Contributions of altered permeability of intestinal barrier and defecation behavior to toxicity formation from graphene oxide in nematode *Caenorhabditis elegans*†

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Graphene oxide (GO) has been extensively studied for potential biomedical applications. Meanwhile, potential GO toxicity arises in both biomedical applications and non-biomedical products where environmental exposures may occur. In the present study, we examined the potential adverse effects of GO and the underlying mechanism using nematode *Caenorhabditis elegans* as the assay system. We compared the *in vivo* effects of GO between acute exposure and prolonged exposure, and found that prolonged exposure to 0.5–100 mg L\(^{-1}\) of GO caused damage on functions of both primary (intestine) and secondary (neuron and reproductive organ) targeted organs. In the intestine, ROS production was significantly correlated with the formation of adverse effects on functions of both primary and secondary targeted organs. GO could be translocated into intestinal cells with loss of microvilli, and distributed to be adjacent to or surrounding mitochondria. Prolonged exposure to GO resulted in a hyper-permeable state of the intestinal barrier, an increase in mean defecation cycle length, and alteration of genes required for intestinal development and defecation behavior. Thus, our data suggest that prolonged exposure to GO may cause potential risk to environmental organisms after release into the environment. GO toxicity may be due to the combinational effects of oxidative stress in the intestinal barrier, enhanced permeability of the biological barrier, and suppressed defecation behavior in *C. elegans*.

Introduction

Graphene, the thinnest known material in the universe and the strongest ever measured, is a single-atom thick, two-dimensional sheet of hexagonally arranged carbon atoms isolated from its three-dimensional parent material, graphite.\(^5\)\(^6\) Graphene oxide (GO), a member of the graphene family of nano-materials (GFNs), has been extensively studied in recent years because of its physiologically stable properties. Biomedical applications of graphene and GO include drug delivery, tissue engineering, and fluorescence-based biomolecular sensing and imaging.\(^2\)\(^4\) A number of companies have been formed worldwide that manufacture and sell GFNs. Meanwhile, GFN research has expanded and engineering applications have emerged. These make the issue of potential GFN toxicity arise not only in biomedical applications but also in non-biomedical products where unintended occupational, consumer, and environmental exposures can occur.\(^2\)

Although many studies have shown that nanomaterials might have side-effects on health,\(^7\)\(^8\) only limited literatures on the toxicity of graphene or GO have been published so far. Studies of *in vitro* and *in vivo* GO toxicity have suggested that generation of reactive oxygen species (ROS) in targeted cells is a potential mechanism.\(^9\)\(^12\) Exposure to graphene or GO resulted in a decrease of cell adhesion, cell apoptosis, alteration of lactate dehydrogenase release, and induction of antifungal activity and immunotoxicity.\(^5\)\(^11\)-\(^16\) The *in vivo* studies have demonstrated the systemic biodistribution of GO following intravenous delivery.\(^13\)\(^17\) Exposure to graphene and GO resulted in pulmonary edema and granuloma formation, increased rate of mitochondrial respiration and ROS production, and activated inflammatory and apoptosis pathways in lung cells.\(^13\)\(^15\)\(^19\)

Nevertheless, the long-term adverse effects of graphene and GO on health still need careful consideration during the design of GO for applications and the release into environment.

Nematode *Caenorhabditis elegans*, a free-living nematode, is of ecological significance and has important roles in nutrient cycling in soil.\(^16\) *C. elegans* is one of the most thoroughly studied...
model animals. Its experimental potential offers a system best suited for asking in vivo questions with relevance at the organism level. The fact that many basic physiological processes, stress responses, signal transduction pathways, and epigenetic marks are conserved between C. elegans and humans enables the corresponding comparison of molecular mechanisms. C. elegans is useful for environmental and toxicological studies of toxicants from whole-animal level down to single cell level. Both lethal and a series of sublethal endpoints have been used in the environmental and toxicological studies. Particularly, because L1-larvae may be more sensitive than L4-larvae and adults, prolonged exposure from L1-larvae to adult (4-day after hatching) can be used to assess the toxicity of specific toxicants at environmental relevant concentrations. So far, C. elegans has been further successfully used for environmental safety evaluation and toxicology of different engineered nanomaterials (ENMs). Recently, C. elegans has been further used to evaluate the oxidative stress-mediated nanotoxicity of GO, and the results indicate that exposure of L4-larvae to 5–20 mg L\(^{-1}\) of GO did not influence the lifespan of animals. Nevertheless, whether prolonged exposure can potentially cause adverse effects on nematodes and the underlying mechanism is still largely unclear.

