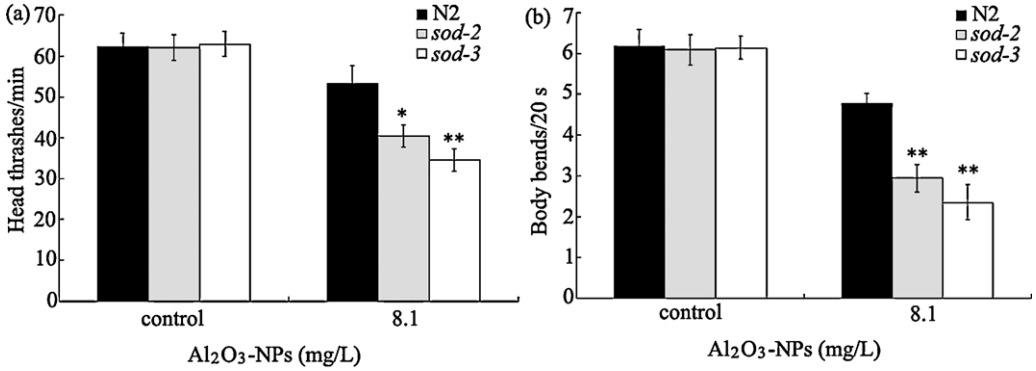


**Fig. 7.** Effects of *sod-2* and *sod-3* mutations on head thrashes (a) and body bends (b) in nematodes chronically exposed to 8.1 mg/L $Al_2O_3$-NPs. $n = 5$. Bars represent mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$.

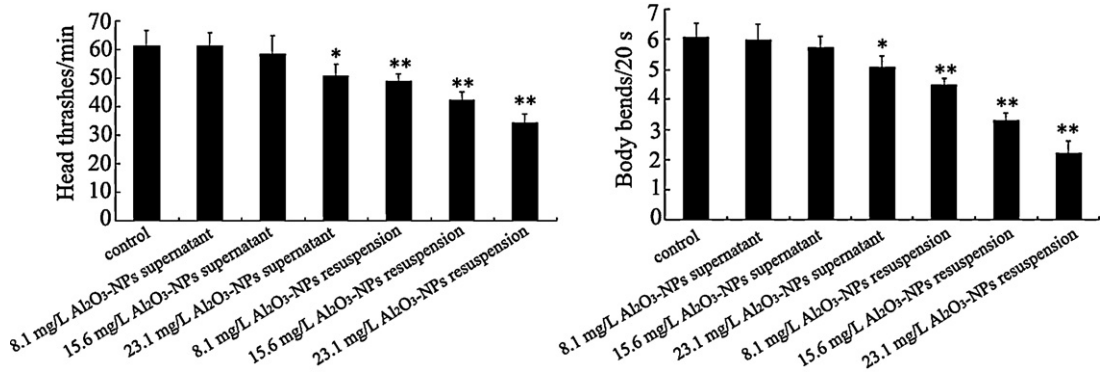


**Fig. 8.** Effects of $Al_2O_3$-NPs supernatants chronic exposure on locomotion behaviors in nematodes. n = 5. Bars represent mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$.

Considering the importance of environmental relevant concentrations in NPs toxicity evaluation, we finally investigated the effects of chronic exposure to $Al_2O_3$-NPs at very low concentrations (0.005–1 mg/L) on locomotion behavior of wild-type N2 nematodes. Chronic exposure to 0.005 mg/L of $Al_2O_3$-NPs did not obviously influence head thrashed and body bends of nematodes; however, exposure to 0.01–1 mg/L of $Al_2O_3$-NPs significantly decreased both head thrashes (0.01 mg/L, $P < 0.05$; 0.5–1 mg/L, $P < 0.01$) and body bends (0.01 mg/L, $P < 0.05$; 0.5–1 mg/L, $P < 0.01$) of nematodes (Fig. 9a and b). Accompanying with the decreases in locomotion behaviors, a noticeable stress response and oxidative damage occurred in nematodes

