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Toxic Assessment of Heavily Traffic-related Fine Particulate Matter Using an *in-vivo* Wild-type *Caenorhabditis elegans* Model

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ABSTRACT

In association with the mortality rate due to air pollution, vehicular emitted fine particles ($PM_{2.5}$) are a threat to public health. $PM_{2.5}$-induced *in-vivo* studies on environmental microorganisms can be used to assess the adverse impacts of $PM_{2.5}$ on human health. In the present study, the toxicity of traffic-related-air-pollutant (TRAP) $PM_{2.5}$ was evaluated in the animal model *Caenorhabditis elegans* (*C. elegans*) using different toxicological endpoints such as lethality, survivability (lifespan), behavioral (head thrashing and body bending), and reproduction (brood size). The TRAP $PM_{2.5}$ sample were collected in Taichung City, Taiwan from Mar 24 to April 15 in 2018. Of these 23 day samples, three samples (Days A, B, and C) were randomly selected. The results showed that no immediate lethality was observed after acute (24 h) exposure of the nematodes. On the other hand, sublethal endpoints of reproduction exhibited statistically significant dose-dependent reduction, although Day A and Day C did not decrease the egg-laying capability of the worms. For the neurological toxicity, it is inferred that the higher the $PM_{2.5}$ concentrations, the more the adverse effects of neurobehavior (head trashing and body bending) it poses on the *C. elegans*. The lifespans of nematodes exposed to heavily TRAP $PM_{2.5}$ were significantly shortened compared with those of untreated ones based on survival rate. The nematodes exposed $PM_{2.5}$ models not only posed potentially adverse health effects on human but also represented ecotoxic impacts on the ecosystem. In conclusion, heavy concentrations of TRAP $PM_{2.5}$ significantly and severely disrupted toxicological endpoints of neurology and reproduction to *C. elegans*. TRAP $PM_{2.5}$ significantly shortened the lifespan of the nematodes compared with the control. TRAP $PM_{2.5}$ might more severely influenced the specific toxic endpoints, such as lifespan and neurobehavira, in this *in-vivo* models compared with the reproductive endpoints.

Keywords: $PM_{2.5}$; Traffic related air pollutant (TRAP); *C. elegans*; Lifespan; Reproduction; Locomotion.

INTRODUCTION

Ambient and indoor air pollution, the most important environmental risk to health, contributed 7 million deaths each year in the past decade (WHO, 2016). The risks that entail $PM_{2.5}$ or fine particulate have threatened the mortality

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of human lives and the adverse health effects associated with $PM_{2.5}$ made it as a major indicator of air particle pollution by the World Health Organization (WHO, 2005). Reports have shown that worldwide exposure to outdoor $PM_{2.5}$ contributed to 4.1 million deaths connected with heart disease and stroke, lung cancer, chronic lung disease, and respiratory infections (Orru *et al.*, 2011; HEI, 2018; Li *et al.*, 2018; Hayes *et al.*, 2019). The increasing epidemiological studies indicate that cardiopulmonary morbidity due to $PM_{2.5}$ exposure play a part in the development of diabetes mellitus and adverse birth outcomes (Hu, 2009; Crouse *et al.*, 2012; Kloog *et al.*, 2012; Chen *et al.*, 2013; Burnett *et al.*, 2014; Zanobetti *et al.*, 2014). $PM_{2.5}$ is accountable for a substantially larger number of attributable deaths than other more well-known life behavioral risk factors such as physical inactivity, alcohol use, and high sodium intake, and it is equivalent to the attributable deaths caused by high cholesterol and high body mass index (HEI, 2018).

$PM_{2.5}$ has chemical constituents such as sulfates, nitrates, and ammonium and its large surface area enable it to carry various toxic compounds such as polycyclic aromatic hydrocarbons (PAHs), black carbon, phthalates, and heavy metals (Yue *et al.*, 2006; Labrada-Delgado *et al.*, 2012; Jiang *et al.*, 2019; Wang *et al.*, 2019; Xing *et al.*, 2020). $PM_{2.5}$ can be emitted directly into the air due to anthropogenic activities or it can be formed in the atmosphere, creating secondary particles (Lu *et al.*, 2019; Lisetskii *et al.*, 2019). $PM_{2.5}$ has also been identified as one of the major contributor in traffic-related air pollution (TRAP) (Chao *et al.*, 2018; Xiang *et al.*, 2019; Min *et al.*, 2020). Motor vehicle traffic is an important source of harmful emissions of $PM_{2.5}$ in the cities of developing countries (Kinney *et al.*, 2011; Brown *et al.*, 2019). According to Kinney *et al.*, (2011), daytime concentrations of $PM_{2.5}$ at the sites adjacent to roadways ranged from 50.7–128.7 μg m^{-3} which were higher than WHO's 24-hour average guideline (25 μg m^{-3}) (WHO, 2005). A similar study conducted by Brown *et al.*, (2019) also reported elevated $PM_{2.5}$ concentrations at the near-road sampling sites in Denver and Indianapolis. It is indicative that traffic emissions contribute to $PM_{2.5}$ levels in the environment and may also cause health damage to people exposed to it. Upon inhalation, $PM_{2.5}$ can readily pass through the nose filtration and then deposit at the end of the respiratory tract, consequently damaging other parts of the body through air exchange (Williams *et al.*, 2019).

Furthermore, $PM_{2.5}$ still presents significant risks to public health even at levels far below the national standards (Elliott and Copes, 2011; Fann *et al.*, 2012). Several *in-vitro* and epidemiological studies showed evidences suggesting the negative health effects that airborne TRAP $PM_{2.5}$ might pose to the general public. The induced exposure to $PM_{2.5}$ of human lung bronchial epithelial cells (BEAS-2B) generated DNA breakage and micronucleus formation indicating that it caused oxidative stress (Oh *et al.*, 2011). The $PM_{2.5}$-induced toxicity in rat lung epithelial cells was found to be cytotoxic and it also caused severe oxidative damage to the cells (Choi *et al.*, 2004). It was also found out that people who were exposed to TRAP $PM_{2.5}$ having higher levels of proinflammatory biomarkers such as TNF-α which is an indicator that $PM_{2.5}$ from traffic sources is a significant contributing factor to the increase of systemic inflammation in humans (Brucker *et al.*, 2013; Zhao *et al.*, 2013; Chao *et al.*, 2018). Additionally, a report indicated strong associations between TRAP $PM_{2.5}$ exposure and adverse health effects like atopic diseases and allergic sensitization (Morgenstern *et al.*, 2008).

The 1 mm long free-living soil nematode used in the animal model *Caenorhabditis elegans* (*C. elegans*) established by Sydney Brenner in 1965 plays a key role in the decomposition and nutrient cycling (Sohlenius, 1980). The *C. elegans in-vivo* system has been used as a model for the assessment of toxic effects of air pollutants (Zhao *et al.*, 2014; Sun *et al.*, 2015, 2016; Yang *et al.*, 2016; Wu *et al.*, 2017; Wang *et al.*, 2018; Chung *et al.*, 2019; Zhao *et al.*, 2019) because it shows much advantages over other animal models: production of large number of progeny (100–200 from a single hermaphrodite), transparency, rapid life cycle (3 days), short lifespan (2–3 weeks), low cost, and easy laboratory cultivation (Brenner, 1974; Nass and Hamza, 2007). Moreover, *C. elegans* follows the widely accepted ethical principles, known as Three Rs (Reduction of the use of higher animals, Refinement of current techniques, and Replacement of animals with alternative methods) (Brenner, 1974).