C. elegans can be successfully used in toxicity assessments in water, sediment, and soil. Thus, in the present study, we first explored the in vivo acute and prolonged exposure assay systems of C. elegans to investigate the possible adverse effects of GO on animals. Again, we examined the in vivo underlying mechanism for the toxicity formation from GO exposure in C. elegans. Our data demonstrate the potential adverse effects of prolonged exposure to GO on environmental organisms. Moreover, our results demonstrate the important contribution of permeability of the intestinal barrier and defecation behavior to GO toxicity in C. elegans.

**Results and discussion**

**Physicochemical properties of prepared GO**

GO was synthesized according to the modified Hummer’s method. The prepared GO was black, and well dispersed. Fig. 1a and b show the representative transmission electron microscopy (TEM) and atomic force microscopy (AFM) images of the prepared GO, respectively. The TEM image of GO confirmed the sheet-like shape of prepared GO (Fig. 1a), indicating that the mono-layer GO sheet was successfully prepared. AFM was further used to measure the thickness of the synthesized GO nanosheets. The height image from AFM indicates that the thickness of the prepared GO was \(\approx 1.0 \text{ nm} \) in topographic height, corresponding to approximately 1 layer (Fig. 1b).

The Fourier transform infrared spectroscopy (FTIR) spectrum of GO indicated that the peak at 3425 cm\(^{-1}\) could be attributed to the O–H stretching vibration, the peak at 1749 cm\(^{-1}\) could be attributed to the C==O stretching vibration, the peak at 1630 cm\(^{-1}\) could be attributed to deformation of C==O, and the peak at 1130 cm\(^{-1}\) could be attributed to the vibration of C–O (alkoxy) (Fig. 1c). Raman spectroscopy is a nondestructive technique that was used to characterize crystallinity and the number of layers. Raman spectroscopy measurement by using 632 nm wavelength excitation showed that D-band signal appeared after treatment with sulfuric acid and KMnO\(_4\), indicating the introduction of disorder into the graphite layer (Fig. 1d). Zeta potential of GO in K medium was measured by a Nano Zetasizer using a dynamic light scattering technique, and the zeta potential of GO in K medium was \(-20.2 \text{ mV}\). Size of GO in K medium was measured by TEM. After sonication, the size of GO in K medium was 72 \pm 11 \text{ nm}\) (Fig. S1†).

**Effects of acute exposure to GO on C. elegans**

A series of studies have suggested that nanomaterials elicit toxicity to C. elegans. We first investigated the possible effects of acute exposure to GO from L4-larvae for 24 h on C. elegans. Acute exposure to GO at concentrations of 0.1–100 mg L\(^{-1}\) did not induce lethality nor influence development in nematodes (Fig. S2†).

Neurons and reproductive organs are important secondary targeted organs for ENMs in C. elegans. Acute exposure to GO at concentrations of 0.1–10 mg L\(^{-1}\) did not alter brood size in nematodes, and acute exposure to GO at concentrations of 0.1–1 mg L\(^{-1}\) did not affect locomotion behavior as reflected by endpoints of head thrash and body bend\(^{44}\) (Fig. 2). In contrast, the significant reduction of brood size was only observed in 100 mg L\(^{-1}\) of GO exposed nematodes, and the significant decrease in locomotion behavior was detected in 10–100 mg L\(^{-1}\) of GO exposed nematodes (Fig. 2).

