Traffic-related-air-pollutant PM$_{2.5}$ Caused Toxicity on *Caenorhabditis elegans* with Cotreatment of High-dose Glucose and Tempeh

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**ABSTRACT**

Rapid economic development and urbanization have significantly increased PM$_{2.5}$-induced and hyperglycemia-induced toxicological effects. Tempeh is a traditional Indonesian food and may be beneficial for patients with type II diabetes. However, the toxicological effects of co-exposure to traffic-related-air-pollutant (TRAP) PM$_{2.5}$ with high glucose and the potential therapeutic effect of tempeh remain unclear. Using *Caenorhabditis elegans* (*C. elegans*) as an in vivo animal model, we found that exposure to 12.74 mg L$^{-1}$ TRAP PM$_{2.5}$ and 80 mM D-glucose could induce toxicity in nematodes that affects growth, reproduction, locomotion behavior, and lifespan. Moreover, TRAP PM$_{2.5}$ and high glucose diet co-treatment reinforced these adverse effects on *C. elegans*. However, pretreatment with 200 µg of tempeh extract had the greatest improvement in the adverse effects of treatment with or without 12.74 mg L$^{-1}$ TRAP PM$_{2.5}$ and with or without 80 mM D-glucose on *C. elegans*. In addition, tempeh treatment also ameliorated the altered mRNA expression of the antioxidant gene in *C. elegans* treated with or without TRAP PM$_{2.5}$ and with or without high-dose glucose diets. These findings reveal that co-exposure to TRAP PM$_{2.5}$ and high-dose glucose causes more serious health effects, while tempeh could modulate oxidative stress which ameliorated TRAP PM$_{2.5}$-induced and hyperglycemia-induced toxicological effects.

**Keywords:** Particulate matter, High-dose glucose, Tempeh, *Caenorhabditis elegans*
1 INTRODUCTION

Public health concerns on fine particulate matters (PM$_{2.5}$ with an aerodynamic diameter < 2.5 µm) have been dramatically raised with the increasingly serious air pollution of PM$_{2.5}$. PM$_{2.5}$ are caused by diverse sources such as internal engines (Chao et al., 2018), incomplete combustion of fossil fuels (Aunan and Wang, 2014), cooking oil fume (Dou et al., 2018), road dust (Martins et al., 2021), open or biomass burning (Wang et al., 2020), metal processing or chemical industries (Shen et al., 2020), and dust storms or monsoon (Yu et al., 2021). They can be directly emitted into the atmosphere or in microenvironment (Chao et al., 2016). Although PM$_{2.5}$ may be generated from natural or anthropogenic activities, vehicular (Chung et al., 2020) and industrial emission (Shen et al., 2020) and long-range transportation from China or transboundary transportation (Chen et al., 2019; Yu et al., 2021) are sources that mainly contributed to PM$_{2.5}$ pollution in Taiwan, particularly in winter and spring in southern Taiwan (Shen et al., 2020). Based on the report of “Particulate Matters (PM$_{2.5}$) Precursor Demonstration Guidance” from the U.S. Environmental Protection Agency (U.S. EPA) (Mathias and Wayland, 2019), sulfur dioxide, oxides of nitrogen, volatile organic carbon, and ammonia, which are correlated with traffic-related-air-pollutants (TRAPs), are precursor pollutants that most easily develop PM$_{2.5}$. TRAP PM$_{2.5}$ has large surface areas to be covered on several organic (i.e., polycyclic aromatic hydrocarbons) or inorganic (i.e., heavy metals) chemicals and some of them are toxic (Dadvand et al., 2015; Sram et al., 2017; Rahmatinia et al., 2021; Hao et al., 2022). TRAP PM$_{2.5}$ may induce inflammatory responses (Chao et al., 2018), DNA adducts or damage (Li et al., 2014), and lung function deterioration in traffic conductors or policemen (Putri Anis Syahira et al., 2020). The negative impact of TRAP PM$_{2.5}$ on the central nervous system and neurobehavior development has been described (Sram et al., 2017). For examples, a positive correlation was observed between children with autism spectrum disorder and children with prenatal exposure to PM$_{2.5}$, especially TRAP PM$_{2.5}$ (Becerra et al., 2013; Raz et al., 2015). In addition, TRAP PM$_{2.5}$ may act as a high-risk factor for Diabetes mellitus (DM) (Esposito et al., 2016).

DM is a major disease affecting people worldwide. According to the World Health Organization (WHO) survey, it is one of the leading causes of death in highly developed countries (Corriere et al., 2013). DM is characterized by a lack of production or cellular uptake of insulin, which depends on various factors (Dong et al., 2019). Type 2 diabetes (T2D) represents 90% of all diabetes cases worldwide (Scully, 2012). T2D is a multifactorial and complex metabolic disease that can occur in a genetic or an acquired manner (Smith-Palmer et al., 2014; Yang et al., 2021). Pancreatic β-cell dysfunction, glucose-stimulated hyperinsulinemia, and insulin resistance are the main factors in the pathogenesis of T2D (DeFronzo, 2004; Zhang et al., 2021a). Impaired insulin secretion and insulin action lead to an accumulation of glucose in the blood (hyperglycemia), with adverse effects on health. Therefore, hyperglycemia in DM patients (e.g., T2D) is characterized by the sum of fasting hyperglycemia and postprandial hyperglycemia (American Diabetes Association, 2014). Clinical features of hyperglycemia and T2D include polyuria, polydipsia, constant hunger, weight loss, vision change, and fatigue (American Diabetes Association, 2009). T2D is a progressive disease that may further contribute to developing neuropathy, retinopathy, cognitive defects, Parkinson’s, cardiovascular disease, and nephropathy (Kuniss et al., 2019; Nijpels et al., 2019). Clinical managements of T2D are various, such as diet, physical activity, behavioral therapy, pharmacotherapy, as well as metabolic surgery (American Diabetes Association, 2021). In recent years, plant-based diets have become popular and are considered an effective tool for the prevention and management of T2D (McMacken and Shah, 2017).