The toxicity effects of $PM_{2.5}$ have been successfully examined using the *C. elegans* animal model. Coal combustion-related $PM_{2.5}$ can induce deficits in the lifespan, development, reproduction, and locomotion behavior of *C. elegans* by altering the expression patterns of genes related to the control of oxidative stress (Sun *et al.*, 2015). Specifically, more severe toxicity was observed for prolonged $PM_{2.5}$ exposures compared to short-term exposures (Sun *et al.*, 2015). In addition, Sun *et al.* (2016) found that heavy metals such as lead, chromium, and copper in coal combustion-related $PM_{2.5}$ induced lower responses on locomotion behavior and lifespan of nematodes, and the combined exposure to these metals caused greater toxicity than single metal exposure. Similarly, Yang *et al.* (2016) pointed out that certain heavy metals in ambient $PM_{2.5}$ possibly caused a decrease in locomotion behavior and production of intestinal reactive oxygen species (ROS) in *C. elegans*; moreover, the gene (*mtl-1* and *mtl-2*) encoded metallothioneins, the proteins involving in the control of stress response to heavy metals in the nematodes, were significantly expressed and could have potential key roles in regulating $PM_{2.5}$ toxicity (Yang *et al.*, 2016). More importantly, a critical finding in *C. elegans* studies is the transgenerational effects of exposure to $PM_{2.5}$. Adverse effects on several toxic endpoints (e.g., reproductive function) were observed not only on the exposed nematodes but also on the later generations of *C. elegans* after exposure to traffic related air pollutants (TRAP) $PM_{2.5}$, which led to the combined effects of oxidative stress, intestinal barrier damage, and abnormal defecation behavior (Zhao *et al.*, 2014) as well as DNA damage (Wang *et al.*, 2019). For the studies of $PM_{2.5}$ toxicity in *C. elegans*, oxidative stress seems to be one of the primary contributing factors to $PM_{2.5}$-mediated toxicity. Oxidative stress was induced via the *mir-231*-SMK-1-SOD-3/SOD-4/CTL-3 signaling pathway after exposure of *C. elegans* to coal combustion-related $PM_{2.5}$ (Wu *et al.*, 2017)

and the expression levels of certain antioxidant enzymes such as glutathione S-transferase (GST-4), superoxide dismutase (SOD-3), glutathione peroxidase (GSH-Px), and catalase (CAT) increased in response to the oxidative stress (Zhao *et al*., 2019). Currently, few studies focused on the toxic effects of TRAP $PM_{2.5}$ on *C. elegans* models: therefore, this study aims to evaluate the toxicity responses of TRAP $PM_{2.5}$ between 24 March and 15 April in 2018 in a major road in Taichung City, Taiwan. Different toxicological endpoints such as survival (lethality and lifespan), behavior or locomotion (head thrash and body bend), and reproduction (brood size) were used in the tested *C. elegans* models.

METHODS

Reagents and Raw Materials

Nematode growth medium (NGM) plates consisted of bacteriological agar and bactopeptone (Laboratories Conda, S.A., Spain) and NaCl (Honeywell Fluka™, New Jersey, USA) were used in this study. Additional components like $CaCl_2$, K_2HPO_4, and cholesterol were all acquired from Sigma-Aldrich (St. Loius, MO, USA), while $MgSO_4$ was acquired from Avantor Performance Materials, Ltd. (Gyeonggi-do, South Korea). Luria-Bertani broth used as a growth medium for *Escherichia coli (E. coli)* was purchased from Sigma-Aldrich. The bleaching solutions utilized in the experiment were NaOCl (J.T. Baker, Central Valley, PA) and KOH (Duksan Pure Chemicals, Gyeonggi-do, South Korea). KH_2PO_4 obtained from Avantor Performance Materials, LLC (Radnor, PA, USA) was used for the phosphate buffer, and Na_2HPO_4 used for the M9 buffer was purchased from Honeywell Fluka™. The components of K-medium were NaCl (Honeywell Fluka™) and KCl (Avantor Performance Materials). All the physiological observations were made using a dissecting microscope (Olympus, SZX10, Wlatham MA, USA).

Air Sampling

The ambient $PM_{2.5}$ air samples were collected from Section 4, Taiwan Avenue, Xitun District, Taichung City. The air sampling site was situated near the Tungdai air monitor site of the Environmental Protection Bureau of Taichung City Government in Tunghai University. Each air sample was collected for 24 hours during the duration from March 24 to April 15 in 2018 in Taiwan through the use of a high-volume air sampler (SIBATA HV-1000R, Japan) following the US EPA Reference Method TO9A or Taiwanese EPA NIEA A205.11C. Quartz fiber filters that had been heated at 600℃ for 2 h before sampling were used to collect $PM_{2.5}$ samples. Each filter paper was conditioned in an electronic desiccator before and after the sample collection for 24 h. It was weighed using a balance with an accuracy of 0.1 mg. The loaded filters were stored in a refrigerator at –20℃ in the National Pingtung University of Science and Technology (NPUST) before extraction in order to limit the possible evaporation of volatile components.

Sample Preparation

Each collected air sample was extracted using a Soxhlet extractor with dichloromethane (DCM) for 24 h. Then, the extract was eluted with 15 mL DCM for the acid-silica column clean-up. The eluate was concentrated to 1 mL then it was placed to a vial. Afterwards, it was further concentrated to near dryness via a nitrogen stream. $PM_{2.5}$ was serially diluted 10X with 1% DMSO for the exposure concentrations. The sample's toxicity tested in the study was controlled in the organic fraction. The samples of 23-day $PM_{2.5}$ samples were prepared for subsequent toxic tests. For the consideration of lifespan more than 22 days, it was decided that the three days, such as Mar 24, April 4, and April 8, were randomly selected from 23 days between Mar 24 and April 15 in 2018.

Age Synchronization of C. elegans

The wild-type N2 strain of *C. elegans* was gifted from the Department of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University (Tainan, Taiwan) and it was maintained in NGM plates seeded with OP50 *E. coli*, which was acquired from the Bioresources Collection and Research Center (Hsinchu, Taiwan), as food and these plates were incubated at 22℃. Then, the plates containing enough eggs and gravid nematodes were chosen and they were washed off with ddH_2O and placed in a centrifuge tube. Afterwards, the gravid nematodes were lysed using the bleaching solution while the eggs remained. The bleaching method lasted for only 6 minutes in order to avoid the eggs to be killed off. Then, the eggs were obtained and placed in a petri dish containing M9 buffer and it was incubated at 22℃ for 12–48 h. After this period, age-synchronized L1 nematode could be produced.

Acute Exposure

The obtained L1 worms were incubated until L3 or young L4 stage. The plates were then gently washed with K-medium in order to get the worms and centrifuged at 2500 x g for 2 minutes. *E. coli* residues were removed by aspirating the supernatant without disturbing the pellet. Afterwards, the pellet was re-suspended in K-medium and the seeding volume (worm μL^{-1}) was calculated by placing two aliquot 10 µL on a glass slide and manually counting the worms per drop. Approximately 200 worms were seeded per well in a 12-well plate containing 1 mL of different exposure concentrations for each samples diluted with K-medium. Then, it was incubated at 22℃ for 24 h without the presence of food.