The intestine is the primary targeted organ for ENMs in C. elegans. We further investigated the effects of GO exposure on intestinal development. Intestinal autofluorescence is caused by lysosomal deposits of lipofuscin, which can accumulate over time in aging nematodes. The significant induction of intestinal autofluorescence was only observed in 100 mg L\(^{-1}\) of GO-exposed nematodes compared with control (Fig. 3). Moreover, we found that the significant ROS production in intestine could be detected in 10–100 mg L\(^{-1}\) of GO-exposed nematodes compared with control (Fig. 4a and b).
In *C. elegans*, lifespan is an important and useful endpoint for GO toxicity assessment, we also examined the effects of acute GO exposure on the lifespan of nematodes. Acute exposure to 0.1–100 mg L$^{-1}$ of GO did not obviously alter the lifespan of nematodes (Fig. S3†).

**Effects of prolonged exposure to GO on *C. elegans***

Formation of toxicity from ENM exposure on environmental organisms may be largely due to their long-term effects. Considering the fact that prolonged exposure assay system in *C. elegans* can be used to assess the toxicity of specific ENMs at environmental relevant concentrations, we next investigated the possible effects of prolonged exposure to GO from L1-larvae-to-adult on nematodes. Similar to the effects of acute exposure to GO, prolonged exposure to GO could not induce lethality of nematodes (Fig. S2a†). However, different from the effects of acute exposure to GO, prolonged exposure to 100 mg L$^{-1}$ of GO significantly reduced body length compared with control, although prolonged exposure to 0.1–10 mg L$^{-1}$ of GO still did...
not obviously influence the body length of nematodes (Fig. S2b†). Moreover, although prolonged exposure to 0.1–10 mg L\(^{-1}\) of GO did not influence the lifespan of nematodes, prolonged exposure to 100 mg L\(^{-1}\) of GO noticeably decreased the lifespan of nematodes (Fig. S3†).

Prolonged exposure to 1–100 mg L\(^{-1}\) of GO further significantly reduced brood size, and prolonged exposure to 0.5–100 mg L\(^{-1}\) of GO significantly decreased locomotion behavior compared with control (Fig. 2). Moreover, prolonged exposure to 1–100 mg L\(^{-1}\) of GO significantly increased intestinal autofluorescence (Fig. 3), and prolonged exposure to 0.5–100 mg L\(^{-1}\) of GO noticeably induced intestinal ROS production (Fig. 4a and b). These results suggest that prolonged exposure to GO may cause damage to the functions of both primary and secondary targeted organs in C. elegans.

**Prolonged GO exposure altered the expression pattern of genes required for oxidative stress**

To examine the molecular basis of oxidative stress in regulating GO toxicity, we further investigated the expression pattern of genes required for oxidative stress (Table S1†) in GO-exposed nematodes. After prolonged exposure, 100 mg L\(^{-1}\) of GO caused a significantly decreased expression level of gas-1 gene, and an increased expression level of sod-1, sod-2, sod-3, sod-4, isp-1, and clk-1 genes (Fig. 4c). In contrast, GO exposure at the concentration of 100 mg L\(^{-1}\) did not influence the expression pattern of sod-5, mev-1, ctl-1, ctl-2, and ctl-3 (Fig. 4c). In C. elegans, sod-1 and sod-4 genes encode copper/zinc superoxide dismutase, sod-2 and sod-3 genes encode manganese superoxide dismutase, isp-1 gene encodes a “Rieske” iron-sulfur protein, gas-1 gene encodes a subunit of mitochondrial complex I, and clk-1 gene encodes a ubiquinone biosynthesis protein COQ7 (Table S1†). These data suggest that the induction of oxidative stress in GO-exposed nematodes at the examined concentration may be associated with the alteration of the activities of superoxide dismutase, “Rieske” iron-sulfur protein, mitochondrial complex I, and ubiquinone biosynthesis protein COQ7.