Tempeh, a popular traditional food in Indonesia, is a source of plant proteins from soybeans fermented by Rhizopus species (Keuth and Bisping, 1993). The fermentation of soybeans promotes the production of proteases, lipases, carbohydrates and phytases. These fungal enzymes increase antimicrobial effects, macromolecules degradation, cell walls and intracellular mater dissolution, reduces antinutritional components, as well as increases levels of some vitamins (e.g., riboflavin, niacin, vitamin B6, and vitamin B12), isoflavones, free amino acids, fatty acids, oligosaccharides, and γ-amino butyric acid (Aoki et al., 2003; Nout and Kiers, 2005). Beneficial effects of tempeh include improving cardiovascular, liver, gut, and bone health, inducing apoptosis in cancerous cell lines, improving cognitive dysfunction and memory, expediting muscle recovery and enhancing
strength, and promoting antioxidant, hypolipidemic, and anti-inflammation activities (Ahnan-Winarno et al., 2021). Tempeh also reduces insulin resistance, HbA1c, and serum glucose levels in streptozotocin combined with high-fat diet-induced T2D rats (Huang et al., 2018). Additionally, our previous results suggested that consuming 2 g of tempeh daily for a period of 3 months attenuated HbA1c and triglyceride levels in participants (Su et al., 2021). These findings suggest that tempeh ameliorates hyperglycemia, hyperlipidemia, and hyperinsulinemia. However, the beneficial effects of tempeh remain unclear.

The nematode of *Caenorhabditis elegans* (*C. elegans*), a non-mammalian model, has been widely used for biomedical and environmental toxicity evaluation because of its short lifespan, high reproduction rate, simple nervous system, adequately described genetics, high level of molecular preservation, transparent body, inexpensive and convenient maintenance, sensitivity to environmental toxins, and without the animal ethics issues (Brenner, 1973; Kaletta and Hengartner, 2006; Leung et al., 2008; Maurer et al., 2015; Hunt, 2017; Chung et al., 2019). However, not many published articles focused on *C. elegans* exposure to PM2.5 and the related adverse effects, especially TRAP PM2.5 (Zhao et al., 2014; Sun et al., 2015; Yang et al., 2015; Sun et al., 2016; Wu et al., 2017; Chung et al., 2019; Zhao et al., 2019; Chung et al., 2020). Studies on adverse effects of TRAP PM2.5 in *C. elegans* models, found that TRAP PM2.5 caused reproductive and neurological toxicity and shortened the lifespan of nematodes after prolonged exposure to TRAP PM2.5 (Chung et al., 2020). In addition, studies have found that dietary glucose affects a variety of physiological and molecular processes in *C. elegans*. A glucose-supplemented diet can increase cellular ROS levels and protein glycosylation, alter gene expression, increase lipid accumulation, alter membrane fluidity, accelerate aging, decrease locomotion capacity, and affect lifespan (Alcantar-Fernandez et al., 2018; Kingsley et al., 2021).

Globally, rapid economic development and urbanization have a significant relationship with PM2.5 concentrations (Wang et al., 2019) and plasma glucose concentrations (Cheema et al., 2014). However, toxicological effects of TRAP PM2.5 co-exposure with high glucose on human health remain unclear. Therefore, the current study aimed to evaluate the possible toxicity of TRAP PM2.5 co-exposure with high glucose in an in vivo *C. elegans* models. The different toxicological endpoints (e.g., growth kinetics, reproduction, locomotion properties, lethality,) and oxidative stress responses of *C. elegans* were investigated.

### 2 MATERIALS AND METHODS

#### 2.1 Air Quality Monitoring and Sampling

Air sampling and pretreatment procedure was described as previously (Chung et al., 2020). Briefly, the PM$_{2.5}$ filters (Quartz fiber filters) were heated at 600°C at least for two hours before the filters were conditioned in the electronic desiccator for one day before and after the on-situ sampling. PM$_{2.5}$ concentrations were determined before and after the filters were weighted using a six-digit balance with an accuracy of 0.1 µg. A high-volume air sampler (SIBATA HV-1000R, Sibata, Japan) was used to collect TRAP PM$_{2.5}$ on Tunghai University campus near the Tungdai air monitor site of the Environmental Protection Bureau of Taichung City Government. The Tungdai air monitor site is next to Section 4, Taiwan Avenue, Xitun District, Taichung City, where traffic is heavy, especially during rush hours. Each TRAP PM$_{2.5}$ sample was gathered for 24 hours between March 24 and April 15 in 2018 followed by the U.S. EPA Reference Method TO9A or Taiwanese EPA NIEA A205.11C. The TRAP PM$_{2.5}$ filters were immediately transferred to the laboratory at National Pingtung University of Science and Technology (NPUST) and refrigerated at −20°C before extraction to prevent loss of volatile portions by evaporation.

For pretreatment, the PM$_{2.5}$ filters were extracted by dichloromethane (DCM) using sonication prior to extraction with the addition of 15 mL DCM passing through the acid-silica column for cleanup, according to our previous report (Chung et al., 2020). All the extracts from the 23-day PM$_{2.5}$ samples were pooled together, and the pooled elute was then concentrated into the volume of 1 mL in a vial. This elute was then concentrated to near dryness under the gentle nitrogen stream. Finally, the elute was redissolved in 1 mL of DMSO.
2.2 Tempheh Extract Preparation

Kaohsiung Number 9 soybeans were fermented to produce tempeh products, as follows. The peeled soybeans were soaked in 1% lactic acid solution (pH 3.5–4.0) for 12 hours at room temperature. After drying, the soybeans were boiled at 100°C for 30 minutes and cooled at room temperature. Spores of R. oligosporus were then added and inoculated in an incubator to ferment at 32°C to 35°C for 48 hours until hyphae covered the soybeans. The fermented soybeans (tempeh) were placed in an oven at 65°C and dried with hot air. The dried tempeh was sterilized by UV irradiation for 12 hours and then ground into powder. Finally, 1 mg of tempeh powder was redissolved with 0.2 mL of DMSO and refrigerated at –20°C for later use.