Lethality Assay

For the lethality assay, 50 exposed worms (per exposure concentration) were transferred into an NGM plate without the presence of food. The viability was assessed by gently poking the worms using a worm picker and the non-responsive nematodes were considered as dead. The assay was done in triplicate for all concentrations.

Locomotion Assay (Head Thrashing and Body Bending)

Head thrashing and body bending behavior were assessed in order to evaluate the motility of the nematodes exposed to different $PM_{2.5}$ concentrations by placing them in a plate containing K-medium. The movement of the head of the worms and/or tail to the same side was counted as one thrash

while the body bending was counted when the worms were able to bend its body in a C-shape. The head thrashing was counted for 1 minute and the body bend for 20 seconds. Thirty worms were assessed and triplicates were made per concentration.

Reproductive Assay (Brood Size)

The brood size or reproductive assay was done by assessing the capability of the L3 or young L4 worms to produce an egg for 4–5 days. One exposed worm is transferred in an NGM plate containing OP50 *E. coli* food and it was incubated at 22℃. After every 2 days, the worms were transferred into a fresh NGM plate until the egg-laying activity ceased. The old plates with eggs were incubated and allowed to grow until L3 for easier counting of progeny. Thirty worms were evaluated for this assay.

Lifespan Assay (Ageing)

Fifty worms obtained from the acute exposure were transferred to NGM plate with food. The worms were transferred every day until the egg-laying period (4–5 days) is done. After that, there was no need to transfer the worms and their viability was assessed every day for 24 days (general lifespan of worms). Then, the worms were scored as alive, censored (lost), and dead. The effects of the different concentrations of $PM_{2.5}$ samples on the lifespan of the nematodes were assessed by constructing the survival plot or Kaplan-Meier plot. Triplicate was done for each exposure concentration.

Statistical Analysis

The data of locomotion and reproduction were found to be not fulfilled normally distribution. The survival plot constructed using Kaplan-Meier were used to evaluate the effects of the $PM_{2.5}$ to the ageing of the worms exposed to different exposure concentrations. The nonparametric Kruskal-Wallis *H* and Mann-Whiney *U* tests were utilized to compare and examine the differences of each concentration to the control. The plots for the lifespan assay were derived using the GraphPad Prism 8 (San, Diego, California, USA) while the significance for each day was defined using the Kruskal-Wallis *H* test. The determined significant days were further tested by comparing them to the control using the Mann-Whitney *U* test. All the statistical analyses were made through the use of Statistical Package for the Social Sciences (SPSS) version 12 (International Business Machines Corp., New York, USA).

RESULTS AND DISCUSSION

Air Pollution in the Highly Heavy Traffic Area

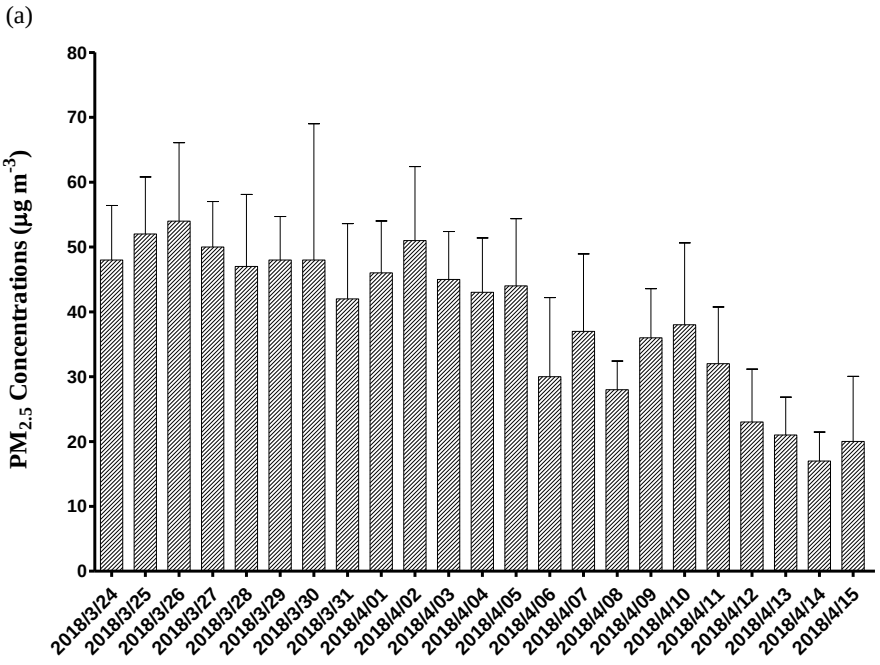
Fig. 1(a) shows the high 24-hour mean levels of $PM_{2.5}$ at the Tungdai air monitor site from March 24 to April 15, 2018 provided from the website of Environmental Protection Bureau, Taichung City. The highest level of $PM_{2.5}$ was 80 μg m^{-3} on April 2 at 1:00 pm and the lowest one was 11 μg m^{-3} on April 6 at 2:00 am. The mean with standard deviation (SD) of 24-hour mean $PM_{2.5}$ for these 23 days was 39.0 ± 3.49 μg m^{-3}. The $PM_{2.5}$ levels in this heavily traffic area during these 23 days were obviously higher than the WHO guideline (25 μg m^{-3}) and most of them exceeded TEPA standard (35 μg m^{-3} for 24-hour mean). High levels of PM_{10}, ozone (O_3), sulfate dioxide (SO_2), nitrogen dioxide (NO_2), and carbon monoxide (CO) were also found in the heavily TRAP area (Figs. 1(b), 2(a), and 2(d)), although the dates of highest levels for these pollutants were different. $PM_{2.5}$ and PM_{10} were highly correlated because they are particulate phase emitted from the TRAP pollution (Fig. 1). Distributed characteristic of $PM_{2.5}$ was inconsistent with those of gas phase pollutants like O_3, SO_2, NO_2, and CO (Figs. 1 and 2).