**Association of intestinal ROS production with adverse effects of GO exposure on C. elegans**

Oxidative stress is a key inducer for toxicity formation of ENMs including GO in C. elegans.\(^{12,55}\) We next investigated the possible association of intestinal ROS production with growth, reproduction, locomotion behavior, and intestinal autofluorescence using linear regression analysis in GO-exposed nematodes. The results of linear regression analysis showed that, after prolonged exposure to GO, intestinal ROS production was significantly correlated with body length (\(R^2 = 0.827, p < 0.05\)), brood size (\(R^2 = 0.839, p < 0.05\)), body bend frequency (\(R^2 = 0.810, p < 0.05\)), head thrash frequency (\(R^2 = 0.929, p < 0.01\)), and intestinal autofluorescence (\(R^2 = 0.966, p < 0.01\)) (Table S2†). Thus, intestinal ROS production may be significantly correlated with growth, reproduction, locomotion behavior, and intestinal autofluorescence in GO-exposed nematodes.

**Distribution of GO in intestinal cells of nematodes**

A previous study demonstrated that GO could be observed through the length of body and in the anterior part of the intestine in C. elegans with the aid of fluorescent labeling.\(^{12}\) Using TEM, we further directly observed that prolonged exposure to GO induced the translocation of GO into intestinal cells of nematodes (Fig. 5). In the intestinal cells, GO was mainly distributed to be adjacent to or surrounding mitochondria (Fig. 5). We did not observe the accumulation of GO in lysosomes. Meanwhile, we observed the disrupted ultrastructure of microvilli and the loss of many microvilli on intestinal cells (Fig. 5). Therefore, the data from TEM assay imply the possible direct damage on intestinal cells and alteration of permeable state for intestinal barrier in GO-exposed nematodes.
Transgenerational effects of prolonged GO exposure on growth of nematodes

Considering the fact that GO could be translocated into the body of nematodes and prolonged exposure to 100 mg L\(^{-1}\) of GO could result in adverse effects on growth (Fig. S2b), we further examined the transgenerational effects of prolonged GO exposure at the concentration of 100 mg L\(^{-1}\) on growth of nematodes. Interestingly, we found that growth of F1 progeny of GO-exposed nematodes was still inhibited, although they were cultured under normal conditions (Fig. S4†). Nevertheless, we did not observe the deficit in growth in F2 progeny of GO exposed nematodes (Fig. S4†), implying the recovery of toxicity on growth from GO exposure in F2 progeny nematodes.

Prolonged GO exposure altered the permeable state for intestinal barrier in nematodes

Considering the key role of primary targeted organs for translocation and distribution of ENMs, we further examined the permeability of primary targeted organs for GO exposed nematodes. After prolonged exposure, we explored the lipophilic fluorescent dye, Nile Red to stain GO exposed nematodes, and found that prolonged exposure to 100 mg L\(^{-1}\) of GO significantly enhanced the relative fluorescence intensity of Nile Red in the intestine compared with control (Fig. 6a and b). Because Nile Red can be used to label fat storage in nematodes, we further stained GO-exposed nematodes with Sudan Black, a general lipid staining probe that required the samples to be permeabilized. Sudan Black staining exhibited no significant difference between control and GO-exposed nematodes (Fig. 6c). Moreover, there was no significant difference of triglyceride content between control and GO-exposed nematodes (Fig. 6d). Based on these data, we concluded the possibility that GO-exposed nematodes may have a hyper-permeable intestinal barrier rather than increased lipid accumulation.

Prolonged GO exposure altered the expression pattern of genes required for intestinal development