2.3 Maintenance of C. elegans

Wild-type N2 nematodes were acquired from the Department of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University (Tainan, Taiwan). Nematodes were maintained on OP50 E. coli-seeded nematode growth medium (NGM) plates at 22°C. NGM plates contained bacteriological agar (Laboratories Conda, S.A., Spain), bactopeptone (Laboratories Conda, S.A., Spain), and NaCl (Honeywell Fluka™, New Jersey, USA). OP50 E. coli cultures were acquired from Bioresources Collection and Research Center (Hsinchu, Taiwan) and were grown in Luria-Bertani broth (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37°C. Nematodes were age-synchronized using the alkaline bleaching method. The bleaching solution contained NaOCl (J.T. Baker, Central Valley, PA) and KOH (Duk san Pure Chemicals, Gyeonggi-do, South Korea). After growth from the synchronized L1 nematodes, the L3 or young-L4 nematodes were rinsed from the plate with K-medium (2.36 g KCl (Avantor Performance Materials, ltd., Gyeonggi-do, South Korea), 3 g NaCl, in 1 L ddH2O) and centrifuged at 2500 × g for 4 min. Finally, L3/young-L4 nematodes were diluted with K-medium and utilized for further experimentation.

2.4 Reproductive Assay of C. elegans

After pretreatment with different concentrations of tempeh extract for 12–16 h followed by 24 h of exposure to with or without TRAP PM2.5 and with or without D-glucose diet, age-synchronized L3/young-L4 nematodes (n = 20) were transferred separately to 12-well NGM plates with fresh OP50 lawns and evaluated for 4 to 5 days of spawning. During the egg-laying, each nematode was transferred to a new plate every 2 days. Old plates containing eggs were hatched and incubated to L4 nematodes to allow easier counting of progeny. Finally, the total number of offspring were calculated for each worm.

2.5 Growth Measurement of C. elegans

The nematodes lengths were measured to evaluate effect of TRAP PM2.5 and/or high glucose exposure on growth. After pretreatment with different concentrations of tempeh extract for 12–16 h followed by exposure to with or without TRAP PM2.5 and with or without D-glucose diet for 24 h, the nematodes were transferred to NGM plates with OP50 lawn and incubated for 48 h until the L4 stage of old age. An Olympus SZX10 dissecting microscope (Olympus, Waltham MA, USA) was used to acquire images, and ImageJ software was used to measure the length of each nematode. For each concentration, 20 worms were measured. These assays were done in triplicate for each concentration.

2.6 Locomotion Assay of C. elegans

Twenty L3/young-L4 nematodes were dispensed on NGM plates (one worm per 12-well and starved for 24 h). 20 worms were pretreated with different concentrations of tempeh extract for 12–16 h followed by 24 h of exposure to with or without TRAP PM2.5 and with or without D-glucose diet. Their locomotive behavior was evaluated by head thrashing and body bending. Head thrashing is defined as a change in the direction of bending in the middle of the body. Body bending is defined as a direction change of the nematode part corresponding to the posterior bulb of the pharynx with respect to the y-axis while the nematode was traveling along the x-axis. Individual animals were picked onto unseeded OP50 NGM plates and the number of head thrashing was scored for 1 min. Body bends were counted in an interval of 20 s when individual animals were picked onto unseeded OP50 NGM plates and pipetted with M9 buffer (7.56 g Na2HPO4 (Honeywell...
Fluka™, Charlotte, NJ, USA), 1.5 g KH2PO4 (Avantor Performance Materials, LLC, Radnor, PA, USA), 2.5 g NaCl, 0.5 mL 1M MgSO4 (Avantor Performance Materials, Ltd.), and 500 mL H2O). Three replicates were performed for each concentration.

2.7 Lifespan Measurements of C. elegans

The synchronized L1 nematodes were grown on OP50 E. coli-seeded NGM plates at 22°C until L3 or young·L4 stage. After rinsed from the plate with K-medium and centrifuged at 2500 × g for 4 min, 200 L3/young·L4 nematodes were dispensed in each well of the 12-well plate and starved for 24 h. The nematodes were pretreated with vehicle or 200 µg tempeh extract for 12–16 h and then exposed for 24 h with or without 12.7 mg L−1 TRAP PM2.5 and with or without 80 mM D-glucose diet. After treatment, the nematodes were transferred onto NGM plates without OP50. For lifespan assay, the nematodes were transferred to fresh NGM plates and observed every day. Worms were considered dead when they did not respond to gentle probing using the worm picker. Survival of the worms was scored for a duration of 25 days. Experiments were performed in triplicate for each concentration.

