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Polyethylene microplastics adversely affect airway patency

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ABSTRACT

Human exposure to microplastics through inhalation has been widely reported in recent years. There is a paucity of work focusing on the direct effect of accumulation of microplastics in airways and how it may impact the respiratory function. This study aimed to investigate whether the exposure of microplastic would change the contractility of isolated airway smooth muscle tissue. Microplastics were obtained through milling of high-density polyethylene (HDPE) pellets by using a centrifugal mill. To confirm that the milled microplastic particle size range fell within the definition of microplastic, field-emission scanning electron microscope (FESEM) was employed. The milled microplastics particle size ranged from 44.2 µm to 552.4 µm. The organ bath technique was employed to study the direct change of tissue contractility of rat isolated tracheal rings. Tracheal rings were incubated with polyethylene microplastics of different concentrations (0.3 mg/ml to 10 mg/ml) for a minimum of 18 hours in physiological Krebs buffer, followed by the construction of concentration-response curves to a contractile agent, carbachol (muscarinic agonist). Exposure to all concentrations of polyethylene microplastics enhanced the contractile responses of the tissues to carbachol. However, the effect was only statistically significant in tissues incubated at 3 mg/ml and above ($p < 0.05$). Findings from this study provide preliminary evidence that exposure to polyethylene microplastics adversely affects airway function. Heightened contractile responses of airways mimic the pathophysiological responses in respiratory diseases such as asthma, chronic cough and chronic obstructive pulmonary disease. Further experiments focusing on the possible mechanism of actions of these microplastics affecting the airway tissue function are now needed.

Keywords: *Microplastics; polyethylene; pollution; airway contractility and inhalation*

INTRODUCTION

Microplastic pollution and its potential impacts on the environment and human health have become a topic of considerable discussion in recent years. Microplastic is defined as any insoluble synthetic solid particle with a regular or irregular shape and with a size ranging from 1 µm to 5 mm, of either primary or secondary manufacturing origin (Frias & Nash, 2019). Studies have shown that the human body is exposed to microplastics through three main routes: inhalation of airborne microplastics, ingestion of food or water containing microplastics and dermal contact with plastic-made products or microplastics-containing dust (Prata et al., 2020). An alarming proposition was recently highlighted that existing non-biodegradable microplastics in the atmosphere will continue to cycle through the earth's systems (Brahney et al., 2021).

Besides the long-standing concerns about the impacts of plastic waste, the COVID-19 pandemic has resulted in an unprecedented increase in plastic usage due to single-use disposable masks, gloves and protective gowns. An extraordinary rise in the production of disposable face masks made from different types of polymeric nanofibers, including polyethylene, polypropylene, polyurethane, polyacrylonitrile or polystyrene, has been reported (Fadare & Okoffo, 2020). These have now become a major contributor to the plastic waste problem (Adyel, 2020). In addition, a recent study has demonstrated the risk of microplastics inhalation through face masks (Li et al., 2021). Although the uptake of microplastics in respiratory tissues and its long-term impact on health is not entirely understood, studies have demonstrated the deposition of inhaled particles in airways (Geiser & Kreyling, 2010; Vianello et al., 2019).

Once inhaled, microplastics can have a direct effect by colliding with the walls of the upper airway (Amato-Lourenço et al., 2020). Some particles may then descend into the lower respiratory tract due to gravity (Amato-Lourenço et al., 2020). Their fate is highly dependent on particle properties such as size and density, and lung physiology (Prata, 2018). These foreign particles are typically removed from the respiratory tract through several clearance mechanisms that include coughing and sneezing, mucociliary escalator as well as macrophage phagocytosis (Thomas, 2013). However, these defence mechanisms are impaired in people with respiratory conditions such as asthma, chronic obstructive pulmonary disease, and primary ciliary dyskinesia (Bustamante-Marin & Ostrowski, 2017). Due to the degradation-resistant nature of microplastics, their long-term accumulation in the respiratory tract could potentially lead to local inflammation and exacerbation of existing respiratory conditions or other health problems in instances where they are translocated (Pang et al., 2021).

Despite the growing evidence of microplastics exposure through inhalation, data on its functional impact on the airway smooth muscle is lacking. The airway patency is tightly controlled by the autonomic nervous system whereby the major contribution of the contraction of the respiratory tract comes from the activation of the muscarinic receptors on the smooth muscles during the activation of the parasympathetic pathway. Thus, in order to study the possible direct impact of the microplastics on airway tissue function, we used the organ bath system and carbachol, a muscarinic agonist, to stimulate airway tissue contraction.