To examine the molecular basis of intestinal barrier in regulating GO toxicity, we further investigated the expression pattern of genes required for intestinal development (Table S3†) in GO-exposed nematodes. After prolonged exposure, 100 mg L\(^{-1}\) of GO caused significantly decreased expression level of par-6 and pke-3 genes, and increased expression level of nhx-2 gene (Fig. 6e). In contrast, GO expression at the concentration of 100 mg L\(^{-1}\) did not influence the expression patterns of ajm-1, egl-8, pho-1, ifb-2, dlg-1, gem-4, esp-8, act-5, vha-6, gtl-1, opt-1, nfm-1, ppg-3, ppg-1, let-413, inx-3, abts-4, nhx-4, mtm-6, par-3, and erm-1 genes in nematodes (Fig. 6e). In C. elegans, par-6, pkc-3, nhx-2, mtm-6, opt-2, pho-1, par-3, ppg-1, ppg-3, vha-6, gtl-1, erm-1, eps-8, act-5, and ifb-2 genes are required for the development of microvilli on intestinal cells; let-413, nfm-1, inx-3, nhx-7, and abts-4 genes are required for development of basolateral domain of intestine; and dlg-1, ajm-1, and egl-8 genes are associated with the development of apical junction of intestine. Our data suggest that the altered permeability of intestine in GO-exposed nematodes at the examined concentration may be associated with the functional changes of PAR-6, PKC-3, and NHX-2 proteins. In C. elegans, the par-6 gene encodes a PDZ-domain-containing protein, pke-3 gene encodes an atypical protein kinase, and the nhx-2 gene encodes a sodium/proton exchanger (Table S3†).

Prolonged GO exposure influenced defecation behavior in nematodes

To further examine the possible mechanism explaining the toxicity formation from GO exposure, we also investigated the effects of GO exposure on defecation behavior in nematodes. Although prolonged exposure to 0.1–0.5 mg L\(^{-1}\) of GO did not obviously alter the mean defecation cycle length, prolonged exposure to 1–100 mg L\(^{-1}\) of GO significantly increased the mean defecation cycle time of nematodes (Fig. 7a). Moreover, we investigated the effects of GO exposure on the expression patterns of genes required for the execution and regulation of defecation behavior (Table S4†). Prolonged exposure to 100 mg L\(^{-1}\) of GO significantly decreased the expression levels of unc-101, itr-1, iri-1, and cab-1 genes, and increased the expression levels of fat-3, isp-1, unc-44, fat-2, clk-1, hlh-8, gar-1, lim-6, unc-93, mlg-2, ced-10, and egl-30 genes (Fig. 7b). These data suggest that the altered defecation state may be associated with the functional changes of UNC-101, ITR-1, SMP-1, IRI-1, CAB-1, FAT-3, ISP-1, UNC-44, FAT-2, CLK-1, HLH-8, GAT-1, LIM-6, UNC-93, MLG-2, CED-10, and EGL-30 proteins. The defecation state may serve as another important contributor to GO toxicity in nematodes.

In the present study, we found that acute exposure to GO at concentrations of 10–100 mg L\(^{-1}\) caused adverse effects on C. elegans. The in vitro study indicated that 100 mg L\(^{-1}\) of graphene increased LDH release, and generation of ROS. Exposure to GO at doses more than 50 mg L\(^{-1}\) exhibited toxicity on human fibroblast cells such as decreasing cell adhesion and inducing cell apoptosis. Therefore, C. elegans may be relatively more sensitive for GO toxicity assessment than other biological assay systems. Moreover, low levels of exposure (<10 mg L\(^{-1}\)) to GO might theoretically be safe in biomedical applications with short-term durations.
In the environment, so far most of the ENMs released are in the range of ng L$^{-1}$ to μg L$^{-1}$. For example, the predicted environmentally relevant concentration of TiO$_2$ nanoparticles (NPs) in sewage treatment effluents is 4 μg L$^{-1}$, and the predicted environmentally relevant concentrations for carbon nanotubes (CNTs) are 6.6–31.5 ng L$^{-1}$ for sewage treatment plant effluent. In the present study, our data show that prolonged exposure to GO at concentrations more than 500 μg L$^{-1}$ would result in the adverse effects on environmental organisms. These data imply that long-term release of GO in the range of certain doses into the environment will cause the risk on environmental health. Nevertheless, we further indicated that prolonged exposure to GO at concentrations less than 500 μg L$^{-1}$ would not influence development and function of primary and secondary targeted organs in nematodes.