2.8 Quantitative Real-Time PCR (qRT-PCR) Assays

500–1000 L3/young·L4 nematodes were dispensed in each 6 cm petri dish and were then exposed to vehicle or 200 µg tempeh extract for 12–16 h followed by exposure to with or without 12.7 mg L−1 TRAP PM2.5 and with or without 80 mM D-glucose diet for 24 h. Worms were collected and total RNA was extracted from each sample using TRIzol Reagent (Gibco, Life Technologies, Carlsbad, CA, USA). For cDNA synthesis, 1000 ng RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription kit (Gibco, Life Technologies, Carlsbad, CA, USA). For real-time PCR, cDNA (50 ng) was amplified with SYBR green PCR master mix (Gibco, Life Technologies, Carlsbad, CA, USA) through a 95°C denaturation stage for 10 min, followed by 40 repetitions at 95°C for 15 s and then 60°C for 1 min using an Applied Biosystems PRISM 7500 fast real-time PCR system. For C. elegans, there was quantification for expression of skinhead-1 (SKN-1), cyp-35A2, cationic amino acid transporter -1 (CAT-1), CAT -4, catalase -1 (CTL-1), CTL-2, CTL-3, Tryptophan Hydroxylase 1 (TPH-1), superoxide dismutase-1 (SOD-1), SOD-2, SOD-3, SOD-4, SOD-5, and ACTIN mRNA. Sequences for the primers used are shown in Table 1.

2.9 Statistical Analysis

Data were analyzed using SAS 9.3 (SAS Institute, Cary, NC, USA). The survival plot (Kaplan–Meier plot) was constructed for lifespan data using GraphPad Prism 6 (San Diego, CA, USA). The length, brood size, and locomotion data were observed to be non-normally distributed using the Shapiro–Wilk test. A survival plot or Kaplan –Meier plot was constructed to evaluate the effects

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<th>Table 1. Primer sequences.</th>
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<tr>
<td><strong>Gene</strong></td>
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<td>C. elegans</td>
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<tr>
<td>SKN-1</td>
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<td>CYP-35A2</td>
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<td>CAT-1</td>
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of different concentrations of toxic solutions on the nematode lifespan. One-way ANOVA and the post hoc Tukey’s honestly significant difference tests were used to make comparisons between subgroups and to calculate $p$-values for comparison. In these analyses, $p < 0.05$ represented a statistically significant result. Values are presented as means ± SD.

### 3 RESULTS AND DISCUSSIONS

**3.1. Effects of TRAP PM$_{2.5}$ on the Reproduction, Growth, and Locomotion Behavior of *C. elegans***

To examine whether TRAP PM$_{2.5}$ exposure affected the reproduction of *C. elegans*, we assayed the brood size after exposure to the different concentrations of TRAP PM$_{2.5}$ (Fig. 1(A)). The results showed that exposure to 0.127–12.7 mg L$^{-1}$ of TRAP PM$_{2.5}$ significantly decreased the brood size of *C. elegans* compared with the control group. A total of 265 ± 48.3 progenies were produced by the control group, which was significantly decreased by 66.7% (177 ± 53.8 progenies), 69.1% (183 ± 35.3 progenies) and 58.0% (154 ± 39.6 progenies) after chronic exposure to 0.127, 1.27, and 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ compared with the control group.

![Fig. 1. Effects of TRAP PM$_{2.5}$ on (A) brood size, (B) body length, (C) head thrashes, and (D) body bends of *C. elegans* after 24-hours exposure. * $p < 0.05$; ** $p < 0.01$ versus the control group.](image-url)
Body length is an important manifestation of *C. elegans* growth and development. To investigate the adverse effect of TRAP PM$_{2.5}$ on growth, the *C. elegans* were exposed to various concentrations of TRAP PM$_{2.5}$ for 24 hours, then transferred nematodes to new NGM plates without exposure to TRAP PM$_{2.5}$ and incubated for 48 hours until the L4 stage. In Fig. 1(B), body length in the control group was 820 ± 80.9 μm, which was significantly increased by 111% (911 ± 122 μm) and 107% (874 ± 98.1 μm) at a concentration of 0.127 and 1.27 mg L$^{-1}$ TRAP PM$_{2.5}$ compared with the control groups. However, the concentration of 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ in treatment group showed the body length was significantly decreased by 90.6% (743 ± 91.9 μm) compared with the control group.

Head thrashing and body bending of the *C. elegans* were selected for the study to assess neurological toxicity based on various concentrations of TRAP PM$_{2.5}$ (Figs. 1(C) and 1(D)). Exposure to 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ significantly decreased the head thrashing of *C. elegans* compared with the control group. Compared with the control group (30.0 ± 3.2 head thrashes per minute), the head thrashing of *C. elegans* decreased by 84.2% (25.2 ± 3.8 head thrashes per minute) at a concentration of 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ (Fig. 1(C)). Additionally, TRAP PM$_{2.5}$ significantly decreased the body bending of *C. elegans* by 94.5%, 87.1%, and 71.2% at concentrations of 0.127, 1.27 and 12.7 mg L$^{-1}$, respectively, compared with the control group (63.1 ± 5.5 body bending per 20 sec) (Fig. 1(D)). These findings suggest that the treatment group of 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ had the most negative effects on the reproduction, growth, and locomotion behavior of *C. elegans*.