MATERIALS AND METHODOLOGY

Polyethylene microplastics preparation and particle size analysis

Bimodal high-density polyethylene pellets (HDPE, Borstar HE6063, Austria) were gifted by Sinowaja (Malaysia) Sdn. Bhd. (Sarawak, Malaysia). The HDPE pellets were ground using a centrifugal mill (Retsch Ultra Centrifugal Mill ZM200, Restch, USA) at 8000 rpm, with a 200 µm ring sieve. The ground material was removed from the collecting cassette and filtered further with a 212 µm stainless steel sieve.

Particle morphology of the HDPE powder was investigated using a Field Emission Scanning Electron Microscope (FESEM) (FEI Quanta 400F, FEI Company, USA). The FESEM was set with the following parameters: voltage = 10kV, spot size = 3.5, magnification = 100x, working distance = 10mm, with large-field detector and low vacuum mode for non-conductive samples. A small amount of the HDPE powder was mounted on the microscope stubs with carbon tape before being inserted into the sample chamber of the FESEM. Then, images displayed on the viewing screen were captured and the diameters of the particles were measured using a measuring tool function available in the FESEM software.

Drug and Krebs-Ringer bicarbonate solution

Carbamylcholine chloride (carbachol) (Nacalai Tesque, Japan) was dissolved in purified water (purified by ELGA PURELAB® flex water purification system) to make a stock concentration of 0.1 M. The Krebs-Ringer bicarbonate solution was freshly prepared daily using purified water, following the composition (in mM): NaCl 120, KCl 5.4, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, $CaCl_2$ 1.26; pH 7.4 and aerated with 95% O2 and 5% CO2.

Tissue preparation and experimental protocol

Ethical approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (AWERB) (Reference: UNMC12). The experiments were conducted with male Sprague-Dawley rats (244 – 591 g; 2 – 3 months old). Animals were purchased from the Faculty of Veterinary Medicine, University Putra Malaysia and sacrificed on the day of the experiment by asphyxiation with carbon dioxide. The trachea was removed and prepared as described in our previous study (Loong et al., 2015). The surrounding connective tissues were removed before excising the trachea into 2 mm rings.

Then, the trachea rings were transferred into glass scintillation vials filled with different amount of HDPE powder in 5 ml of aerated Krebs solution. The concentration of HDPE microplastics used in this study were 0.3 mg/ml, 1.0 mg/ml, 3.0 mg/ml, 5.0 mg/ml and 10.0 mg/ml. These concentrations were achieved by measuring 1.5 mg, 5.0 mg, 15.0 mg, 25.0 mg and 50.0 mg of HDPE microplastics into the glass scintillation vials and adding 5 ml of aerated Krebs solution into the vials. The tissues were then stored at 4 °C for 18 hours. Glass scintillation vials

were used to avoid risk of plastic leaching from plasticwares (Honeycutt et al., 2017).

Following overnight incubation (18 hours), each ring was placed in a glass chamber filled with 10 ml of Krebs solution. The temperature was maintained at 37 °C and tissues were supplied with 95% O_2 and 5% CO_2 throughout the experiments. Changes in muscle tension were detected by a force transducer (MLTF050/ST, ADInstruments, USA). Data were recorded with a PowerLab data acquisition system (LabChart v7.3.8). After the application of tension of 9.8 mN (equivalent to 1 g), tissues were left to equilibrate to bath condition for at least 30 minutes. Next, the tissues were exposed to 60 mM potassium chloride (KCl) two to three times to assess tissue viability. Any tissue with an average KCl-induced tone of < 3.92 mN was considered to be non-responsive (Loong et al., 2015). Subsequently, cumulative concentration-response curves (CRCs) to carbachol (0.1 nM to 300 µM) were constructed at time intervals of 5 minutes or until the response plateaued. The degree of contraction was measured as a fraction of the contraction from basal tension and expressed as a percentage of 60 mM KCl-induced tone.

Statistical analysis

The diameter (width) of the polyethylene microplastic particles was measured using the measuring software following the FESEM (FEI Quanta 400F). Data obtained from the organ bath experiments were expressed as mean ± standard error of mean (SEM) of n number of animals. GraphPad Prism version 8.4.3 for Windows (GraphPad software, USA) was utilised for data analysis and graphical visualisation of the data. The maximum response (E_{max}) and pEC_{50} values were derived from non-linear regression analysis of the obtained CRC. The pEC_{50} value is the negative logarithm of EC_{50} where EC_{50} is the concentration of drug that produces 50% of its maximum response. Statistical comparisons of E_{max} and pEC_{50} between the control and HDPE-treated group were performed using unpaired t-test (two-tailed) and the results were considered statistically significant if p-value < 0.05.