Previous in vitro assays demonstrated that graphene and GO can cause a dose-dependent oxidative stress in cells. Here we further provided the in vivo evidence that GO exposure can induce oxidative stress, which was correlated with the altered growth, reproduction, locomotion behavior, and intestinal autofluorescence in GO-exposed nematodes. Especially, our data imply that superoxide dismutase, iron-sulfur protein, subunit of mitochondrial complex I, and ubiquinone biosynthesis protein COQ7 may be involved in the control of GO toxicity in nematodes. Therefore, we hypothesize that oxidative stress in the intestine may serve as a key activator for adverse effects on the functions of primary and secondary targeted organs in GO-exposed nematodes.

Fig. 6 Effects of GO exposure on intestinal permeability in C. elegans. (a) Nile Red staining of nematodes exposed to GO. (b) Comparison of relative fluorescent intensities of Nile Red in nematodes exposed to GO. (c) Sudan Black staining of nematodes exposed to GO. (d) Comparison of triglyceride amount of nematodes exposed to GO. (e) Expression pattern comparison of genes required for intestinal development in GO-exposed nematodes. The final results were expressed as the relative expression ratio (between targeted gene and tba-1 reference gene). Prolonged exposure to 100 mg L$^{-1}$ of GO was performed from L1-larvae to adult. Bars represent means ± SEM. **p < 0.01.

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Previous studies demonstrated that GO can accumulate in several targeted organs of mice. In human fibroblast cells, GO can enter into lysosomes, mitochondria, endoplasm, and cell nuclei. In this study, our data demonstrated that GO could be translocated into the body of nematodes by crossing the intestinal barrier. Moreover, we observed the large accumulation of GO in intestinal cells, and GO was adjacent to or surrounded mitochondria in intestinal cells. These data imply that GO may cause the adverse effects on nematodes by negatively influencing development or function of mitochondria, such as mitochondrial generation of ROS or mitochondrial respiration. Previous studies demonstrated that GO exposure increased the rate of mitochondrial respiration in the lung cells of mice or altered mitochondrial activity in adherent human skin fibroblast. It was further reported that GO could induce mitochondrial generation of ROS.

For the underlying mechanism explaining the GO toxicity in C. elegans, we hypothesized here that GO toxicity may be explained by the combination of enhanced intestinal permeability and suppressed defecation behavior. Loss of many microvilli on intestinal cells, increased Nile Red staining, and expression pattern alteration of genes required for intestinal development demonstrated the possible hyper-permeable state of the intestinal barrier in GO-exposed nematodes. The increased mean defecation cycle length and the expression pattern alteration of genes required for defecation implies the suppression of defecation behavior in GO-exposed nematodes. Nevertheless, prolonged exposure to GO at the examined
concentrations did not completely block the defecation behavior, implying that a certain amount of GO can still be removed from the body by nematodes.

**Conclusion**

In conclusion, we compared the possible *in vivo* effects of GO between acute exposure and prolonged exposure using *C. elegans* as the assay system. Our data demonstrated that prolonged exposure to 0.5–100 mg L$^{-1}$ of GO might induce the adverse effects on environmental organisms. The induced adverse effects from GO exposure contained functional alterations of both primary and secondary targeted organs in *C. elegans*. For the mechanism explaining the toxicity of GO, we raise a hypothesis here that GO toxicity may due to the combinational effects of enhanced permeability of the intestinal barrier and suppressed defecation behavior. Our data imply the possible risk of GO to environmental organisms after release into the environment. Our results will shed light on the further design of surface modifications, such as PEGylation, to reduce or prevent the adverse effects of GO on environmental organisms.

**Experimental**

**Reagents and preparation of GO suspensions**

GO was prepared from natural graphite powder by the modified Hummer’s method. Graphite (2 g) and sodium nitrate (1 g) were added to a 250 mL flask, and then concentrated H$_2$SO$_4$ (50 mL) was slowly added with stirring on ice. After stirring the mixture for 30 min, KMnO$_4$ (7 g) was added to the mixture over 1 h, and 90 mL of H$_2$O was slowly added dropwise to cause an increase in temperature to 70 °C after the temperature of the mixture was warmed to 35 °C. The diluted suspension was stirred at 70 °C for another 15 min, and further treated with a mixture of 7 mL of 30% H$_2$O$_2$ and 55 mL of H$_2$O. The resulting warm suspension was filtered to result in a yellow-brown filter cake, which was washed three times with a solution of 3% HCl, followed by drying at 40 °C for 24 h. Finally, GO was obtained by ultrasonication of as-prepared graphite oxide in water for 1 h.

