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An epigenetic signal encoded protection mechanism is activated by graphene oxide to inhibit its induced reproductive toxicity in *Caenorhabditis elegans*

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ABSTRACT

Although many studies have suggested the adverse effects of engineered nanomaterials (ENMs), the self-protection mechanisms for organisms against ENMs toxicity are still largely unclear. Using *Caenorhabditis elegans* as an *in vivo* assay system, our results suggest the toxicity of graphene oxide in reducing reproductive capacity by inducing damage on gonad development. The observed reproductive toxicity of GO on gonad development was due to the combinational effect of germline apoptosis and cell cycle arrest, and DNA damage activation might act as an inducer for this combinational effect. For the underlying molecular mechanism of reproductive toxicity of GO, we raised a signaling cascade of HUS-1/CLK-2-CEP-1-EGL-1-CED-4-CED-3 to explain the roles of core apoptosis signaling pathway and DNA damage checkpoints. Moreover, we identified a miRNA regulation mechanism activated by GO to suppress its induced reproductive toxicity. A *mir-360* regulation mechanism was activated by GO to suppress its induced DNA damage-apoptosis signaling cascade through affecting component of CEP-1. Our identified epigenetic signal encoded protection mechanism activated by GO suggests a novel self-protection mechanism for organisms against the ENMs toxicity.

1. Introduction

Engineered nanomaterials (ENMs), defined as materials possessing at least one dimensional structure and diameters less than 100 nm, have been widely used in a broad range of areas in human endeavors. Graphene oxide (GO), a member of graphene family, can be potentially applied in biomedicine including drug delivery, tissue engineering, biosensor, and imaging due to its properties of single-atom thick and two-dimensional sheet with hexagonally arranged carbon atoms [1–4]. Meanwhile, both *in vitro* and *in vivo* studies have suggested that GO exposure would result in the adverse effects on human health and environmental organisms. GO exposure could be translocated into the targeted organs and caused the generation of reactive oxygen species (ROS), induction of cell death, pulmonary toxicity, and immunotoxicity [5–12]. More recently, it was reported that GO could result in the reproductive toxicity in animals [13,14].

Caenorhabditis elegans (*C. elegans*), a free-living nematode, has been developed as a useful non-mammalian alternative toxicity assay model [15]. *C. elegans* has the developmental properties of short lifespan, short and prolific life cycle, small and transparent body, ease of culture and maintenance, and well-characterized genetic background [16,17]. *C. elegans* is sensitive to environmental toxicants based on toxicity assessment using some sub-lethal endpoints such as lifespan, development, reproduction, and locomotion behavior [17–19]. *C. elegans* has been used for translocation and toxicological studies of carbon-based ENMs including fullerene, carbon nanotubes, graphite, graphene quantum dots, and GO [8,20–25]. Previous studies have suggested that prolonged or chronic exposure to GO caused several aspects of toxicity on nematodes [8,26–30]. GO exposure at the concentration of 100 mg/L could cause the reduced body length and lifespan, and GO exposure at concentrations of 0.1–100 mg/L did not induce the lethality of nematodes [26]. Moreover, GO exposure at concentrations more than 1 mg/L led to the reproductive toxicity by decreasing the brood size in nematodes [26]. GO could be translocated into both the primary targeted organs such as intestinal cells and the secondary targeted organs such as reproductive organs of gonad and spermatheca [26,31,32]. Meanwhile, some

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microRNAs (miRNAs) were suggested to be involved in the control of GO toxicity in nematodes [32]. Nevertheless, the molecular basis for GO toxicity on reproductive organs is still largely unclear.

Considering the crucial functions of reproductive organs during the development, using *C. elegans* as an *in vivo* assay system, we examined the molecular basis of GO toxicity on reproductive organs of animals. Among the targeted reproductive organs, we focused on the gonad to investigate the adverse effects of GO in inducing germline apoptosis and the underlying molecular mechanism. Moreover, we identified an epigenetic signal encoded protection mechanism was activated by GO to be against its induced reproductive toxicity, which implies a novel self-protection mechanism for organisms to be against ENMs toxicity.

2. Materials and methods

2.1. Preparation and characterization of GO

GO was prepared from natural graphite powder as described previously [33,34]. Graphite (2 g) and sodium nitrate (1 g) were added into a 250-mL flask, and concentrated H_2SO_4 (50 mL) was added on ice. After the addition of $KMnO_4$ (7 g), 90 mL of H_2O was slowly dripped into the paste. The diluted suspension was stirred at 70 °C for 15 min, and then treated with a mixture of 7 mL of 30% H_2O_2 and 55 mL of H_2O. After filtering the resulting warm suspension, the obtained yellow-brown filter cake was washed for three times with a solution of 3% HCl, followed by drying at 40 °C for 24 h. The GO will be finally obtained by ultrasonication of as-made graphite oxide in water for 1 h. GO was dispersed in K medium to prepare stock solution (1 mg/mL), which was sonicated for 30-min (40 kHz, 100 W) and diluted to the used concentrations (1, 10, 100, and 1000 mg/L) with K medium just prior to exposure. All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

GO was characterized by transmission electron microscopy (TEM, JEM-200CX, JEOL, Japan), atomic force microscopy (AFM, SPM-9600, Shimadzu, Japan), and zeta potential analyzed by the Nano Zetasizer using a dynamic light scattering technique.

