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Toxicity of Low-dose Graphene Oxide Nanoparticles in an *in-vivo* Wild Type of *Caenorhabditis elegans* Model

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ABSTRACT

Carbon-based engineered nanomaterials, such as graphene oxide nanoparticles (GO NPs), are widely available for application, but their potentially adverse health effects on humans still require investigation. In this study, the environmental levels of GO NPs are addressed to examine whether GO leads to adverse effects on an *in-vivo* model of *Caenorhabditis elegans* (*C. elegans*). Nematodes with prolonged exposure (L1 larvae to young adult) to GO NPs at 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1} were used to evaluate the potential toxic effects, including lethality (acute toxicity), reproductive (brood size) and neurological (locomotion including head thrash and body bend) responses, longevity (lifespan), and oxidative stress (gene expression of sod-1, sod-3, and clt-2). Prolonged exposure to GO NPs was not found to induce lethality at the selective levels. In the brood-size and head-thrash tests, the biological responses in nematodes were significantly reduced at 0.0100-1.00 ng L^{-1} GO NP exposure as compared with the untreated control. The nematodes exposure to GO NPs at 0.00100–1.00 ng L^{-1} exhibited significant delays in body bending behavior compared with the control. In the examination of the longevity of nematodes,

it was found that the lifespan of all GO NP-exposed worms was significantly shortened as compared to the untreated worms. Gene expression of sod-1, sod-3, and ctl-2 presented significantly higher induction folds in the exposed worms compared with the controls. Consequently, prolonged exposure to the low-dose GO NPs might be associated with disruption of reproduction and locomotion, attenuation of longevity, and induction of oxidative stress in nematodes.

Keywords: Graphene oxide, *Caenorhabditis elegans*, Reproductive toxicity, Neurobehavioral toxicity, Oxidative stress

1 INTRODUCTION

Graphene oxide nanoparticles (GO NPs), one of the most promising derivatives of graphene, comprise a monolayered engineered nanomaterial (ENM) with high oxygen-containing functional groups such as carboxyl, epoxy, carbonyl, and hydroxyl (Bianco *et al.*, 2013; Li *et al.*, 2015). Graphene oxide (GO) is known to have excellent dispersibility in many solvents, chemical reactivity, and the capacity for chemical functionalization (Konios *et al.*, 2014; De Marchi *et al.*, 2018). The adsorption capacity of GO NPs has been taken advantage of for environmental remediation applications such as in GO-based membranes, which remove gaseous contaminants such as sulfur dioxide and hydrogen sulfide (Fakhri, 2017). In addition, they also act as adsorbents for elimination of various aqueous contaminants owing to their high content of functionalized oxygen groups available to interact with metal ions (Li *et al.*, 2019). Furthermore, GO NPs can be applied in nanoelectronics, catalysis, nanocomposites, sensor technology, water purification and desalination, and drug delivery (Zhang *et al.*, 2011; Pan *et al.*, 2012; Giust *et al.*, 2018; Prasad *et al.*, 2020). According to studies from Yang *et al.* (2015) and Maharubin *et al.* (2016), GO can be utilized for hydrogen storage (anode, cathode, and lithium sulfur batteries) and supercapacitor management. Furthermore, GO composites are used as antimicrobial agents for water disinfection to remove organic molecules and waterborne pathogens (Upadhyay *et al.*, 2014). Several studies used the environmental levels at μg L^{-1} to mg L^{-1} of GO NP contamination to test the *in-vivo* models (He *et al.*, 2017; Zhang *et al.*, 2017; Li *et al.*, 2019).

The global market for graphene-based products, such as GO, is increasing. The demand is expected to be $675 and 987 million by 2020 and 2022, respectively (Ahmed and Rodrigues, 2013). Due to their potential for both production and application, GO materials are expected to be released in the environment during their lifecycle and eventually be generated in landfills and wastewater treatment plants (Du *et al.*, 2017; Suárez-Iglesias *et al.*, 2017; Jamialahmadi *et al.*, 2018). GO can be released into the water environment through the development of its composites as adsorbents for aqueous contamination, membranes for water filtration and purification, and catalysts for environmental decontamination (Zhao *et al.*, 2014; Goodwin *et al.*, 2018). Several studies have reported that the dispersion and long retention time of GO within microbial communities can lead to serious negative effects on wastewater microbial flora due to its hydrophility (Lyon and Alvarez, 2008; Kang *et al.*, 2009; Rodrigues and Elimelech, 2010). The predicted environmental concentrations of GO can be correlated with those of multi-walled carbon nanotubes because both have relatively similar properties, such as nanometer size, a carbon-based structure, and applications in consumer electronic devices (Zhang *et al.*, 2017).

Few epidemiological studies focused on human exposure to GO particularly for the highly exposed population. For the *in-vivo* models including rats, mice, zebra fish, nematodes, and daphnia, the animals could induce nanotoxicity including acute, developmental, neurological, reproductive, immunological, and neurobehavioral toxicity as well as shortened longevity after they were exposed to GO NPs (Sanchez *et al.*, 2012; Patlolla *et al.*, 2017; Qu *et al.*, 2017; Souza *et al.*, 2017; Kim *et al.*, 2018; Qu *et al.*, 2019; Kim *et al.*, 2020). In the past years, *in vivo* and *in vitro* GO NPs toxic effects, including immunotoxicity, activation of inflammation, induction of reactive oxygen species (ROS), generation of oxidative stress, apoptosis, and potential GO exposure mechanisms have been investigated (Guo and Mei, 2014; Bengtson *et al.*, 2017; Pelin *et al.*, 2018; Tang *et al.*, 2018). The accumulation of GO in the cytoplasm causes dramatic morphological alterations and reduces the ability of toll-like receptor 4 (TLR4) for phagocytosis (Qu *et al.*, 2013a). However, an increase in intracellular ROS contributes to necrotic cell death in macrophages (Qu *et al.*, 2013a). Previous