GO was dispersed in K medium ([50 mM NaCl, 30 mM KCl, 10 mM NaOAc], pH 6.0) to prepare the stock solution (1.0 mg mL$^{-1}$). Stock solution was sonicated for 30 min (40 kHz, 100 W) and diluted to different concentrations with K medium.

**Fig. 7** Effects of prolonged exposure to GO on defecation behavior in *C. elegans*. (a) Comparison of mean defecation cycle length in GO-exposed nematodes. (b) Expression pattern comparison of genes required for defecation in GO-exposed nematodes. The final results were expressed as the relative expression ratio (between targeted gene and *tba-1* reference gene). Prolonged exposure to GO was performed from L1-larvae to adult. Bars represent means ± SEM. **p < 0.01.
just prior to exposure. All the other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Characterization of GO

The prepared GO materials were characterized by TEM (JEM-200CX, JEOL, Japan), AFM (SPM-9600, Shimadzu, Japan), FTIR (Avatar 370, Thermo Nicolet, USA), Raman spectroscopy (Renishaw Invia Plus laser Raman spectrometer, Renishaw, UK), and Nano Zetasizer (Malvern Instrument Ltd., UK). TEM images were collected on the filed emission JEM-200CX transmission electron microscopy, equipped with a CCD camera. A few drops of GO suspension solution were deposited on a TEM grid, dried, and evacuated before analysis. To perform the AFM measurement, a few drops of the GO suspension solution were pipetted on Si substrates, and substrates were air-dried and placed directly under AFM tip for morphology analysis. Zeta potential was analyzed by the Nano Zetasizer using a dynamic light scattering technique.

Strain preparation

Nematodes used in the present study were wild-type N2, originally obtained from the Caenorhabditis Genetics Center (funded by the NIH National Center for Research Resource, USA). They were maintained on nematode growth medium (NGM) plates seeded with Escherichia OP50 at 20 °C as described. Gravid nematodes were washed off plates into centrifuge tubes, and were lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl). Age synchronous populations of L1-larvae and L4-larvae nematodes were obtained as described. Exposures to GO were performed on L4-larvae nematodes for 24 h (acute exposure), or from L1-larvae to adult (prolonged exposure) in 12-well sterile tissue culture plates at 20 °C in the presence of food. The exposed nematodes were used for toxicity evaluation using lethality, growth, reproduction, locomotion behavior, intestinal autofluorescence, and intestinal ROS production as endpoints when they developed into adults.

Lethality, growth, reproduction, and locomotion behavior

For lethality assay, a 1.0 mL aliquot of test solution for GO was added to each well of tissue culture plate, which was subsequently loaded with approximately 50 nematodes for each concentration. Following exposure, wells were observed under a dissecting microscope, where inactive ones were scored. Nematodes were judged to be dead if they did not respond to stimulus using a small, metal wire. Lethality was evaluated by the percentage of survival animals. Five replicates were performed.

Growth was assessed by body length, which was determined by measuring the flat surface area of nematodes using the Image-Pro® Express software. Ten nematodes were used for each growth assay.

Reproduction was assessed by brood size. To assay brood size, the number of offspring at all stages beyond the egg was counted. Ten nematodes were used for each reproduction assay. Five replicates were performed.

Locomotion behaviors of nematodes were evaluated by head thrash and body bend. To assay head thrash, the examined nematodes were washed with K medium, and transferred into a microtiter well containing 60 μL of K medium on the top of agar. After a 1 min recovery period, head thrashes, defined as a change in the direction of bending at the mid body, were counted for 1 min. To assay body bend, the examined nematodes were picked onto a second plate and scored for the number of body bends in an interval of 20 s. A body bend was counted as a change in the direction of the part of nematodes corresponding to the posterior bulb of pharynx along the y axis, assuming that nematode was traveling along the x axis. Twenty nematodes were used for each locomotion behavior assay.