Lethal Effects of $PM_{2.5}$ to C. elegans

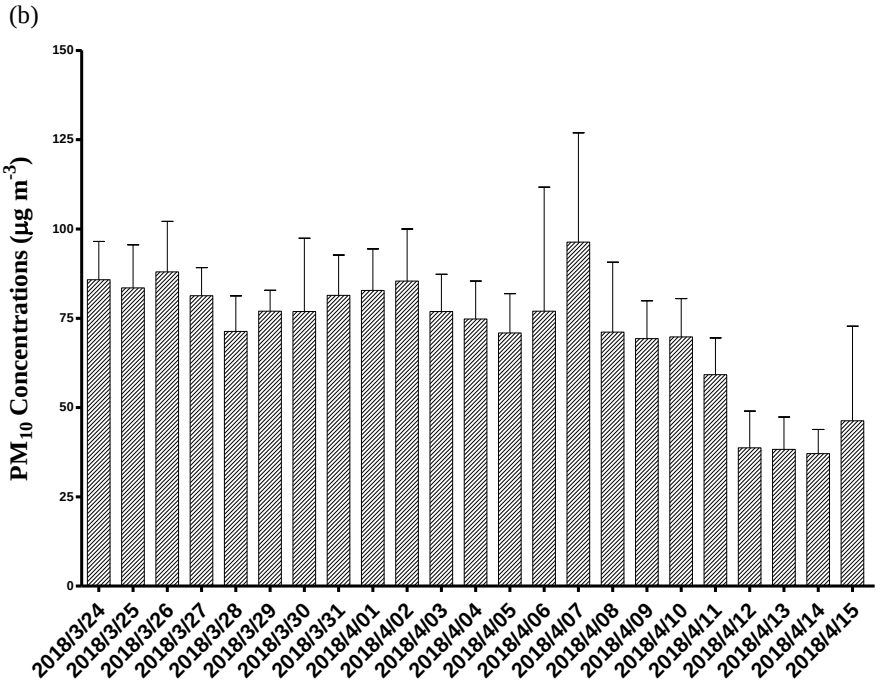
After 24-h exposure, the lethality values of different $PM_{2.5}$ concentrations to the age-synchronized worms were inspected. It was observed that all the samples with different $PM_{2.5}$ concentrations (Day A [March 25 2018] B [April 4 2018], and C [April 8 2018]) did not exhibit any lethal effects on the young L4 nematodes (Figs. 3(a), 3(b), and 3(c), respectively). A similar result obtained by Sun *et al.* (2015) who demonstrated that coal combustion related $PM_{2.5}$ had no lethal effects on exposed nematodes even after prolonged exposure. *Zhao et al.*, (2014) also found that TRAP $PM_{2.5}$ did not induce any adverse effects on the *C. elegans* after acute and prolonged exposure. One our previous report also showed no lethality for any ambient $PM_{2.5}$ sample collected from rural areas including NPUST (0.00144–1440 mg L^{-1}) and Linluo Junior High School (0.00115–1150 mg L^{-1}) (Chung *et al.*, 2019). Further tests were made in order to confirm that $PM_{2.5}$ did not cause immediate mortality to the exposed nematodes. Environmental toxicants, such as TRAP $PM_{2.5}$, can enter the nematode's intestines thereby affecting the intestinal cells and making them as the primary targeted organs. However, they can also be translocated into other parts of the body such as the reproductive organs and even the neurons (Nouara *et al.*, 2013; Zhao *et al.*, 2015). Thus, it is important to perform toxicity assay regarding the locomotion and reproduction in order to determine whether $PM_{2.5}$ caused an adverse effect on the worms.

Effects of TRAP $PM_{2.5}$ on the Brood Size of C. elegans

Brood size assay or the egg laying capability of the nematodes was assessed after exposure to different $PM_{2.5}$ concentrations (Figs. 4(a)–4(c)). The worms exposed to 7.43, 74.3, and 743 g L^{-1} concentrations of Day B $PM_{2.5}$ sample had a significant reduction on their brood size or their egg number; however, those exposed to Days A and C $PM_{2.5}$ samples did not present any effect on their reproduction. Although this result is still unclear, further studies are necessary to identify the underlying mechanism. One our previous study also showed that high ambient $PM_{2.5}$ concentration had a negative effect on the brood size of the *C. elegans* (Chung *et al.*, 2019). Moreover, several studies have demonstrated the dose-dependent manner reduction of the brood size of the nematodes exposed to different toxic compounds, such as $PM_{2.5}$ from coal combustion (Sun *et al.*, 2015), cadmium (Wang *et al.*, 2018), zearalenone (Yang *et al.*, 2018), and silver nanomaterial (Moon *et al.*, 2019). Hodgkin and Barnes (1991) reported that the optimum progeny of a hermaphrodite



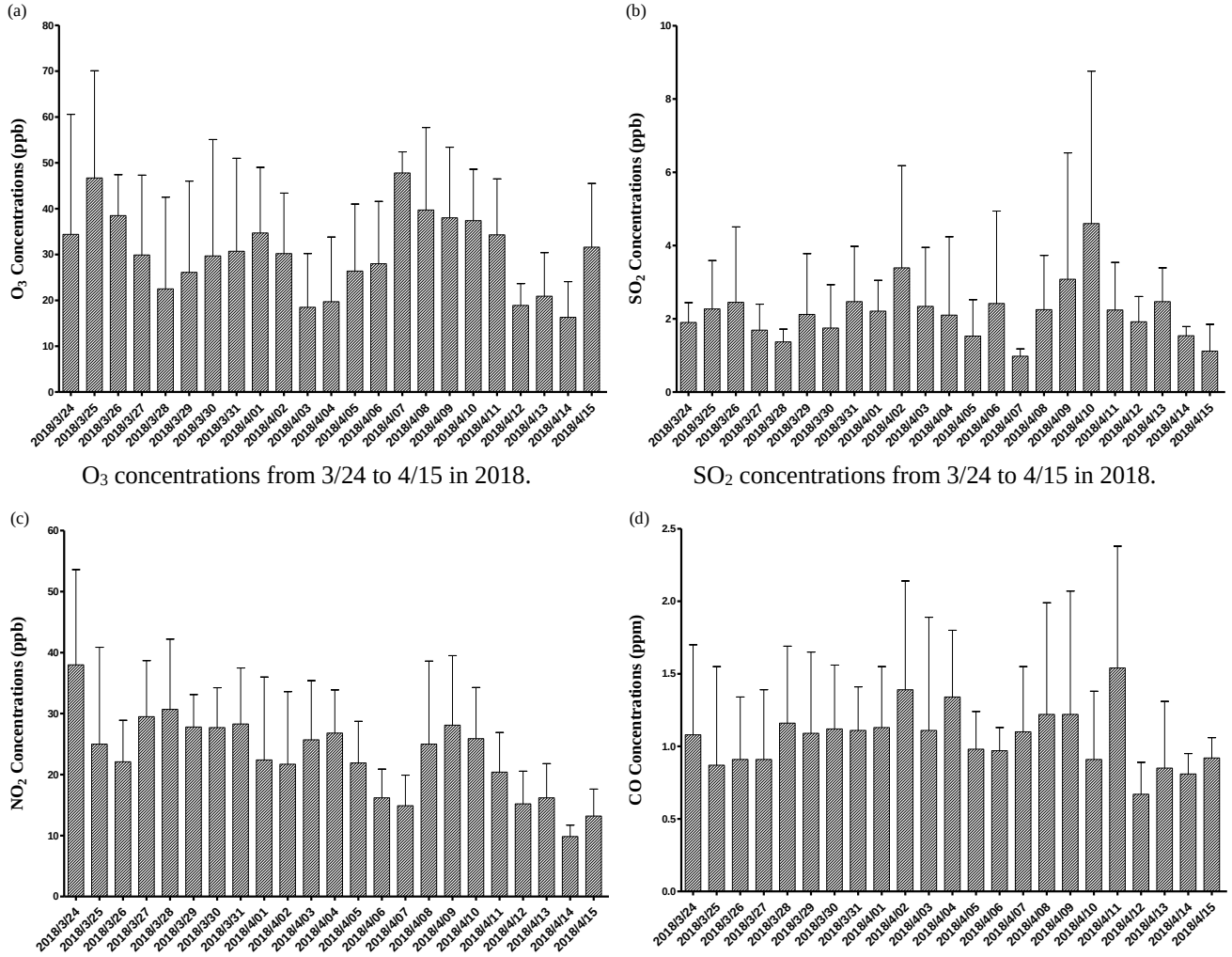
24-hour mean $PM_{2.5}$ concemtrations between March 25 and April 15 in 2018.