studies have also reported that GO promotes cell growth inhibition, hatching delay, ROS generation, and damages the circulatory system of zebrafish embryos (Liu *et al.*, 2014; Chen *et al.*, 2016; Souza *et al.*, 2017). In mice, GO can accumulate in organs such as the liver, lungs, spleen, and kidneys, which may induce organismal toxicity through intracellular oxidative stress caused by the accumulation of ROS (Qu *et al.*, 2013b; Yang *et al.*, 2013). GO can enter the human body through inhalation and may be deposited in regions of the respiratory tract. When deposited in alveolar regions, it may impair clearance, form granulomas, and possibly produce fibrosis (Sanchez *et al.*, 2012).

The *in vivo* model used in this study was the transparent nematode, *Caenorhabditis elegans* (*C. elegans*), which has been successfully used in toxicological evaluation of various nanomaterials such as GO NPs (Zhang *et al.*, 2012; Wu *et al.*, 2013; Piechulek and von Mikecz, 2018). Advantages of *C. elegans* as an in-vivo model system were as the following: (1) simple anatomy, (2) transparent, (3) invariant cell lineage, (4) short life cycle with large brood size, (5) easily accessible embryos, (6) easy and cheap maintenance in lab, and (7) powerful experimental tool (Brenner, 1974; Hunt, 2017). The *C. elegans* model is not a mammal model to unavailable to examine several toxic endpoints like blood sugar and pressure, tissues in the skin, probiotic system in the intestine, heart and cardiovascular diseases. *C. elegans* is considered to be a novel tool for *in-vivo* techniques, and testing of *C. elegans* is known to be analogous to mammalian neurotoxin testing (Cole *et al.*, 2004). Recently, scientists have focused on the disruption of biological effects from ROS, reproductive effects, gene expression, neurological development, and neurobehavior with treatment of GO NPs in *C. elegans* models (Wu *et al.*, 2013; Qu *et al.*, 2017; Kim *et al.*, 2018; Rive *et al.*, 2019; Kim *et al.*, 2020; Zhao *et al.*, 2020). Zhang *et al.* (2012) indicated no negative impact on longevity after exposing L4-larva-to-young-adult *C. elegans* to GO NPs at concentrations ranging from 5 to 20 mg L^{-1}. Inversely, *C. elegans* with prolonged exposure (from L1 larva to young adult) to 0.5–100 mg L^{-1} of GO NPs presented adverse effects on primary (digestive organs such as the intestine) and secondary (neurological tissues such as neurons and reproductive organs) target organs (Wu *et al.*, 2013). GO NPs possibly shortened lifespan by influencing the expression of the DAF-2-AGE-1-AKT-1/2-DAF-16 signaling cascade in the intestine of the nematodes (Zhao *et al.*, 2016b). After GO NP exposure, expression of neuronal substances may decrease ROS generation and reduce locomotion behavior in nematodes (Zhao *et al.*, 2020). GO NPs probably caused damage to the dopaminergic and glutamatergic neurons in *C. elegans* after chronic exposure to GO NPs for 6 days, from L1 larvae to the adult stage (Li *et al.*, 2017). Liu *et al.* (2020) observed that GO NPs induced intestinal barrier dysfunction in *C. elegans*. Rive *et al.* (2019) proposed that worms chronically (or prolongedly) exposed to GO NPs (levels of 100 and 200 mg L^{-1}) were significantly shortened in size and developed morphological abnormalities in the pharynx and intestine. Kim *et al.* (2018) found accumulation of GO NPs in the reproductive organs of *C. elegans* using Raman spectrometry. Also, GO NP exposure promoted reproductive toxicity by suppressing spermatogenesis of *C. elegans* during development, resulting in decreased sperm numbers and progeny numbers (Kim *et al.*, 2018). This study was aimed toward evaluating the effect of environmentally-relevant concentrations of GO NPs in *C. elegans* by assessing toxicological endpoints including acute lethality, reproduction, locomotion, lifespan, and gene expression.

2 METHODS

2.1 Preparation of Graphene Oxide Nanoparticles

GO NPs were prepared from expandable graphite using a modified Hummers' method (Yan *et al.*, 2014). In brief, 1 g graphite power and 50 mL sulfuric acid (H_2SO_4, 98%) were poured into a 250 mL flask, followed by the addition of 0.5 g $NaNO_3$. The mixture was mechanically agitated for 30 min in an ice bath. For further oxidation, 5 g of potassium permanganate ($KMnO_4$) was added while slowly stirring the mixture for 4 h. Subsequently, H_2O_2 was added to MnO_2 until the mixture became yellow. Afterward, 1% HCl was added, and the mixture was centrifuged at 8000 rpm for 5 min, followed by washing 3 times with distilled water to dilute the acid solution.

2.2 Reagents, Chemicals, and Nematode Cultivation

The GO NPs underwent sonication for 30 min (40 kHz and 100 W) to disperse them in K medium (50 mM, 30 mM KCl, 1.0 mg mL^{-1} and PH of 6.0) as the stock solution (200 mg L^{-1}) following the

methods in previous studies (Wu *et al.*, 2013; Zhao *et al.*, 2016b). The stock solution was diluted to various concentrations using K medium prior to exposure.