According to the latest revision of WHO air quality guidelines in September 2021, PM$_{2.5}$ values should not exceed 5 μg m$^{-3}$ per year and 15 μg m$^{-3}$ per day (WHO, 2021). Previous studies have shown that short-term exposure to PM$_{2.5}$, with a 10 μg m$^{-3}$ increase in PM$_{2.5}$ concentration, increases mortality and the incidence of cardiovascular disease, respiratory system disease, diabetes, low birth weight, male reproductive toxicity, and negative effects on the patients’ central nervous system and neurobehavior development (Becerra et al., 2013; Raz et al., 2015; Esposito et al., 2016; Xing et al., 2016; Sram et al., 2017; Li et al., 2019; Ban et al., 2021). Animal studies have also indicated that exposure to PM$_{2.5}$ at 500–1000 μg m$^{-3}$ every three days for 28 days (3.8% of the average lifespan) induces apoptosis of ovarian granulosa cells and oocytes in female mice, leading to the disruption of embryonic development and female fertility (Liao et al., 2020). Exposure to 59.8 and 484 μg m$^{-3}$ of PM$_{2.5}$ for 6 h a day and 5 days a week for 2-month (8.3% of the average lifespan) induced spermatocyte damage and energy metabolism disorder in male mice (Shi et al., 2022). In addition, exposure to 218 ± 74.8 μg m$^{-3}$ of PM$_{2.5}$ for 8 h day$^{-1}$, 5 days week$^{-1}$ for 6 weeks (5.8% of the average lifespan) disrupted normal structure and spiral artery remodeling of placenta, ultimately leading to low birth weight and fetal growth restriction (Tao et al., 2022). Furthermore, gestational exposure to 30 μL PM$_{2.5}$ suspension of 3.46 μg μL$^{-1}$ every 3 days for 21 days (2.9% of the average life span) affected apoptosis and neuroinflammation, as well as neurogenesis in the hippocampus, eventually causing spatial memory dysfunction and neurodevelopmental impairment of mouse offspring (Zheng et al., 2018). In the present study and our previous study (Chung et al., 2019), exposure to PM$_{2.5}$ concentrations greater than 1 μg L$^{-1}$ for 1 day (4.2% of the mean life span) was observed to cause toxicities in reproduction, growth, and locomotive behavior.

### 3.2 TRAP PM$_{2.5}$ and High Glucose Diet Co-treatment Enhance Adverse Effects

Since PM$_{2.5}$ exposure is a risk factor for T2D (Esposito et al., 2016; Chilian-Herrera et al., 2021), our study next evaluated possible toxicity of TRAP PM$_{2.5}$ co-exposure with high glucose in *C. elegans*. To examine the effects of glucose on the reproduction, growth, and locomotion behavior of *C. elegans*, 20, 40, 80, and 120 mM of D-glucose were added to their diet. In Fig. 2(A), the reproductive assay showed that exposure to 20, 40, 80, and 120 mM of D-glucose significantly reduced the brood size of *C. elegans* by 37.7% (100 ± 49.3 progenies), 12.1% (32.0 ± 21.4 progenies), 29.4% (78.1 ± 24.8 progenies), and 39.4% (104 ± 35.6 progenies), respectively, compared to the control group (265 ± 49.2 progenies). Growth measurement revealed that exposure to 40, 80, and 120 mM of D-glucose significantly decreased the body length of *C. elegans* by 72.1% (591 ± 117.0 μm), 61.2% (501 ± 78.6 μm), and 72.4% (594 ± 88.8 μm), respectively, compared to the control group (820.1 ± 80.9 μm) (Fig. 2(B)). Additionally, a decreased percentage of the head thrashing (94.6%, 25.2 ± 3.8 head thrashes per minute vs. 30.0 ± 3.2 head thrashes per minute in control) was found in 80 mM D-glucose treated *C. elegans* (Fig. 2(C)). High glucose diet also...
Fig. 2. Effects of with or without exposure to 12.74 mg L⁻¹ TRAP PM₂.₅, and with or without 80 mM D-glucose diets on (A, E) brood size, (B, F) body length, (C, G) head thrashes, and (D, H) body bends of *C. elegans* after 24-hours exposure. * *p* < 0.05; ** *p* < 0.01 versus the control group by Shapiro–Wilk test. # *p* < 0.05; ## *p* < 0.01 by one-way variance analysis followed by Tukey’s pairwise comparison.
significantly decreased the body bending of *C. elegans* by 90.9% (57.4 ± 9.0 body bending per 20 sec) and 92.1% (58.1 ± 6.7 body bending per 20 sec) at a concentration of 80 and 120 mM, respectively, compared with the control group (63.1 ± 5.5 body bending per 20 sec) (Fig. 2(D)). Accordingly, 80 mM D-glucose treatment was used as the further experimental paradigm.

Next, TRAP PM$_{2.5}$ and high glucose diet co-treatment enhancements of adverse effects on *C. elegans* were evaluated. *C. elegans* were treated with or without 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ and with or without 80 mM D-glucose. In Figs. 2(E–H), the brood size, body length, head thrashes and body bends for control *C. elegans* were 311 ± 70.8 progenies, 1160 ± 175 μm, 29.8 ± 3.3 head thrashes per minute and 56.3 ± 4.9 body bending per 20 sec, respectively. Treatment with 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ or 80 mM D-glucose reduced the brood size, body length, head thrashes and body bends in *C. elegans*. Adverse effects of 80 mM D-glucose on brood size (152 ± 45.6 progenies), body length (875 ± 94.7 μm), and body bends (43.4 ± 9.5 body bending per 20 sec) of nematodes were greater than those of 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ (221 ± 55.7 progenies, 1120 ± 118 μm body length, and 48.5 ± 8.0 body bending per 20 sec). However, TRAP PM$_{2.5}$ had a greater negative effect on nematode head thrashes (26.3 ± 2.9 head thrashes per minute) than 80 mM D-glucose exposure (28.3 ± 3.8 head thrashes per minute). We further found that TRAP PM$_{2.5}$-D-glucose co-treatment enhanced adverse effects on the brood size (119 ± 54.3 progenies), body length (859 ± 103 μm), head thrashes (25.0 ± 4.0 head thrashes per minute) and body bends (39.8 ± 6.9 body bending per 20 sec) in *C. elegans*.

TRAP PM$_{2.5}$ may induce endothelial dysfunction, inflammation, visceral adipose tissue dysregulation, insulin resistance, and elevated hemoglobin A1c level, which act as high-risk factors for T2D (Esposito et al., 2016; Chilian-Herrera et al., 2021). Animal studies indicated that pre-exposure to 14–289 mg m$^{-3}$ of PM$_{2.5}$ for 12 weeks promoted β-cell damage in mice upon streptozotocin (Zhang et al., 2021b). STZ-stimulated rats exposed to 1 mg kg$^{-1}$ PM$_{2.5}$ every 2 days for 12 days revealed that short-time PM$_{2.5}$ exposure enhances insulin resistance and raises inflammatory factors in T2D rats (Long et al., 2020). The current study demonstrates that using short-time PM$_{2.5}$ exposure aggravates high glucose diet-induced toxic effect on head thrashes in *C. elegans*.