24-hour mean PM_{10} concemtrations between March 25 and April 15 in 2018.

Fig. 1. 24-hour mean levels of (a) $PM_{2.5}$ and (b) PM_{10} from March 24, 2018 to April 15, 2018 in the Tungdai air monitor site.

nematode was around 300, but in one our previous report (Chung *et al.*, 2019) and the present study, the fecundity of the *C. elegans* was far below the optimum number reported by Hodgkin and Barnes (1991). Factors such as dietary intake and temperature have been found to have correlation with the delayed and altered reproductive capabilities of the worms (McMullen *et al.*, 2012; Petrella, 2014; El-Hajj and Newman, 2015). Wang *et al*. (2019) indicated that the exposure of *C. elegans* to diesel particulate matter (DPM) (a dominant PM in $PM_{2.5}$) increased a significantly dose-dependent matter in germ line cell apoptosis and exhibited a significantly dose-independent decrease in brood size from 0 to 100 μg mL^{-1}. Zhao *et al.* (2014) revealed that $PM_{2.5}$ at 100 mg L^{-1} from urban areas significantly decreased the brood size of *C. elegans* in the acute exposure scenario and those between 1.00 and 100 mg L^{-1} in the prolonged exposure



O_3 concentrations from 3/24 to 4/15 in 2018.

SO_2 concentrations from 3/24 to 4/15 in 2018.

NO_2 concentrations from 3/24 to 4/15 in 2018.

CO concentrations from 3/24 to 4/15 in 2018.

Fig. 2. (a) Levels of ozone (O_3), (b) sulfur dioxide (SO_2), (c) nitrogen dioxide (NO_2), and (d) carbon monoxide (CO) from Mar 24, 2018 to April 15, 2018 in the Tungdai air monitor site.

scenario. The incubation temperature of the present study in the *C. elegans* model was 22℃. According to Petrella (2014), temperature may affect the worm laying eggs if the incubation temperature is higher than 20℃. Previous studies showed the brood size number between 250 and 300 in the incubation at 20℃ in the control group (Zhao *et al.*, 2014; Wang *et al.*, 2019). It is worth noting that the incubation temperature of 22℃ in the present study made it easy to account the brood size number compared with that of 20℃ due to low number of brood size at 22℃. The results of the brood size numbers in this study is inconsistent with those of two previous studies (Zhao *et al.*, 2014; Wang *et al.*, 2019), particularly for treatment with the toxicants like $PM_{2.5}$, indicating that the incubating temperature was an important factor which influenced the variation of brood size.

Effects of TRAP $PM_{2.5}$ on the Locomotion Behavior of C. elegans

The locomotion assay was performed to investigate the neurological toxicity of TRAP $PM_{2.5}$ and analyze the endpoints of neurobehavior, such as body bending and head thrashing. Highly significant dose-dependent reductions of the head thrashing and body bending of nematodes were observed after subjecting the worms to the $PM_{2.5}$ of Day A (Figs. 5(a1) and 5(a2), respectively). Similar results were also found for the nematodes after exposure to the Days B and C $PM_{2.5}$ samples with different concentrations (Figs. 5(b1), 5(b2), 5(c1), and 5(c2), respectively). The results presented that the higher the $PM_{2.5}$ concentrations was, the more likely the motor neurons of the exposed nematodes were affected by the TRAP $PM_{2.5}$. Even at the lowest concentration (1.29 or 1.67 g L^{-1}), $PM_{2.5}$ still negatively affected the head thrashing and body bending of Days A and C samples although it did not show significant result for the body bending endpoint for Day B sample (the lowest dose = 0.734 g L^{-1}). Also, the body bending values of the nematodes after exposure at the highest $PM_{2.5}$ concentrations of Days A, B, and C were significantly reduced by more than 50% compared with those after exposure at the control. According to our previous study (Chung *et al.*, 2019), there was an obvious reduction

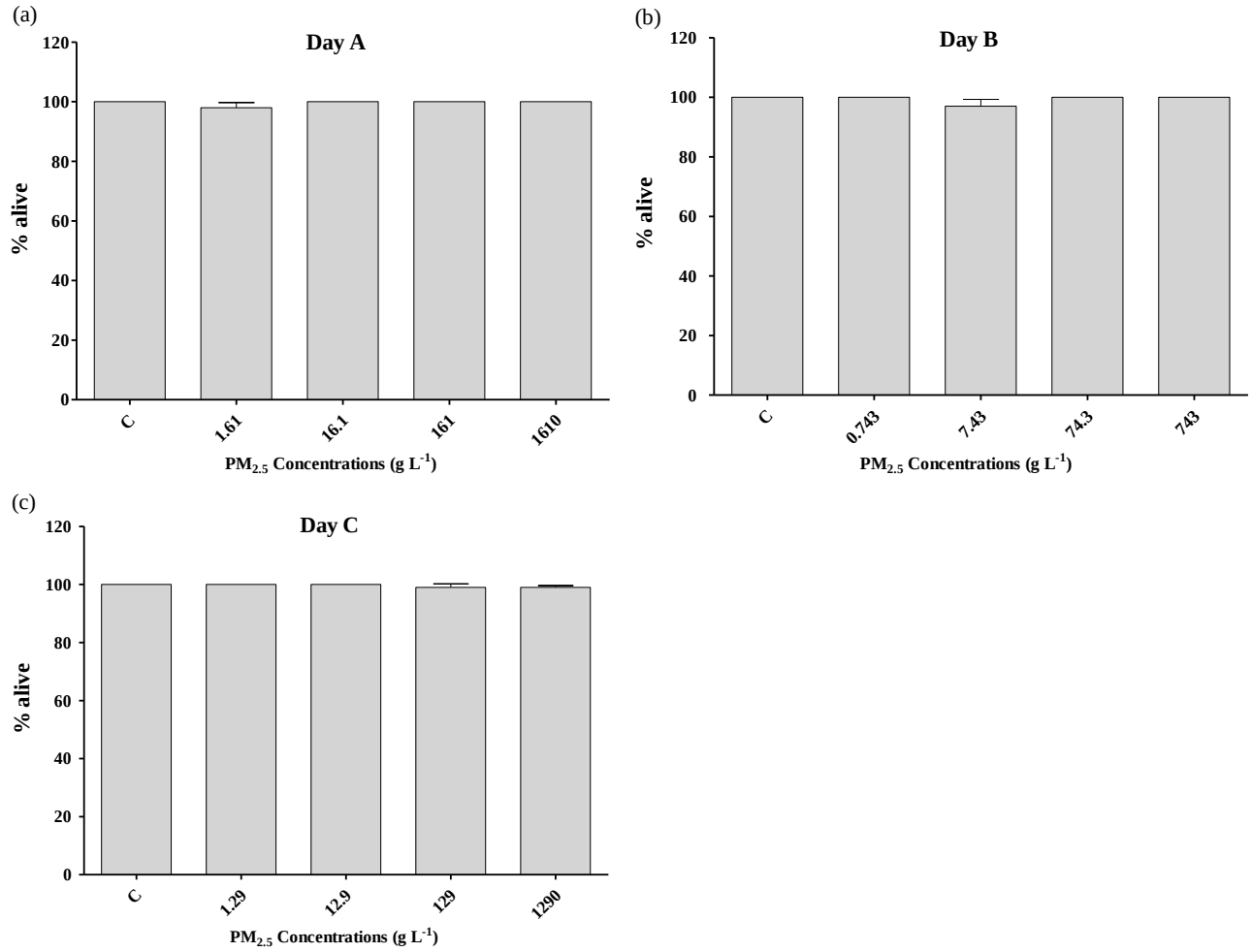


Fig. 3. Survival rates of *C. elegans* after 24 h exposure to heavily TRAP $PM_{2.5}$ obtained by selected three days (Days (a) A, (b) B, and (c) C).