The wild-type N2 *C. elegans* strain was gifted from Dr. Chang-Shi Chen in the Department of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University (Tainan, Taiwan). *C. elegans* was maintained on nematode growth medium (NGM) seeded with OP50 *Escherichia coli* (*E. coli*) cultures from the Bioresources Collection and Research Center (Hsinchu, Taiwan), and Luria-Bertani broth was obtained from Sigma-Aldrich (St. Louis, MO, USA). The NGM plates contained bacteriological agar and bactopeptone, which were obtained from Laboratories Conda (S.A., Spain). The NaCl was obtained from Honeywell Fluka™ (New Jersey, USA). Age-synchronized worms were collected using a bleaching mixture that contained NaOCl obtained from J.T. Baker (Central Valley, PA) and KOH obtained from Duksan Pure Chemicals (Gyeonggi-do, South Korea). Supplemental reagents such as $CaCl_2$, K_2HPO_4, and cholesterol were obtained from Sigma-Aldrich (St. Louis, MO, USA); $MgSO_4$ was acquired from Avantor Performance Materials, ltd. (Gyeonggi-do, South Korea); the KH_2PO_4 used for the phosphate buffer was acquired from Avantor Performance Materials, LLC (Radnor, PA, USA), and the Na_2HPO_4 used for the M9 buffer was obtained from Honeywell Fluka™ (New Jersey, USA). All physiological observations were done under a dissecting microscope (Olympus, SZX10, Waltham MA, USA). The experimental protocols in the nematode model followed those used in our previous study (Chung *et al.*, 2019, 2020). The lethality, growth, reproduction, locomotion behavior examinations followed the protocols previously published, with minor modifications (Chung *et al.*, 2019, 2020).

2.3 Lethality and Lifespan Assay

The nematodes were exposed to GO NPs (control, 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1}) for 48 h (prolonged exposure) from L1-larvae to young adults incubated at 20°C. Prolonged exposure was performed in a fresh plate with an OP50 *E. coli* lawn. After treatment, the lethal toxicity of the samples was evaluated by softly poking them using a worm picker. The worms that did not respond were considered dead. Three biological replicates were performed, and a total of 150 worms were assayed.

The worms evaluated for the lifespan assay were exposed for a prolonged period of time to the different GO NP concentrations (the untreated control, 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1}) from L1 to the mature stage for the lifespan test. Fifty worms were transferred to fresh plates every other day for 4–5 days of egg-laying. Live and dead nematodes were evaluated daily by softly poking them with a worm picker. Three biological replicates were performed, and a total of 150 worms were evaluated. Several lifespan indicators (mean lifespan, day of 50^{th} percentile death, day of 75^{th} percentile death, day of 95^{th} percentile death, and day of all death) were evaluated by following the Chung's study (Chung *et al.*, 2020).

2.4 Reproductive Assay and Locomotion Assay

The brood size (reproductive assay) of the L3 or young L4 nematodes was assessed for 4–5 days after prolonged exposure to different GO NP concentrations (control, 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1}) at 20°C. Each worm was transferred to a fresh plate and transferred again until the egg-laying period stopped. The plates with eggs were incubated until the progeny could be easily counted. A total of 30 worms was evaluated for the reproductive assay.

Locomotion behavior including head thrashing and body bending in the nematode models was expressed as the motor neuron function (Qu *et al.*, 2019; Zhao *et al.*, 2020). The body bending and head thrashing of the nematodes (locomotion assay) were evaluated after prolonged exposure to various concentrations of GO NP (control, 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1}). The body bending was evaluated by transferring the exposed worm onto a fresh plate. After one day, the body bending of the worms was counted for 20 secs. The head thrashing was evaluated by placing the exposed worm on a glass slide containing an adequate amount of K-media. The head thrashing of the worms was counted for 1 min. Three biological replicates were performed, where 60 worms were evaluated for body bending, and 30 worms were evaluated for head thrashing.

2.5 Gene Expression Tests

C. elegans in the different treatment groups (untreated control, 0.00100, 0.100, and 1.00 μg L^{-1})

were collected from three replicates for RNA extraction after exposure. Trizol reagent (TIANGEN, China) was used to extract total RNA in accordance with the manufacturer's standard protocol. RNA concentrations were measured by the absorbance at 260 nm, and the RNA purity was evaluated based on the ratio of the optical densities from RNA samples measured at 260 and 280 nm. The first-strand cDNA synthesis reaction was conducted with 500 ng of purified RNA using a Fast Quant RT Kit (with gDNase) according to the manufacturer's protocol (TIANGEN, China). Specific superoxide dismutase genes, including (sod) 1 (sod-1), sod-3, and catalase 2 (ctl-2) were detected in the present study. The data were analyzed using the 2-ΔΔCt method, as previously reported (Zhou *et al.*, 2016), and the mRNA expressions were normalized based on the act-1 mRNA. For each tested gene, a qRT-PCR analysis was conducted in triplicate (technical replicates).

2.6 Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) version 12 software (International Business Machines Corp., New York, USA) was used to perform all statistical analyses. All data was checked to normality, and the Shapiro-Wilk test was used determined the normal and non-normal distribution. A one-way ANOVA was used to analyze the significance levels of the differences between treatments. The plots and figures were made using GraphPad Prism 6 (San Diego, California, USA).