RESULTS

Particle size distribution of HDPE microplastics

The shape and size of the milled and sieved microplastics varied, as evident from the FESEM (Figure 1). The particle size ranged from 44.2 µm to 552.4 µm, with more than 70% of the pieces being smaller than 200 µm (Table 1). Some particles appeared round while others were long and irregular. Those with a round shape tended to have smaller diameter than those with irregular dimensions.

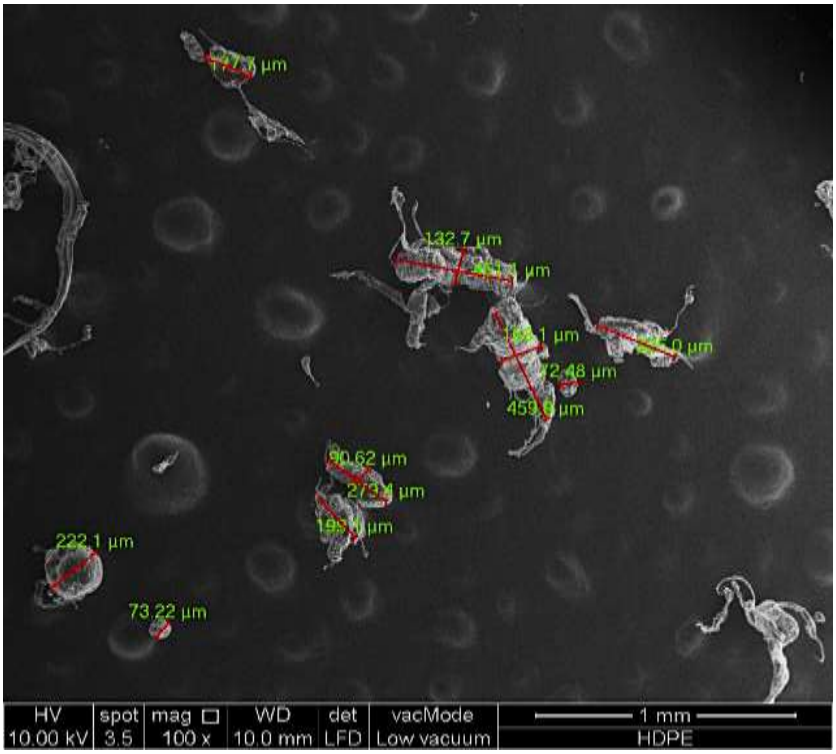

Figure 1: A representative image of the polyethylene microplastics viewed under field emission scanning electron microscope (FESEM). Abbreviations: HV= voltage, spot = spot size, mag = magnification, WD = working distance, det = detector, LFD = large-field detector (pre-selected for non-conductive samples), vacMode = vacuum mode, HDPE = high density polyethylene (sample name).

Table 1: Particle size distribution of the milled and sieved polyethylene microplastics. The FESEM imaging was conducted four times and the particle diameters were grouped into three categories (1 – 200 µm, 201 – 400 µm and 401 – 600 µm). The mean diameters of the particles were measured with the FESEM measuring software.

Sample diameter (µm)	Mean diameter (µm)	Percentage (%)
1 – 200	119.5	70.00
201 – 400	272.5	23.33
401 – 600	534.3	6.67

Polyethylene microplastics potentiated carbachol-induced contractions

Figure 2 and Table 2 show that at all concentrations of the microplastics tested, airway responses to carbachol were affected. Significant changes were observed in tissues exposed to microplastics at higher concentrations (3 mg/ml, 5 mg/ml and 10 mg/ml) whilst a marginal increase in the maximum response to carbachol was detected in the lower concentrations (0.3 mg/ml to 1 mg/ml), as compared to the control. E_{max} and pEC_{50} values of carbachol derived from the respective CRC are displayed in Table 2.