in the head thrashing movement and body bending ability of the nematodes after they had acutely exposed to ambient $PM_{2.5}$ samples collected from a rural area, implying that a higher exposure level was negatively correlated with the disruption of the neurosensory system of *C. elegans.* Moreover, the prolonged exposure to $PM_{2.5}$ (concentrations ranging from 0.1 or 1.0 to 100 mg L^{-1}) had rendered more toxicity on the locomotion behavior of the nematodes as compared to the acute exposure which only the highest $PM_{2.5}$ concentration of 10 or 100 mg L^{-1} resulted in an altered locomotion (Zhao *et al.*, 2014; Sun *et al.*, 2015; Yang *et al.*, 2016). Similarly, two studies proposed that the prolonged exposure to 1 or 100 mg L^{-1} of coal combustion related $PM_{2.5}$ could induce significant reductions on the head thrash and body bending of the nematodes (Sun *et al.*, 2015, Wu *et al.*, 2017). In this study, the nematodes with acute exposure to extremely high levels of TRAP $PM_{2.5}$ from 0.743 to 1290 g L^{-1} also showed consistent results with those of some previous reports (Zhao *et al.*, 2014; Sun *et al.*, 2015; Yang *et al.*, 2016), supporting the fine particulate disrupted behavior of head thrashing and body bending for *C. elegans* after their acute exposure to high levels of TRAP and coal combustion related $PM_{2.5}$.

Effects of TRAP $PM_{2.5}$ on the Life Span of C. elegans

The lifespan or ageing of nematodes is an important toxicological endpoint for assessing the long term effects of the different concentrations of TRAP $PM_{2.5}$ obtained by Days A, B, and C. The three days' samples were pooled together before the test. The expected lifespan of a nematode is usually 24 days. The nematodes exposed to varying concentrations (1.21, 12.1, 121, and 1210 g L^{-1}) of $PM_{2.5}$ had smaller survival rates (Fig. 6(a)). The trend of survival rate data in the present study is in agreement with those of previous studies which linked $PM_{2.5}$ concentration with the decreasing population of exposed nematodes (Zhao *et al.*, 2014; Sun *et al.*, 2015, 2016; Chung *et al.*, 2019; Wang *et al.*, 2019; Zhao *et al.*, 2019). Sun *et al.* (2015) also reported that acute exposure of *C. elegans* to coal combustion related-$PM_{2.5}$ at 100 mg L^{-1} significantly decreased the population of the exposed nematodes.

The nematodes exposed to varying concentrations (1.21, 12.1, 121, and 1210 g L^{-1}) of $PM_{2.5}$ also exhibited shorter lifespans as compared to the control ones (Figs. 6(b)–6(e)). The results were similar to that of one our previous report (Chung *et al.*, 2019), revealing that the highest concentrations of ambient $PM_{2.5}$ from rural areas (Chung *et al.*, 2019) and

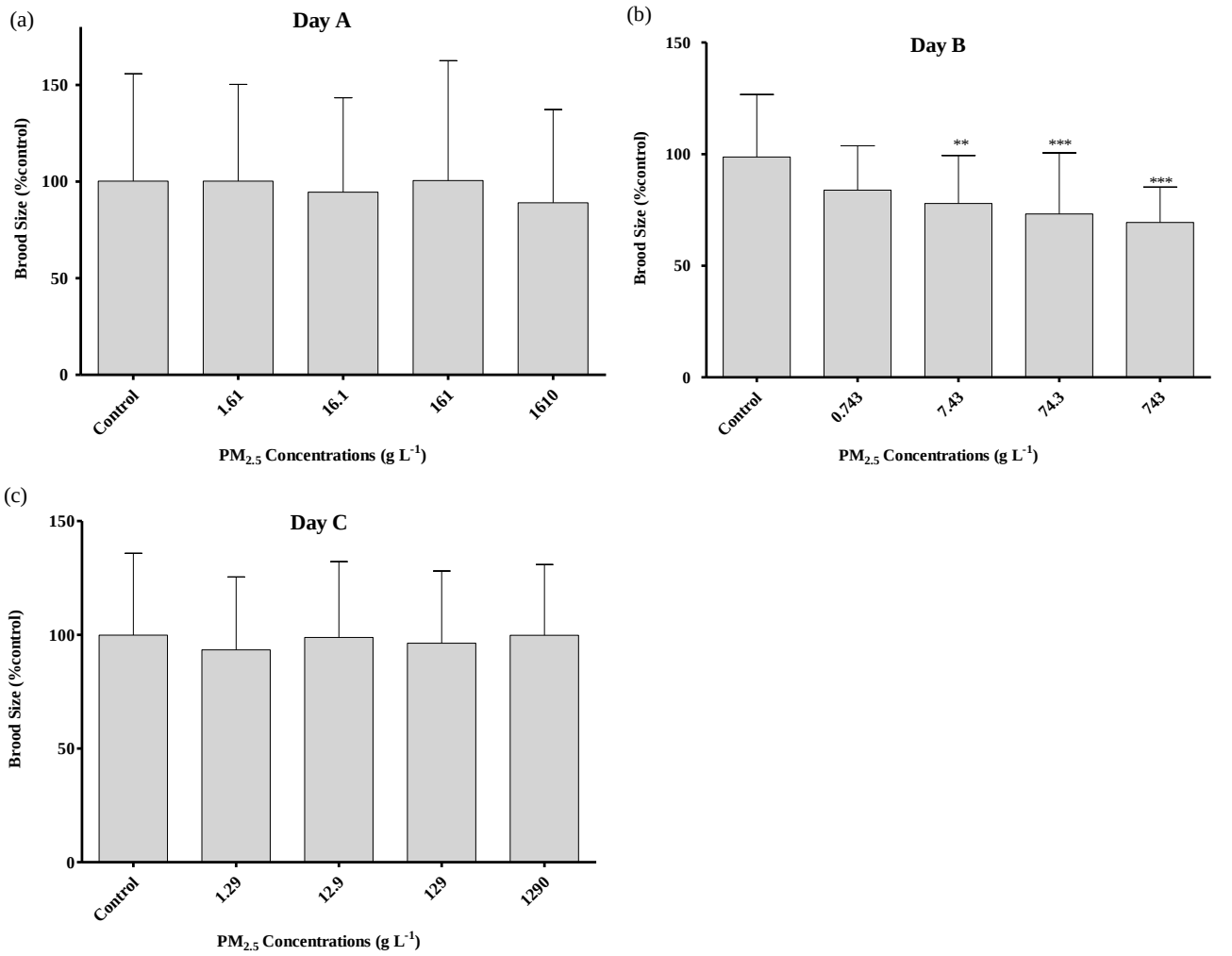


Fig. 4. Reproductive effects of brood sizes in the *C. elegans* models after 24-hr exposure to heavily TRAP $PM_{2.5}$ obtained by selected three days (Days (a) A, (b) B, and (c) C).

the $PM_{2.5}$ with various concentrations in the present study significantly accelerated the ageing of the nematodes to have lifespans shorter than their typical ones. Zhao *et al.* (2019) indicated that ambient $PM_{2.5}$ on a college campus at the concentrations of 10 and 100 mg L^{-1} caused the oxidative stress activation, increased metabolic enzyme response, induced unfold protein response (UPR), and decreased the lifespan of the nematodes. Furthermore, the antioxidant, such as N-acetylcysteine, could be treated in *C. elagans* to suppress the oxidative stress from UPR and then to recovery the lifespan attenuation induced by fine particulate (Zhao *et al.*, 2019).