3 RESULTS AND DISCUSSION

3.1 GO NPs Lethality

C. elegans was evaluated through a variety of toxicological endpoints, including lethality, reproduction, locomotion, lifespan, and oxidative stress (gene expression of sod-1, sod-3, and clt-2) in the present study. This study is the first time that low doses of GO NP (approximately at least 1000-fold lower compared to those used in the previous studies) has been used to examine nanotoxicity in a *C. elegans* model. Exposure from L1-larvae to young adult was performed to assess the effects of prolonged GO exposure on both larvae and adult nematodes. As shown in Fig. 1, the survival rate of the nematodes after prolonged exposure to GO did not indicate significant lethal effects. No significant between-group differences in mortality were observed in the

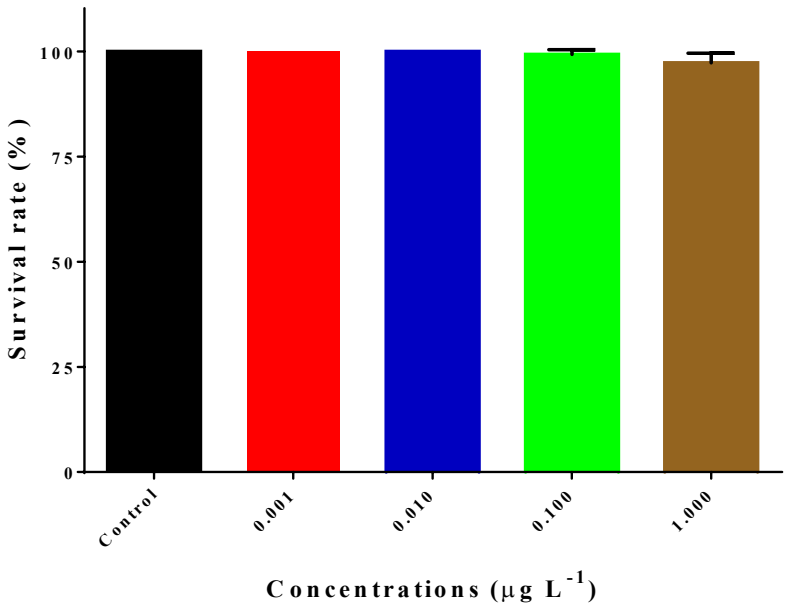


Fig. 1. Survival rates of *C. elegans* after prolonged exposure to GO NPs at concentrations of 0.00100, 0.0100, 0.100, and 1.00 and the untreated control. Bars shown as mean ± SD. Significant differences were expressed as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

control group and in the group treated with concentrations ranging from 0.00100 to 1.00 μg L^{-1}. Few studies have examined acute toxicity of GO NPs in N_2 *C. elegans* models (Wu *et al.*, 2013, 2014; Li *et al.*, 2019). Wu *et al.* (2013) indicated that no lethality was observed at GO NP concentrations from 0.100 to 100 mg L^{-1} after the worms were acutely or prolongedly exposed to these carbon-based ENMs. On the contrary, a study examining acute toxicity with treatment of high doses of 5, 10, 50, and 100 mg L^{-1} of GO NPs in nematodes found that GO concentrations higher than 5 mg L^{-1} may cause lethality, where no worms survived at a dosage of 100 mg L^{-1} (Li *et al.*, 2017). Wu *et al.* (2014) also examined low levels of GO NPs from 0.00100 to 1.00 mg L^{-1} and found no significant differences in lethality except in the case of the highest concentration of 1.00 mg L^{-1} after the nematodes were chronically exposed to these carbon-based ENMs from L1 larvae to adult-day 8. Most GO NP studies refer to Wu's study (Wu *et al.*, 2013) and use similar dosage levels (ppm levels) to examine neurological, reproductive, neurobehavioral, and immunological toxicity, and inflammatory responses.

3.2 Reproductive Toxicity of GO NPs

Reproduction in nematodes is a vital endpoint because it has been shown to be sensitive to lower concentrations of chemical stressors than those that impair the behavior and viability of nematodes (Wu *et al.*, 2019). The results in Fig. 2 show that prolonged exposure to GO NPs in the nematodes reduced brood size production. A significant decrease was observed in the progeny number at concentrations of 0.0100 (p = 0.036), 0.100 (p = 0.008), and 1.00 ($p < 0.001$) μg L^{-1} of GO NPs. The reduction rates in the brood size at these three concentrations compared to the control group were 15.2, 20.1 and 27.3%, respectively. The results showed that higher concentrations of GO NPs induced more reproductive toxicity based on our experiments on brood size number in nematodes. Our results were consistent with most GO NP studies reporting that GO exposure can cause adverse effects through damaging the fertility and egg ejection behavior of nematodes (Wu *et al.*, 2013; Zhao *et al.*, 2016a; Kim *et al.*, 2018; Rive *et al.*, 2019). Wu *et al.* (2013) showed that *C. elegans* with prolonged exposure to 1–100 mg L^{-1} exhibited significantly decreased brood size compared to the control, but there were no significant between-group differences at 0.1 and 0.5 mg L^{-1}. A similar result was also found in a previous study (Rive *et al.*, 2019), indicating that prolonged exposure to GO NPs at 100 and 200 mg L^{-1} significantly decreased egg-laying rates compared to the untreated control. Kim *et al.* (2018) revealed that accumulation of GO NPs (10 mg L^{-1}) in the reproductive organs, which might be