Intestinal autofluorescence

Images were collected for fluorescence in the endogenous intestine using a 525 nm bandpass filter and without automatic gain control to preserve the relative intensity of the fluorescence of different animals. Fluorescence was recorded and color images were taken for the documentation of results with Magnafire® software (Olympus, Irving, TX, USA). Lipofuscin levels were measured using ImageJ software (NIH Image, Bethesda, MD, USA) by determining the mean pixel intensity in the intestine of each nematode. Twenty nematodes were used for each intestinal autofluorescence assay.

Intestinal ROS production

The examined nematodes were transferred to 1 mL of M9 buffer containing 1 μM CM-H$_2$DCFDA in 12-well sterile tissue culture plates to pre-incubate for 3 h at 20 °C, and then mounted on 2% 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. The relative fluorescent intensities of intestines were semiquantiﬁed, and the semiquantiﬁed ROS was expressed as relative fluorescent units (RFU). Twenty nematodes were used for each intestinal ROS production assay.

Defecation

To assay mean defecation cycle length, an individual animal was examined for a ﬁxed number of cycles, and a cycle period was deﬁned as the interval between initiations of two successive posterior body-wall muscle contraction steps. Thirty nematodes were used for each mean defecation cycle length assay.

Nile Red and Sudan Black staining

Staining experiments were performed as previously described. Nile Red (Molecular Probes, Eugene, OR) was dissolved in acetone to produce a 0.5 mg mL$^{-1}$ stock solution and stored at 4 °C. Stock solution was freshly diluted in 1× PBS to 1 μg mL$^{-1}$, and 150 μL of the diluted solution was used to stain nematodes. Twenty nematodes were used for each Nile Red staining.

To perform Sudan Black staining, the examined nematodes were washed in M9 buffer and fixed in 1% paraformaldehyde in M9 buffer. Nematodes were then subjected to 3 freeze–thaw cycles and dehydrated through an ethanol series. Nematodes were stained overnight in a 50% saturated solution of Sudan
Black in 70% ethanol, rehydrated, and then photographed. Twenty nematodes were used for each Sudan Black staining.

Analysis of triglyceride content

Lipids of nematodes were extracted by the method of Bligh and Dyer. Triglyceride content was measured using an enzymatic kit (Wako Triglyceride E-test, Wako Pure Chemical Ltd., Osaka, Japan). Ten plates of nematodes were used for each triglyceride content assay. Ten replicates were examined per treatment.

Electron microscopy

Nematodes were fixed in 0.5% glutaraldehyde and 2% osmium tetroxide. The fixed nematodes were embedded in araldite resin following the infiltration series (30% araldite/acetone for 4 h, 70% araldite/acetone for 5 h, 90% araldite/acetone overnight, and pure araldite for 8 h). Serial sections were collected using an Ultracut E microtome. Images were obtained on a Hitachi H-7100 electron microscope using a Gatan slow scan digital camera.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using RNeasy Mini Kit (Qiagen). Total nematode RNA (~1 μg) was reverse-transcribed using cDNA Synthesis kit (Bio-Rad Laboratories). Quantitative reverse transcription PCR (RT-PCR) was run at the optimized annealing temperature of 58 °C. The relative quantification of targeted genes required for oxidative stress, intestinal development or defecation behavior in comparison to the reference tba-1 gene, encoding the tubulin, was determined. The final results were expressed as the relative expression ratio (between targeted gene and reference gene). The designed primers for targeted genes and reference tba-1 gene were shown in Table S5.

Statistical analysis

All data in this article were expressed as means ± standard error of the mean (SEM). Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA). Probability levels of 0.05 and 0.01 were considered statistically significant. Associations of intestinal ROS production with growth, reproduction, locomotion behavior, and intestinal autofluorescence were assessed with linear regression analysis. The independent variable was intestinal ROS production, and the dependent variables were growth, reproduction, locomotion behavior, and intestinal autofluorescence.

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