In comparison with that of the control (24 days), the longest lifespans of the nematodes with acute exposure to $PM_{2.5}$ of 1.21, 12.1, 121, and 1210 g L^{-1} were shortened to 20, 18, 17, and 16 days, respectively. For the mean lifespan, the nematodes in the control groups performed a significantly longer mean lifespan (14.2 ± 0.577 days) compared with the those after exposure to TRAP $PM_{2.5}$ (11.0 ± 0.500, 9.50 ± 0.860, 9.67 ± 0.289, 9.33 ± 0.287 days at 1.21, 12.1, 121, 1210 g L^{-1}, respectively) (Fig. 6(b)). Some previous studies addressed that no significant differences in mean or median lifespans were presented for the nematodes exposed to a low $PM_{2.5}$ concentration (0.1 or 1 mg L^{-1}), but untreated nematodes had significantly longer lifespans compared with the nematodes exposed to the $PM_{2.5}$ concentration of 10.0 or 100 mg L^{-1} (Zhao *et al.*, 2014; Sun *et al.*, 2015, 2016; Zhao *et al.*, 2019). The days for the 50^{th}, 25^{th}, and 5^{th} percentile of survival rates (or the 50^{th}, 75^{th}, and 95^{th} percentile death days in Figs. 6(c)–6(e), respectively) also showed that the nematodes exposed to TRAP $PM_{2.5}$ had the shorter percentile death days than those of the untreated nematodes. The 50^{th}, 75^{th}, 95^{th} percentile death day were expressed as the distribution of survival rates for the untreated and treated $PM_{2.5}$ nematodes. This information is very important for the descriptive statistics of survival rates and death days. Compared with untreated nematodes, 50^{th} and 75^{th} percentile death days didn't have larger change in the treated models in Figs. 6(c) and 6(d). It was worth observing that 95^{th} percentile death days of the treated models were notably and significantly decreased compared with that of the untreated model.

The *C. elegans* were treated with the high levels of TRAP $PM_{2.5}$ in the present study. $PM_{2.5}$-bounded contaminants are diverse and variant up to the sources. Yang *et al.* (2016)

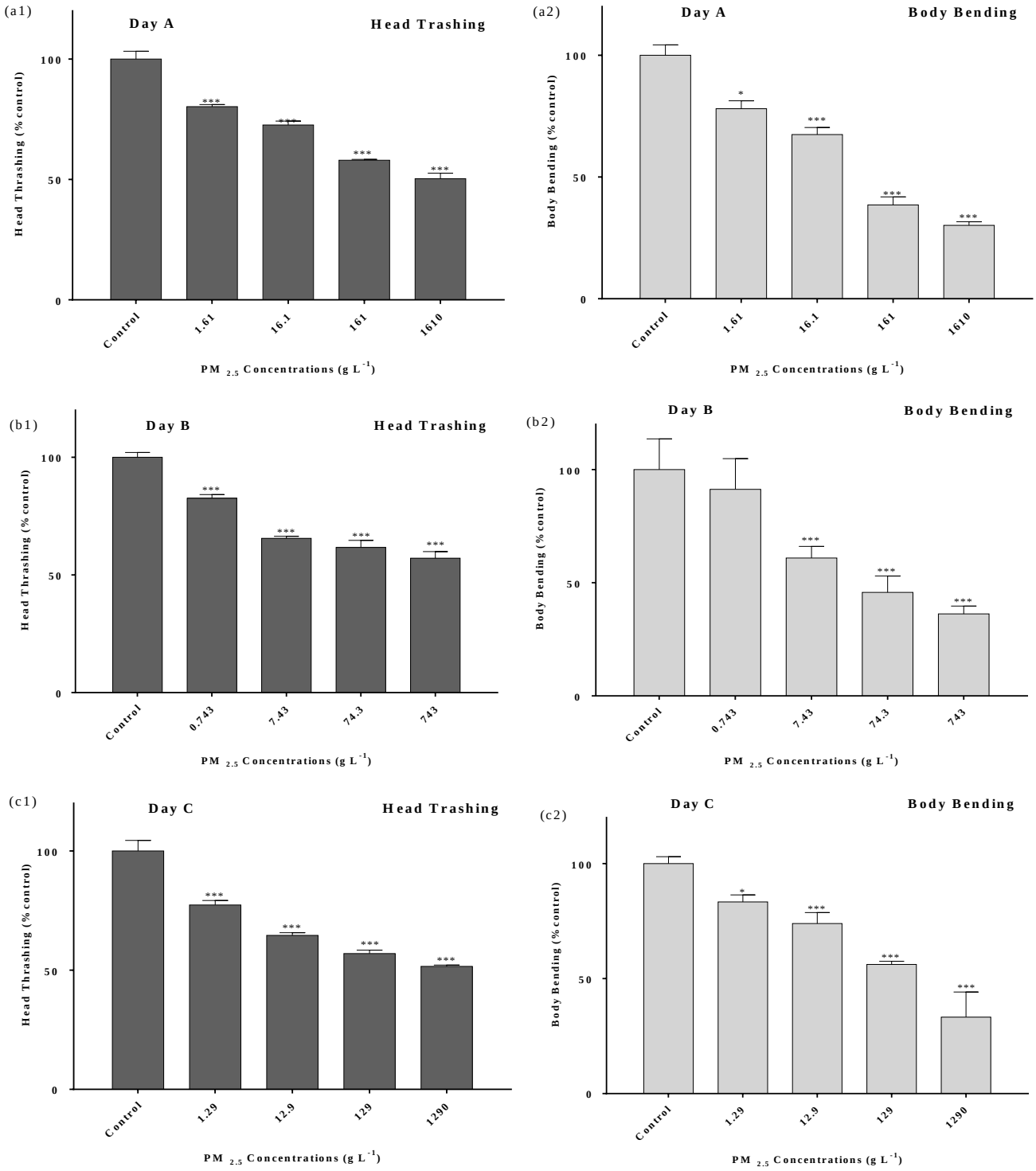


Fig. 5. Dose-dependent reductions of locomotion ((1) head thrashing and (2) body bending) in the *C. elegans* models after 24-hr exposure to heavily TRAP $PM_{2.5}$ obtained in selected three days ((a1 and a2) Days A, (b1 and b2) B, and (c1 and c2) C).