2.2. C. elegans strain preparation

Nematodes used in the present study were wild-type N2, mutants of MT1522/*ced-3(n717)*, MT2547/*ced-4(n1162)*, MT8735/*egl-1(n1084n3082)*, MT4770/*ced-9(n1950)*, WS2277/*hus-1(op241)*, TJ1/*cep-1(gk138)*, SP506/*clk-2(mn159)*, MT15018/*mir-360(n4635)*, MT12969/*mir-259(n4106)*, and MT13954/*mir-81&82(nDf54)*, and transgenic strains of MD701/*bcIs39*[P*lim-7-ced-1::GFP*], WS1433/*hus-1(op241)opIs34*[*hus-1::GFP*], and *Ex*(P*mir-360-mir-360*). Some of the strains were originally obtained from *Caenorhabditis* Genetics Center (CGC, funded by NIH Office of Research Infrastructure Programs (P40 OD010440)). Nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C as described previously [16]. Gravid nematodes collected from NGM plates by washing were lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl), and age synchronous populations of L1-larvae were obtained as described [35]. Prolonged exposure to GO was performed from L1-larvae to young adults in 12-well sterile tissue culture plates at 20 °C in the presence of food of OP50.

2.3. Germline apoptosis assay

Germline apoptosis assay was performed with a modified acridine orange (AO) staining as described previously [36]. The examined nematodes were transferred into a Costar 24-well plate containing aliquots of 25 μg/mL of AO. To facilitate uptake of dye, OP50 was added prior to staining. Nematodes were incubated at 20 °C for 60 min, and allowed to recover for 60 min on bacterial lawns to repel excessive dye in intestine. Nematodes were mounted onto agar pads on slides, and examined under an epifluorescence microscopy (Olympus BX41, Olympus Corporation, Japan) equipped with an AxioCam MRm camera. Only the gonad arm in the posterior part of the body was scored. Twenty nematodes were examined per treatment, and three replicates were performed.

2.4. Assay of the number of apoptotic cells per gonad arm using CED-1::GFP transgenic strain

Apoptotic cells were also measured using MD701 transgenic nematodes, which can help us directly visualize apoptotic cells with the aid of CED-1::GFP fusion protein [37]. In MD701, CED-1::GFP is expressed in the nuclei of gonadal sheath cells [37]. After exposure, CED-1::GFP transgenic nematodes were mounted onto agar pads on slides in 5 M NaN_3 and examined under a fluorescence microscope (Olympus BX41, Olympus Corporation, Japan). The apoptotic cells were labeled with a bright GFP-positive circle, while intact cells were uniformly green in color. The number of apoptotic cells in the germline meiotic region of one gonad arm was scored. Twenty nematodes were examined per treatment, and three replicates were performed.

2.5. Fertility ability assay

Fertility ability was assessed by the endpoints of brood size, and number of oocytes. Brood size was counted as the number of offspring at all stages beyond the egg was counted [38]. Number of oocytes was counted as the oocyte number in single gonad arm, because in most conditions the intestine would shade the gonad arm [39]. Egg ejection was also determined by count the eggs on the plate every hour after removing the single laying nematode to a new NGM plate [40]. Embryonic lethality was analyzed by dividing the number of eggs remaining by the total number plated. Twenty nematodes were examined per treatment, and three replicates were performed.

2.6. Assay of DNA damage using HUS-1::GFP transgenic strain

DNA damage was analyzed using WS1433 transgenic strain with the aid of HUS-1::GFP fusion protein [41]. HUS-1::GFP can be localized in the nuclei of proliferating and meiotic germ cells [41]. HUS-1::GFP foci were quantified by counting the number of bright foci present in the middle/late pachytene germ cells under a fluorescence microscope (Olympus BX41, Olympus Corporation, Japan). Twenty nematodes were examined per treatment, and three replicates were performed.

2.7. Assay of 4′,6-diamidino-2-phenylindole (DAPI) staining

DAPI can be used to assess nuclear morphology and/or quantify the number of mitotic cells in germline [42]. Transfer the examined nematodes into the NaN_3 drop on a microscope slide. Using of syringe needle cut the pharynx in order to outflow the gonad. After removing NaN_3 using filter paper, add 10 μL of Carnoy' fluid (ethanol:trichloromethane:acetic acid = 6:3:1) for fixation. Remove the Carnoy' fluid after 15 min, and then add DAPI at a final concentration of 2 ng/μL in M9 buffer containing 0.5% Tween 20. Place a coverslip and then fix it at both sides with nail polish. Slides were observed under a fluorescence microscope (Olympus BX41, Olympus Corporation, Japan) after approximately 5 min staining. Six replicates were performed.