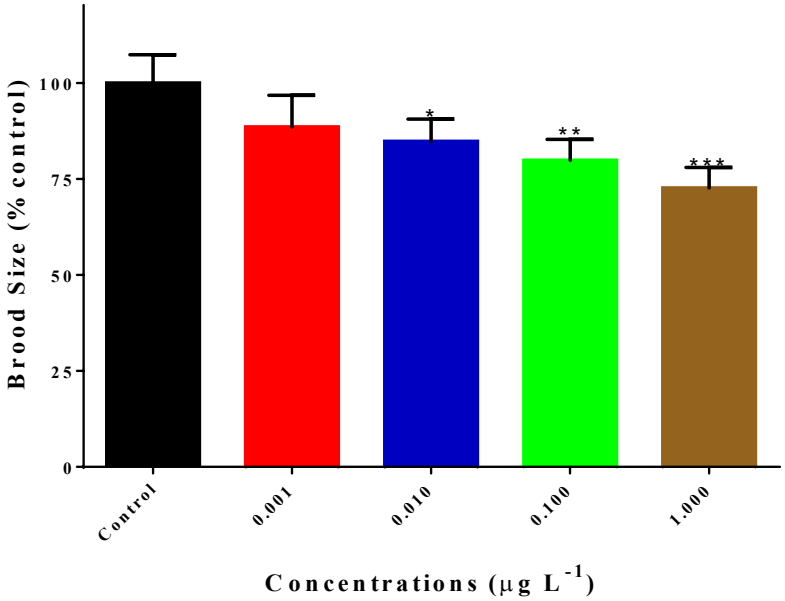


Fig. 2. Effects of the brood size in the *C. elegans* after prolonged exposure to GO NPs at concentrations of 0.00100, 0.0100, 0.100, and 1.00 and the untreated control. Bars shown as mean ± SD. Significant differences were expressed as *$p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.

the direct cause of reproductive toxicity, could reduce brood size and sperm count by suppressing spermatogenesis of the hermaphrodite nematodes at the GO levels of 5 or 10 mg L^{-1}. However, the negative impact of GO NP exposure on the reproductive function in the present and published studies (Wu *et al.*, 2013; Zhao *et al.*, 2016a; Kim *et al.*, 2018; Rive *et al.*, 2019), as well as our results, suggest that prolonged exposure to GO NPs at low doses from 0.0100 to 1.00 μg L^{-1} could decrease progeny number or fecundity in N_2 *C elegans* models.

3.3. GO NP Exposure Affects Locomotive Behavior

Locomotive behavior assays are well-established methods for studying nematode neurotoxicity. After prolonged exposure, GO induced obvious decreases in both head thrashing and body bending in nematodes (Fig. 3). In the head thrash examination, 0.0100, 0.100 and, 1.00 μg L^{-1} concentrations of GO NPs significantly decreased head thrashing by 12.0, 5.41, and 19.8%, respectively, compared

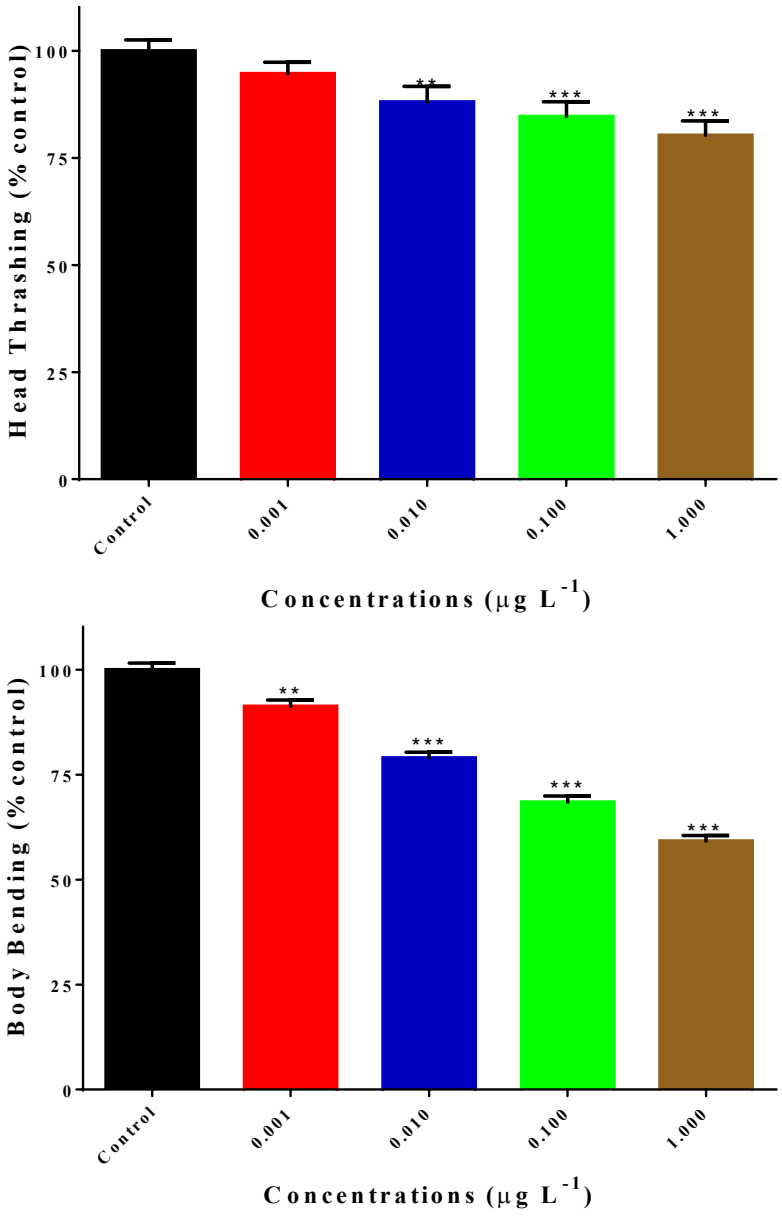


Fig. 3. Effects of head thrashing and body bending in the nematodes after prolonged exposure to GO NPs at concentrations of 0.00100, 0.0100, 0.100, and 1.00 and the untreated control. Bars shown as mean ± SD. Significant differences were expressed as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