indicated that nematodes with acute exposure to the ambient $PM_{2.5}$ collected during the Chinese Spring Festival in Beijing at a level of 10 mg L^{-1} as well as prolonged exposure to those of 0.100–100 mg L^{-1} disrupted their locomotion behavior and generated the obvious activation of ROS production in the nematodes' intestine. According to our previous study (Chung *et al.*, 2019), $PM_{2.5}$ with concentrations of 2.5 to 4.5 μg Nm^{-3} collected in rural areas, caused the neurobehavioral toxicity of nematodes at the low level of 0.115 or 0.144 mg L^{-1}. For the *C. elegans* models, fine particulate is probably more sensitive to neurological development compared with reproductive development and lifespan. Most published articles addressed to treatment with $PM_{2.5}$ in *C. elegans* models at the concentrations of 10.0 or 100 mg L^{-1}

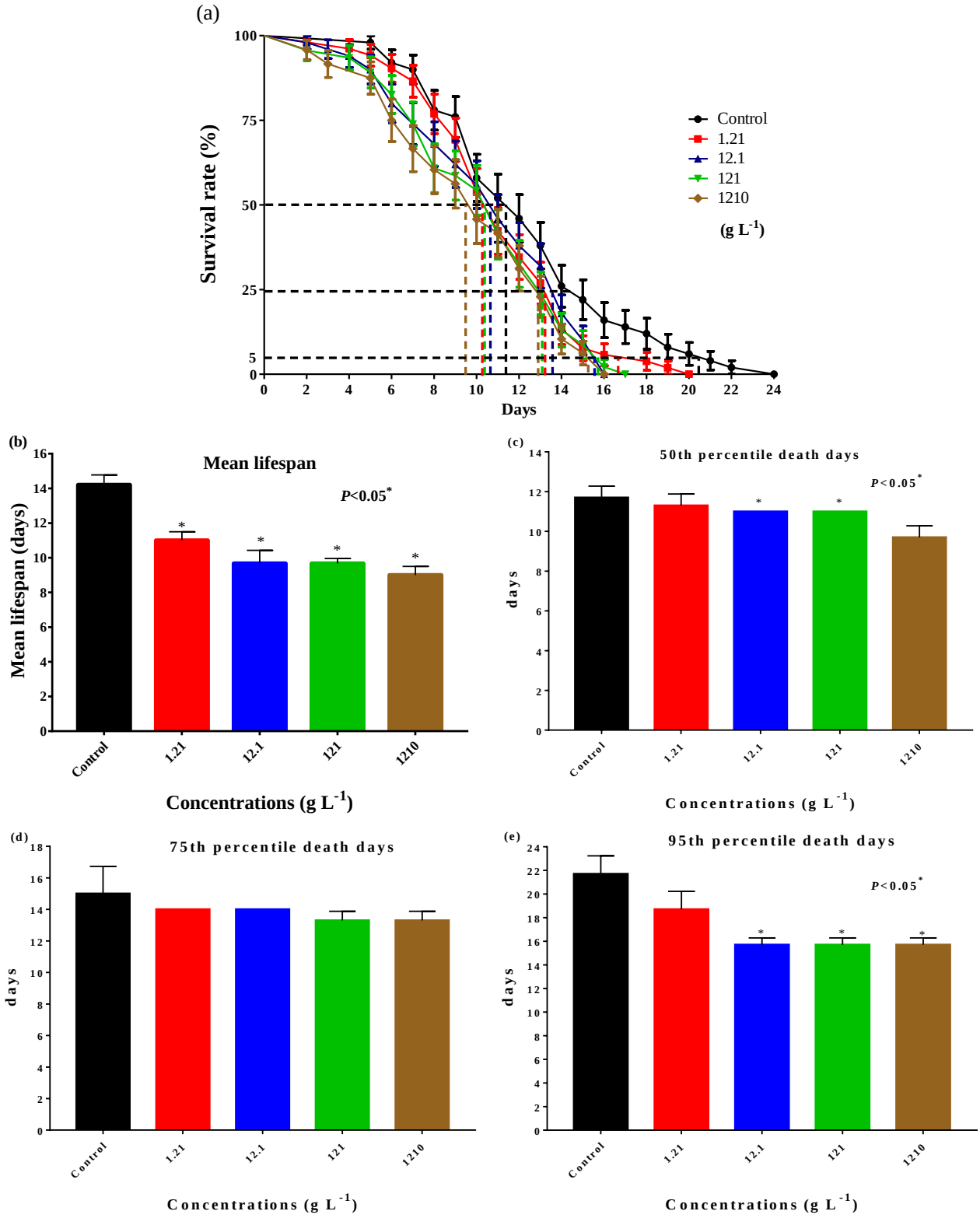


Fig. 6. (a) Survival rates (%), (b) mean lifespans, (c) 50th percentile death days, (d) 75th percentile death days, and (e) 95th percentile death days of *C. elegans* after 24-hr exposure to heavily TRAP $PM_{2.5}$ obtained by selected three days (a mixture of Days A, B, and C).

to present the disrupting effects of neurodevelopment, reproduction, lifespan, oxidative stress activation, and metabolic enzyme response (Zhao *et al.*, 2014; Sun *et al.*, 2015, 2016; Zhao *et al.*, 2019). The nematodes exposed to extremely high levels of TRAP $PM_{2.5}$ (e.g., 1610 g L^{-1}) didn't cause the acute toxicity based on our results. It is

interesting to examine whether the adverse effects of head thrashing, body bending, brood size, and lifespan were affected after the nematodes were exposed to high concentrations of TRAP $PM_{2.5}$. According to our results, it was showed that high levels of TRAP $PM_{2.5}$ obviously disrupted the neurological and reproductive function and lifespan in the *C. elegans* models. Furthermore, the severe TRAP pollution was found in Figs. 1 and 2. For the epidemiological studies, it is demonstrated that TRAP species caused the adverse health effects, but their composition and distribution are complicated. It is very difficult to distinguish what kind of the air pollutants or aerosol affect the adverse effects. Although the concentrations of TRAP species were variant and severe in the present study, it makes it clear to show the toxic effects for the nematodes by treating $PM_{2.5}$ model. Our result might be reflected to the epidemiological studies. The negative impacts of $PM_{2.5}$ on environmental organisms may eventually lead to probable health risk to humans. Further studies are still needed in order to assess whether the impact of $PM_{2.5}$ on environmental organisms such as the *C. elegans* is the same to that on human health.

CONCLUSIONS

Heavy TRAP $PM_{2.5}$ can disrupt multiple toxic endpoints such as locomotion, reproduction, and lifespan in the *in-vivo* model *C. elegans*. The locomotion and reproduction of tested nematodes were negatively affected by TRAP $PM_{2.5}$. TRAP $PM_{2.5}$ entered the body to cause adverse effects specially for the systematic effects, such as locomotion and reproduction, indicating the possible deterioration of secondary targeted organs (neurological or reproductive system) of the nematodes. Also, the lifespans of the nematodes dramatically decreased after it was exposed to the TRAP $PM_{2.5}$ compared with those in the untreated ones. Therefore, heavy TRAP $PM_{2.5}$ could shorten the nematodes' lifespan.

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DISCLAIMER

The authors of this paper declare no conflict of interest.

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