### 3.3 Tempeh Extract Pretreatment Ameliorates TRAP PM$_{2.5}$-induced and High Glucose Diet-induced Adverse Effects on *C. elegans*

The results of our previous clinical study showed that tempeh attenuates HbA1C and triglyceride levels in participants (Su et al., 2021). Therefore, we next investigated whether tempeh would have any effect on worms exposed to TRAP PM$_{2.5}$ and fed high glucose. In Fig. 3, *C. elegans* were pretreated with 50, 100, and 200 μg tempeh extract for 12–16 h and then fed with 80 mM D-glucose. The brood size, body length, head thrashes and body bends for control *C. elegans* were 243 ± 44.7 progenies, 1110 ± 127 μm, 39.9 ± 8.1 head thrashes per minute and 28.1 ± 3.2 body bending per 20 sec, respectively. Treatment with 80 mM D-glucose reduced brood size by 0.63-fold (152 ± 28.7 progenies), body length by 0.90-fold (994 ± 108 μm), head thrashes by 0.80-fold (32.1 ± 9.1 head thrashes per minute) and body bends by 0.89-fold (25.0 ± 4.2 body bending per 20 sec) in *C. elegans* compared with the control group (Fig. 4). Compared with the 80 mM D-glucose-fed group, there were no significant differences in brood size for any of the pretreatments with tempeh concentrations (50–200 μg) (Fig. 3(A)). However, the body length of *C. elegans* pretreated with 200 μg tempeh increased significantly by 103% (1020 ± 112 μm) compared with the 80 mM D-glucose-fed group (Fig. 3(B)). Head thrashes were significantly increased by 1.27-fold (40.8 ± 6.3 head thrashes per minute), 1.22-fold (39.2 ± 9.4 head thrashes per minute), and 1.31-fold (42.3 ± 9.0 head thrashes per minute) in *C. elegans* pretreated with 50, 100, and 200 μg tempeh compared to the 80 mM D-glucose-fed group (Fig. 3(C)). Besides, body bends was also significantly increase by 1.12-fold (28.1 ± 4.2 body bending per 20 sec), 1.15-fold (28.7 ± 3.9 body bending per 20 sec), and 1.17-fold (29.2 ± 4.2 body bending per 20 sec) in *C. elegans* pretreated with 50, 100, and 200 μg tempeh compared to the 80 mM D-glucose-fed group (Fig. 3(D)). These findings suggest that pretreatment with 200 μg of tempeh had the greatest improvement in the adverse effects on *C. elegans* caused by a high glucose diet.

To investigate whether 200 μg of tempeh extract would have any effect on worms exposed to TRAP PM$_{2.5}$, reproduction, growth, and locomotive behavior assays of *C. elegans* were evaluated.
Fig. 3. Effects of 80 mM D-glucose diets with/without tempeh pretreatment on (A) brood size, (B) body length, (C) head thrashes, and (D) body bends of *C. elegans* after 24-hours exposure. ** *p* < 0.01 versus the control group; *#* *p* < 0.05; **#** *p* < 0.01 versus the glucose group.

by pretreating with or without 200 µg tempeh for 12–16 h, followed by exposure with or without 12.74 mg L\(^{-1}\) TRAP PM\(_{2.5}\). As shown in Fig. 4, the 12.7 mg L\(^{-1}\) TRAP PM\(_{2.5}\) treatment group had a negative effect on brood size (212 ± 64.5 progenies), body length (1180 ± 102 µm), head thrashes (37.0 ± 10.4 head thrashes per minute), and body bends (24.9 ± 4.5 body bending per 20 sec) of *C. elegans* compared to the control group (314 ± 55.0 progenies, 1313.2 ± 121.3 µm body length, 54.6 ± 8.6 head thrashes per minute, and 29.5 ± 3.5 body bending per 20 sec). In comparison with the 12.74 mg L\(^{-1}\) TRAP PM\(_{2.5}\) treatment, there was no meaningful difference in head thrashes for 200 µg tempeh pretreatment (Fig. 4(C)). However, 200 µg tempeh pretreatment was significantly increased brood size (268 ± 39.6 progenies) and body bends (29.5 ± 3.5 body bending per 20 sec) by 1.27-fold and 1.18-fold, but body length (1090 ± 115 µm) decreased 0.92-fold in *C. elegans* compared to the 12.7 mg L\(^{-1}\) TRAP PM\(_{2.5}\) treatment group (Figs. 4(A), 4(B), and 4(D)).

In Fig. 5, the brood size, body length, head thrashes and body bends for control *C. elegans* were 268 ± 49.5 progenies, 1320 ± 129 µm, 35.9 ± 9.6 head thrashes per minute and 58.0 ± 9.3 body bending per 20 sec, respectively. TRAP PM\(_{2.5}\)-D-glucose co-treatment reduced brood size
Fig. 4. Effects of 12.74 mg L\(^{-1}\) TRAP PM\(_{2.5}\) with/without 200 \(\mu\)g of tempeh pretreatment on (A) brood size, (B) body length, (C) head thrashes, and (D) body bends of \textit{C. elegans} after 24-hours exposure. ** \(p < 0.01\) versus the control group; * \(p < 0.05\); ** \(p < 0.01\) versus the TRAP PM\(_{2.5}\) group.

by 0.48-fold (128 ± 72.7 progenies), body length by 0.63-fold (837 ± 100 \(\mu\)m), head thrashes by 0.75-fold (26.9 ± 10 head thrashes per minute) and body bends by 0.55-fold (32.1 ± 4.8 body bending per 20 sec) in \textit{C. elegans} compared with the control group. Similarly, compared to the TRAP PM\(_{2.5}\)-D-glucose co-treatment group, \textit{C. elegans} pretreated with 200 \(\mu\)g tempeh significantly increased body length (887 ± 127 \(\mu\)m) and head thrashes (33.9 ± 10 head thrashes per minute) by 1.06-fold and 1.26-fold, respectively (Figs. 5(B) and 5(C)), but there was no meaningful difference in brood size (138 ± 39.5 progenies) and body bends (32.6 ± 6.7 body bending per 20 sec) (Figs. 5(A) and 5(D)).