to the untreated control. Furthermore, body bending was significantly reduced at 0.00100, 0.0100, 0.100 and 1.00 μg L^{-1} GO NPs by 8.78, 21.2, 31.5, and 40.8%, respectively, in comparison with the control groups. Our results were consistent with those in most published articles, implying that GO NP exposure damages the neurological functions and negatively disrupts head thrashing and body bending behavior (Wu *et al.*, 2013, 2014; Zhao *et al.*, 2015, 2016c; Chen *et al.*, 2017; Li *et al.*, 2017; Qu *et al.*, 2017; Kim *et al.*, 2018; Rive *et al.*, 2019; Zhao *et al.*, 2020). In Wu's report (Wu *et al.*, 2014), head thrash and body bend locomotion was significantly reduced at 0.0100, 0.100, and 1.00 mg L^{-1} levels compared with an untreated control. Li *et al.* (2017) indicated that prolonged exposure to GO NPs (5.00–100 mg L^{-1}) significantly reduced body bending, head thrashing, pharynx pumping frequency, mean speed, bending angle-frequency, and the wavelength of the crawling movement of nematodes. GO NPs also induced damage to dopaminergic and glutamatergic neurons in nematodes (Li *et al.*, 2017). Kim *et al.* (2020) also proposed that GO significantly accumulated in the head regions, generated ROS induction, reduced neurotransmitter substances in dopaminergic and glutamatergic neurons, and damaged AFD neurons, which are the main thermosensors in *C. elegans*, after the nematodes were exposed to GO NPs (10 mg L^{-1}). In a Korean study, Kim *et al.* (2018) also found that neurotransmitters, such as dopamine, γ-Aminobutyric acid (GABA), tyramine, tryptophan, and tyrosine, were reduced in nematodes exposed to GO NPs. According to the current data, including the present study (Wu *et al.*, 2013, 2014; Zhao *et al.*, 2015, 2016a; Chen *et al.*, 2017; Li *et al.*, 2017; Qu *et al.*, 2017; Kim *et al.*, 2018; Rive *et al.*, 2019; Zhao *et al.*, 2020), it has been concluded that GO NPs exposure causes adverse effects on the neurological system of *C. elegans* particularly in terms of damage to neurons, influences on neurotransmitter neurodisruptions, and delays in neurobehavioral development. In the present study, environmental levels (0.0100–1.00 μg L^{-1}) of GO NP doses were used to treat the nematodes to determine the negative impact on their locomotion behavior.

3.4 Effect of GO NPs on Lifespan

In *C. elegans* models, lifespan is an important endpoint for assessment of toxicants. After prolonged exposure in nematodes, GO NPs at concentrations of 0.00100–1.00 μg L^{-1} led to shorter lifespans than was the case for the untreated controls (Fig. 4). Several indicators of lifespan, including mean lifespan (Fig. 4(b)), mean day of median (50^{th} percentile) death (Fig. 4(c)), mean day of 75^{th} percentile death (Fig. 4(d)), mean day of 95^{th} percentile death (Fig. 4(e)), and the day of all death (Fig. 4(a)) indicated significantly longer longevity in the untreated control as compared to in GO NP-exposed nematodes ($p < 0.001$). The mean lifespan and the day of all death were 13.9, 7.01, 6.23, 6.94, and 6.35 days in the untreated control and 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1}, respectively, and 20, 16, 14, 14, 16 days in the untreated control and 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1}, respectively. After 6 days, the percent survival rate of nematodes decreased to as much as 50% of the total population. It was also observed that the nematodes treated with GO NPs exhibited faster reductions in lifespan than the control group. In summary, Fig. 4 indicates that prolonged exposure to GO NPs reduces the lifespan of nematodes ($p < 0.001$). According to the current data from previous reports (Zhang *et al.*, 2012; Zhao *et al.*, 2016b, 2016c; Qu *et al.*, 2017; Rive *et al.*, 2019), contradictory results were obtained, where two studies indicated that GO NP exposure, including both acute and prolonged exposure didn't have effects on longevity (Zhang *et al.*, 2012; Rive *et al.*, 2019), and other studies obtained different results indicating that the worms with prolonged exposure to GO NPs exhibited significantly reduced longevity (Zhao *et al.*, 2016b, 2016c; Qu *et al.*, 2017) at GO NP concentrations between 1.00 and 200 mg L^{-1}. Two molecular mechanisms of intestinal insulin signaling may be involved in the shortened longevity of nematodes exposed to an GO NP concentration of 100 mg L^{-1} due to association with suppression of DAF-16 and sod-3 functions (Zhao *et al.*, 2016b). Based on our results, the low dose of 0.00100 μg L^{-1} significantly reduced the nematodes' longevity.

3.5 Gene Expression after GO NPs Exposure

The sod genes encode superoxide dismutases (SODs), which comprise an antioxidant system for *C. elegans* against oxidative stress after GO NP exposure (Ren *et al.*, 2018). SODs which exist in three isoforms of sod1, sod2, and sod-3 are a class of the antioxidant protein. The increased folds in expression from the induced sod-1, sod-3, and ctl-2 genes after *C. elegans* had undergone