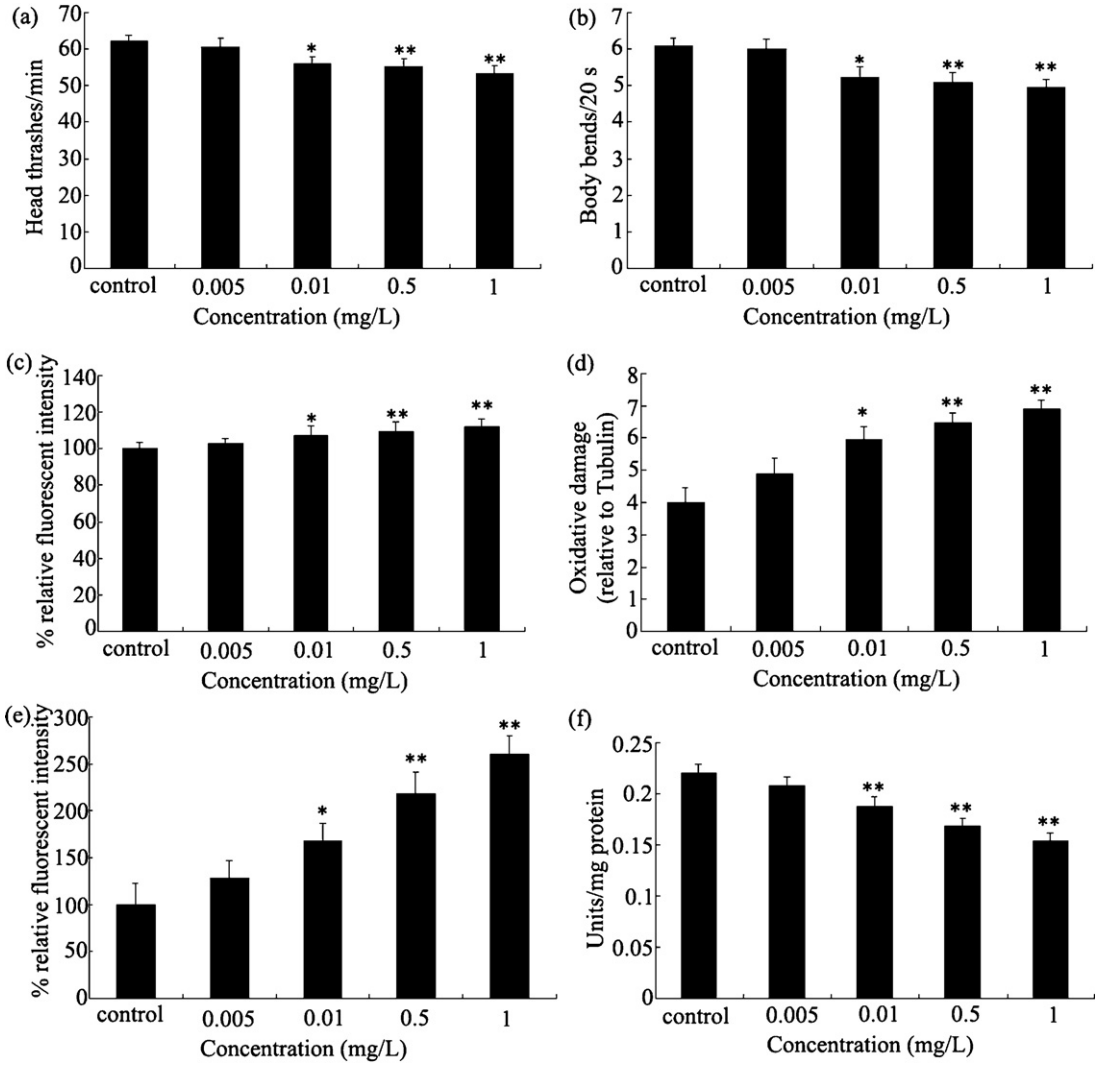


**Fig. 9.** Effects of chronic $Al_2O_3$-NPs exposure at very low concentrations on head thrash (a), body bend (b), stress response (c), oxidative damage (d), ROS production (e), and SOD activity (f) in nematodes. Increased relative fluorescent intensities for *Phsp-16.2::gfp* expression were observed to reflect stress response. Examination of carbonylated proteins reveals increased oxidative damage. CM-$H_2$DCFDA was used to detect ROS production, and semiquantified ROS was expressed as relative fluorescent units (RFU). $n = 5$. Bars represent mean ± S.E.M. $^*P < 0.05$; $^{**}P < 0.01$.

chronic exposed to 0.01–1 mg/L of $Al_2O_3$-NPs (Fig. 9c and d). Moreover, we observed significant induction of ROS production and reduction in SOD activities in nematodes chronic exposed to 0.01–1 mg/L of $Al_2O_3$-NPs (Fig. 9e and f).

The detection limits for most methods are not sufficiently low to detect environmentally relevant concentrations of engineered NPs in the range of ng/L to pg/L [28]. In the present study, the examined $Al_2O_3$-NPs concentrations, such as 0.01 mg/L, are relatively lower concentrations that still have toxic effects on organisms compared with the concentrations in our previously study [3]. We did not detect possible adverse effects of $Al_2O_3$-NPs on nematodes in the range of ng/L as detected with $CeO_2$-NPs in nematodes [29]. Published data on the possible adverse effects of nanomaterials on organisms at the environmental relevant concentrations are still very limited.

## 4. Conclusions

In the present study, our data confirmed the possible size effects of $Al_2O_3$-NPs on nematodes to a certain degree compared with bulk $Al_2O_3$; however, we found that exposure to bulk $Al_2O_3$ also caused the neurotoxic effects on nematodes. Therefore, so far we still cannot exclude such a possibility that the observed toxicity in $Al_2O_3$-NPs exposed nematodes may also largely due to the difference of transfer or distribution from that in bulk $Al_2O_3$ exposed nematodes. In addition, the relatively large surface area of $Al_2O_3$-NPs compared with bulk $Al_2O_3$ may also participate in the explanation of the differences of toxicity between $Al_2O_3$-NPs and bulk $Al_2O_3$.

Our data further suggest that chronic exposure to $Al_2O_3$-NPs at concentrations more than 0.01 mg/L induced decrease in locomotion behaviors of nematodes. This concentration is much lower than those examined in other organisms [30–33]. Considering the fact that *sod-2* and *sod-3* mutants are more sensitive than wild-type N2 to the neurotoxicity from chronic exposure to $Al_2O_3$-NPs, lower concentrations than 0.01 mg/L may be detected to have toxic effects on organisms.

Moreover, we found that decreases in locomotion behaviors in nematodes chronically exposed to $Al_2O_3$-NPs are associated with both increase in ROS generation and disruption of ROS defense mechanisms. Furthermore, chronic exposure to $Al_2O_3$-NPs may increase the ROS generation and disrupt the ROS defense mechanisms through suppressing the activities of Mn-SODs.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2012.03.083.

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