Tempeh may have various biological activities, including cancer suppression, skeletal muscle recovery, modulation of colonic environment, improvement in malnutrition, cognitive function, lung health, cardiovascular health, and liver health (Ahnan-Winarno \textit{et al.}, 2021). Additionally, the tempeh treatment (40 mg kg body weight\(^{-1}\) day\(^{-1}\)) for four weeks altered the internal microbiota, leading to the inhibition of cholesterol synthesis and promotion of lipolysis, ultimately modulating
Fig. 5. Effects of 12.74 mg L⁻¹ TRAP PM₂.₅-80 mM D-glucose co-treatment with/without 200 µg of tempeh pretreatment on brood size (A), body length (B), head thrashes (C), and body bends (D) of *C. elegans* after 24-hours exposure. ** *p* < 0.01 versus the control group; *p* < 0.05; ** *p* < 0.01 versus the TRAP PM₂.₅-D-glucose co-treatment group.

Serum glucose and lipid levels in STZ-induced T2D rats (Huang *et al.*, 2018). In STZ-induced T2D rats, the 30-day tempeh treatment also significantly decreased hyperglycemia-induced advanced glycation end-product (AGE) production and receptor expression in testicular organs and improved sperm quality (Gofur *et al.*, 2020). In senescence-accelerated mouse prone 8 mice, tempeh regulates Nrf2 gene expression via the MAPK pathway, which attenuates antioxidant imbalance, beta-amyloid and cognitive deficit (Chan *et al.*, 2018). In the primary culture of nerve cells dissected from fetal mice brains, trans-resveratrol isolated from tempeh exerts neuroprotective and neurorestorative effects by reducing 2-methoxyethanol-induced beta-amyloid neurotoxicity (Irnidayanti *et al.*, 2021). Our results showed that the tempeh pretreatment ameliorates the toxic effects on the growth and locomotor behavior of *C. elegans* induced by a high glucose diet. Combination of those studies with our findings suggested that tempeh also improves T2D and protects nerve cells. Moreover, the current study also showed that the tempeh pretreatment could ameliorate TRAP PM₂.₅-induced and TRAP PM₂.₅-D-glucose co-treatment-induced adverse effects on *C. elegans*. 
3.4 Effect of Nematode Exposure to TRAP PM$_{2.5}$, High Glucose Diet and Tempeh on their Lifespan

To determine the possible effects of TRAP PM$_{2.5}$, high glucose diet and tempeh on nematodes, the toxicological endpoint lifespan was also investigated in this study for a duration of 24 days (Fig. 6). There was no meaningful difference in the lifespan of *C. elegans* with 200 µg tempeh pretreatment compared to the control group. The lifespan of *C. elegans* was significantly shorter following 12.7 mg L$^{-1}$ TRAP PM$_{2.5}$ treatment, 80 mM D-glucose treatment, and TRAP PM$_{2.5}$-D-glucose co-treatment, compared with the control group. However, 200 µg tempeh pretreatment prolonged the lifespan of nematodes after TRAP PM$_{2.5}$ treatment, high glucose diet treatment, and TRAP PM$_{2.5}$-D-glucose co-treatment.

3.5 Alteration of Antioxidant Gene Expression in *C. elegans* Following Exposure to TRAP PM$_{2.5}$, High Glucose Diet and Tempeh