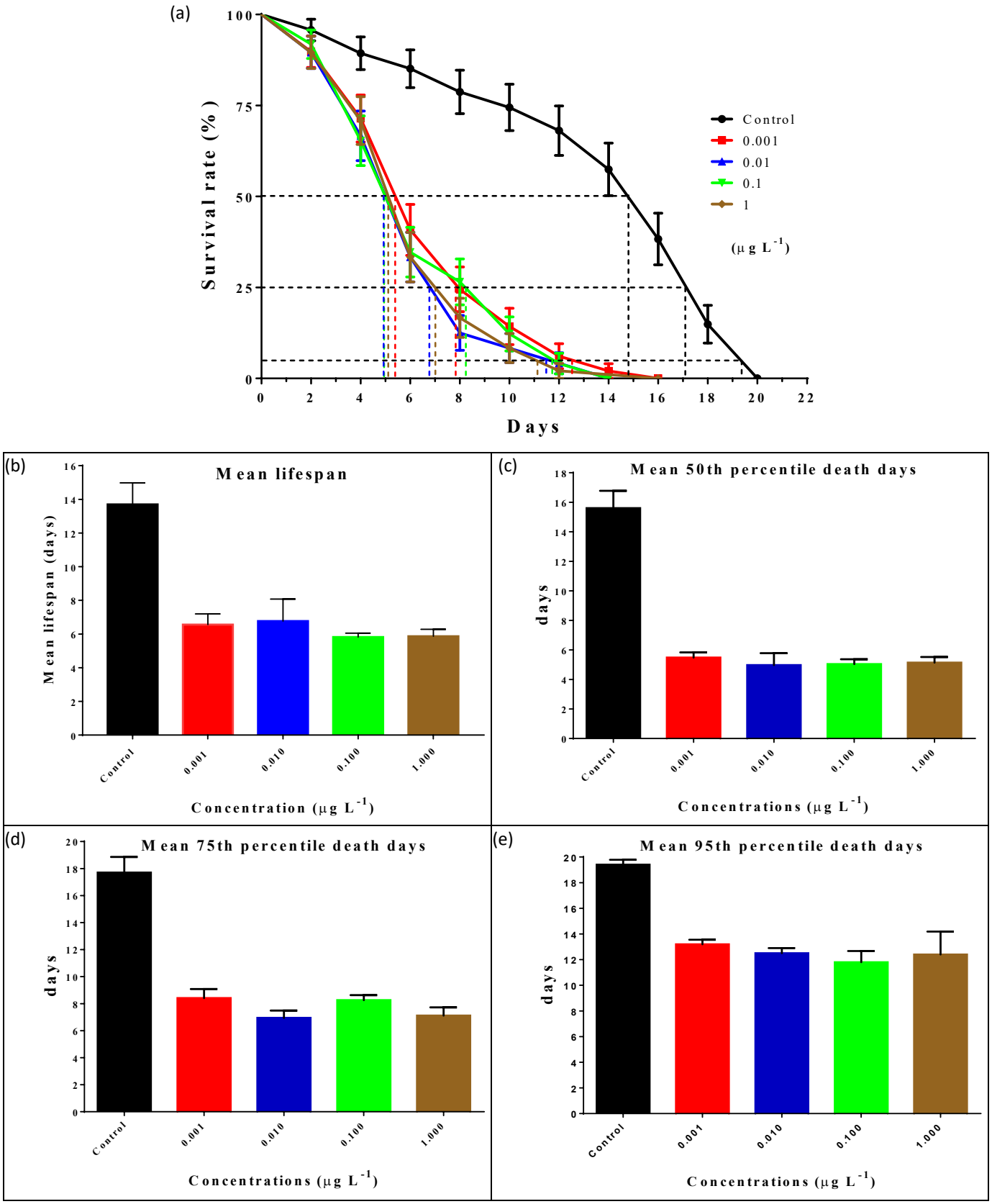
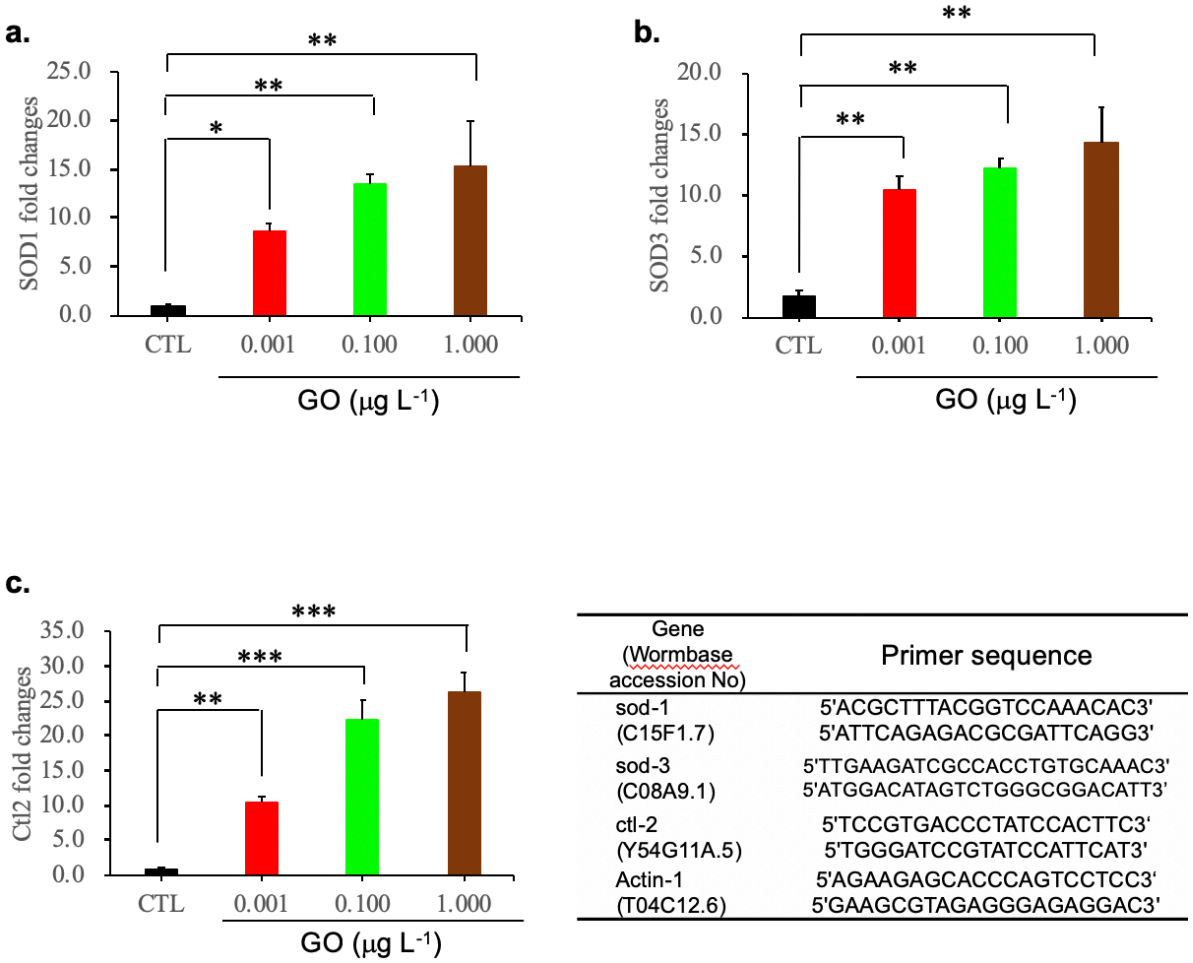


Fig. 4. Lifespan of *C. elegans* after prolonged exposure to GO NPs at levels of 0.00100, 0.0100, 0.100, and 1.00 μg L^{-1} and the control (a) nanotoxic assessment of worms with prolonged GO exposure for lifespan, (b) mean lifespan, (c) mean day of 50th percentile death, (d) mean day of 75th percentile death, and (e) mean day of 95th percentile death. Bars shown as mean ± SD. Significant differences were expressed as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

prolonged exposure to 0.00100, 0.100, and 1.00 μg L^{-1} GO NP compared with the untreated control are shown in Fig. 5. The activated expressions of sod-1, sod-3, and ctl-2 at the concentrations of 0.00100, 0.100, and 1.00 μg L^{-1} in the GO NP-exposed *C. elegans* were significantly higher than those in the untreated control. SOD is a key enzyme in the detoxification function of free radicals. It removed free radicals generated from GO NPs in extracellular sources in nematodes. Results similar to those found in the present study were also found in previous studies (Wu *et al.*, 2013; Zhao *et al.*, 2016b), which indicates that GO NPs could induce sod-1 or sod-3 activation. The findings from Wu's study suggested that oxidative stress induced in the treated GO NP nematodes may be related to changes of SOD activities (Wu *et al.*, 2013). Based on these findings, it can be inferred that oxidative stress is a possible mechanism causing adverse effects on neurodevelopment and neurobehavioral development after prolonged GO NP exposure, as suggested in previous reports (Wu *et al.*, 2013; Zhao *et al.*, 2016b), in combination with the results of induced SOD activation and neurotoxicity in the GO-exposed nematodes in the present study (Figs. 3 and 5). Furthermore, sod-1, sod-3, and ctl-2 activation may be associated with the shortened longevity in the GO-exposed worms, based on Figs. 4 and 5. In Zhou's study (Zhou *et al.*, 2016), *C. elegans* ctl-2 gene encoded peroxisomal catalase was linked to environmental oxidative stress after worms were exposed to bisphenol A. Few studies have addressed to link between ctl-2 expression and GO exposure in *C. elegans*. Although a positive association between ctl-2 expression and GO NP exposure was shown in the present study, the mechanism is still unclear.

Finally, it was concluded in the present study that extremely low doses of GO NPs, compared with the dosages discussed in recent published articles, can cause reproductive and neurobehavioral



Gene (Wormbase accession No)	Primer sequence
sod-1 (C15F1.7)	5'ACGCTTTACGGTCCAAACAC3' 5'ATTCAGAGACGCGATTCAGG3'
sod-3 (C08A9.1)	5'TTGAAGATCGCCACCTGTGCAAAC3' 5'ATGGACATAGTCTGGGCGGACATT3'
ctl-2 (Y54G11A.5)	5'TCCGTGACCCTATCCACTTC3' 5'TGGGATCCGTATCCATTCAT3'
Actin-1 (T04C12.6)	5'AGAAGAGCACCCAGTCCTCC3' 5'GAAGCGTAGAGGGAGAGGAC3'

Fig. 5. Gene expression in *C. elegans* with prolonged exposure to GO NPs at the levels of the untreated control, 0.00100, 0.100, and 1.00 μg L^{-1} (a) SOD-1 (C15F1.7), (b) SOD-3 (C08A9.1), and (c) ctl-2 (Y54G11A.5); Actin-1 (T04C12.6) as the internal control. Bars shown as mean ± SD. Significant differences were expressed as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

toxicity and induce several-fold increases of sod-1, sod-3, and clt-2 gene expression. It is worth noting that in the present study, the potentially toxic effects of environmental levels of GO NPs in *in-vivo C. elegans* models were evaluated to show the negative impacts on reproduction, neurobehavioral development, and oxidative stress. It is thus reiterated that based on our findings, GO NPs at environmental levels may cause chronically toxic effects.

4 CONCLUSIONS

It is the first time to use the low dosage of GO NPs treating in the *in-vivo* model to find the adverse effects in nematodes. Based on our findings, prolonged exposure to GO NPs causes reproductive effects, generates neurotoxicity, shortens longevity, and induces oxidative stress in *C. elegans*. It is reiterated that low-dose GO NPs at environmental levels from 0.00100 to 1.00 μg L^{-1} caused significantly negative impacts on nematodes in contrast to the current published data. Thus, the adverse effects of low-level GO NPs on human health should be evaluated in the future.

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DISCLAIMER

The authors of this paper declare no conflict of interest.

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