PM$_{2.5}$ and hyperglycemia exposure can trigger oxidative stress and inflammatory response, which may contribute to the apoptosis. However, increasing expression of the antioxidant gene may alleviate the cell apoptosis caused by exposure to PM$_{2.5}$ and hyperglycemia (Nna et al., 2019; Li et al., 2021). To investigate possible mechanisms of toxicity and repair, *C. elegans* were exposed to TRAP PM$_{2.5}$, high glucose diets and tempeh in their adult stage for 24 hours, and then the expression of antioxidant gene was analyzed via real-time PCR. Skn-1 (the *C. elegans* functional ortholog of the mammalian Nrf transcription factors), CYP35A2, CAT1, CAT4, CTL1, CTL2, CTL3, TPH-1 (Fig. 7), SOD1, SOD2, SOD3, SOD4 and SOD5 (Fig. 8) genes showed no statistically significant differences between the control and 200 µg tempeh pretreatment groups. In Fig. 7 and Fig. 8, 12.74 mg L$^{-1}$ TRAP PM$_{2.5}$ or 80 mM D-glucose treatment significantly increased the Skn-1 (3.4-fold and 11.7-fold), CYP35A2 (13.1-fold and 82.4-fold), CAT1 (10.5-fold and 112.7-fold), CAT4 (3.4-fold and 19.4-fold), CTL1 (2.7-fold and 20.5-fold), CTL2 (8.1-fold in glucose treatment group), CTL3 (5.9-fold in glucose treatment group), TPH-1 (22.8-fold and 194.1-fold), SOD1 (3.8-fold and 5.4-fold), SOD2 (2.2-fold in glucose treatment group), SOD3 (17-fold and 151.8-fold), SOD4 (3.2-fold and 5.4-fold), and SOD5 (13.1-fold and 124.2-fold) mRNA expression compared to the control.
Fig. 7. Effects of with or without exposure to 12.74 mg L⁻¹ TRAP PM₂.⁵, as well as with or without 80 mM D-glucose diet, and with or without 200 µg tempeh on *C. elegans* (A) Skn-1, (B) CYP35A2, (C) CAT1, (D) CAT4, (E) CTL1, (F) CTL2, (G) CTL3, and (H) TPH1 mRNA expression after 24-hour exposure. * p < 0.05; ** p < 0.01 by one-way variance analysis followed by Tukey’s pairwise comparison.
Fig. 8. Effects of with or without exposure to 12.74 mg L⁻¹ TRAP PM₂.₅, as well as with or without 80 mM D-glucose diets, and with or without 200 µg tempeh on *C. elegans* (A) SOD1, (B) SOD2, (C) SOD3, (D) SOD4, and (E) SOD5 mRNA expression after 24-hour exposure. *p < 0.05; **p < 0.01 by one-way variance analysis followed by Tukey’s pairwise comparison.
group. These results indicated that enhancing the expression of SKN-1/Nrf2-mediated antioxidant pathway may protect against oxidative stress induced by TRAP PM$_{2.5}$ and high glucose diets, thereby regulating of the brood size, egg-laying capacity, vulval development, peroxisomal morphology, lifespan and the metabolizing of both endogenous compounds (e.g., signaling molecules), and exogenous compounds (e.g., xenobiotic chemicals) in *C. elegans* (Eom *et al.*, 2015; Schaar *et al.*, 2015; Tullet *et al.*, 2017).

TRAP PM$_{2.5}$-D-glucose co-treatment enhanced the mRNA expression of these antioxidant genes compared to the TRAP PM$_{2.5}$ group, except for the CTL3 gene. However, TRAP PM$_{2.5}$-D-glucose co-treatment significantly reduced the mRNA expression of these antioxidant genes, except for the CTL3 and SOD4 genes, compared with the 80 mM D-glucose group. 200 µg tempeh pretreatment ameliorated the alterations in mRNA expression of these antioxidant genes induced by high glucose diets or TRAP PM$_{2.5}$-D-glucose co-treatment in *C. elegans*. Although 200 µg tempeh pretreatment also ameliorated the altered expression of Skn-1, CYP35A2, CAT1, TPH-1, SOD3, SOD4, and SOD5 genes but CAT4, CTL1, CTL2, CTL3, SOD1, and SOD2 genes were not induced by the treatment of 12.74 mg L$^{-1}$ TRAP PM$_{2.5}$ in *C. elegans*. Interestingly, tempeh attenuates antioxidant imbalance, beta-amyloid and cognitive deficit by enhancing Nrf2 mediated-SOD/CAT antioxidant pathway in senescence-accelerated mouse prone 8 mice (Chan *et al.*, 2018). However, the current study revealed that tempeh pretreatment ameliorated the adverse effects on growth, reproduction, locomotion behavior, and lifespan in *C. elegans* caused by TRAP PM$_{2.5}$ and high glucose diet. The possible mechanism is certain that ROS play an important role in cell signaling (e.g., acting as second messengers) that contribute to the development, reproduction, adaptation, and survival (e.g., killing the invading pathogen) of *C. elegans* acting as to regulate (Miranda-Vizuete and Veal, 2017). TRAP PM$_{2.5}$-induced and high glucose diet-induced overexpression of antioxidant genes may suppress endogenously produced ROS, which can negatively affect reproduction, growth, and locomotor behavior in *C. elegans*. Tempeh pretreatment may maintain appropriate endogenous ROS generation and thus ameliorates TRAP PM$_{2.5}$-induced and high glucose diet-induced adverse effects on *C. elegans*. The previous study indicated that the nematodes of *C. elegans* share several fundamental physiological and stress response processes with their homologues in most human genes (60%–80%), including multiple signal transduction pathways (Kaletta and Hengartner, 2006). Additionally, our previous clinical study also suggested that tempeh attenuated HbA1C and triglyceride levels in participants (Su *et al.*, 2021). Therefore, we suggest *C. elegans* could give a different perspective or provide a more accessible or reliable assay to evaluate the possible toxicity of TRAP PM$_{2.5}$ co-exposure with high glucose the toxic effect and infer that tempeh may be beneficial for patients with TRAP PM$_{2.5}$ or hyperglycemia exposure. However, these need further investigation.

This study has several limitations. First, the *C. elegans* system seems to serve as an initial screening, but the toxic effects of TRAP PM$_{2.5}$ and high glucose diet co-treatment and the protective effect of tempeh should be confirmed in a more complex system, such as mammalian. Second, the experimental exposure methods in the current study are not real-world PM$_{2.5}$ exposures. Another important limitation of this study is the lack of results on larval exposure, so it cannot be used for inferences of early childhood exposure to TRAP PM$_{2.5}$ and high glucose diet co-treatment and tempeh application.

4 CONCLUSIONS

The present study revealed that TRAP PM$_{2.5}$ and high glucose diet co-treatment reinforced TRAP PM$_{2.5}$-induced or high glucose diet-induced adverse effects on growth, reproduction, locomotive behavior, lifespan and altered antioxidant mRNA expression in *C. elegans*. However, the tempeh pretreatment improved these adverse effects in *C. elegans* treated with or without TRAP PM$_{2.5}$ and with or without high glucose diet. These findings reveal that co-exposure to both TRAP PM$_{2.5}$ and high glucose causes more serious health effects, while tempeh may be beneficial nutritional supplements to ameliorate both TRAP PM$_{2.5}$-induced and hyperglycemia-induced toxicological effects.
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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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