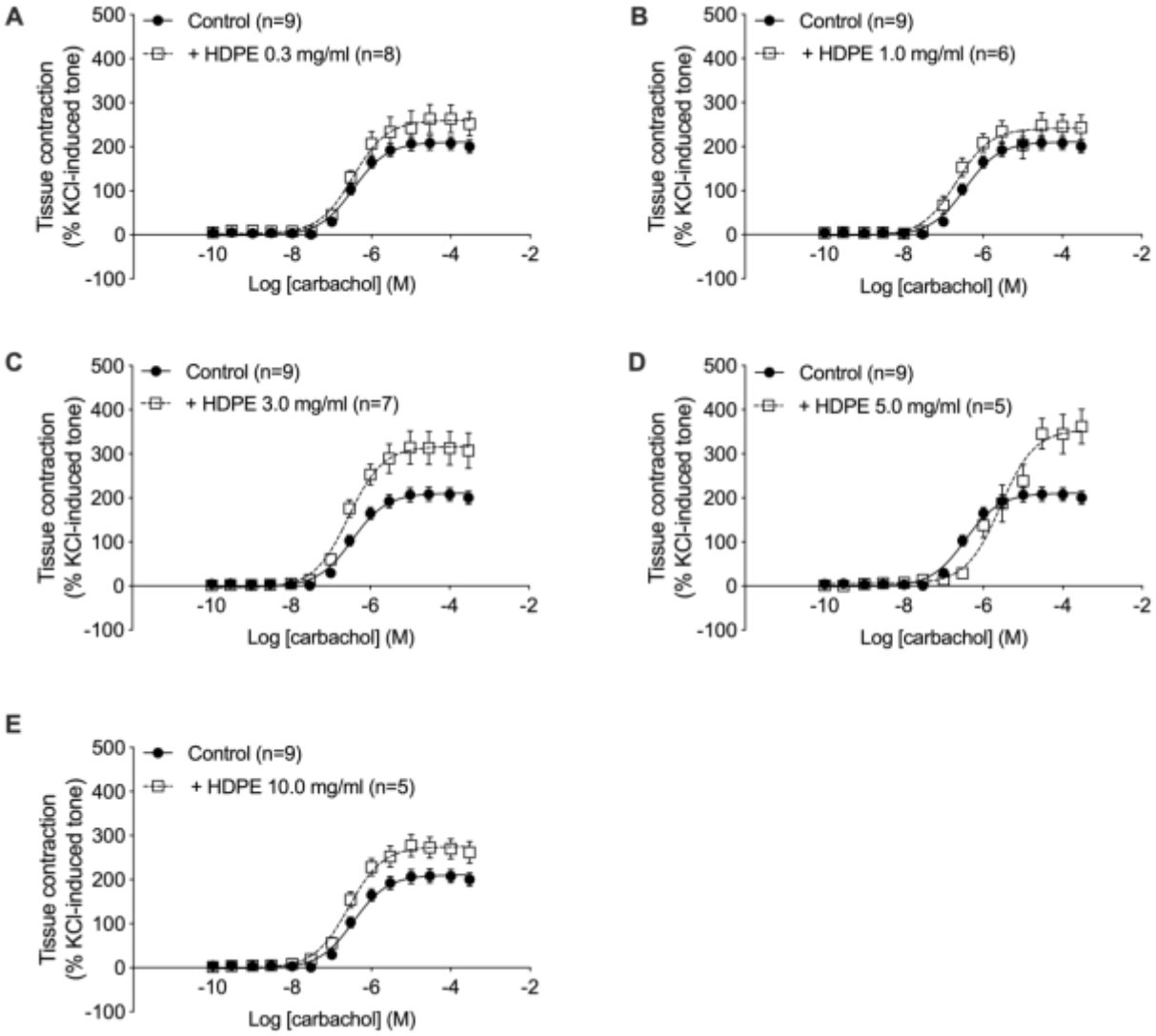

Figure 2: Effect of overnight incubation with high-density polyethylene (HDPE) microplastics on carbachol-induced contractions in isolated rat trachea. Tissues were incubated overnight in Krebs solution (control) or Krebs solution with HDPE microplastics (A) 0.3 mg/ml, (B) 1 mg/ml, (C) 3 mg/ml, (D) 5 mg/ml, (E) 10 mg/ml. Cumulative concentration-response curves to carbachol were constructed following overnight incubation. Tissue contractions were expressed as a percentage of 60 mM KCl-induced tone and shown as mean ± SEM of n number of animals.

Table 2: The maximum response (E_{max}) and pEC_{50} of carbachol were derived from the respective concentration response curve. Unpaired t-test where comparison of the mean was made between control and the HDPE-treated group; *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. Data were shown as mean ± SEM of *n* number of animals.

Concentration of HDPE (mg/ml)	*n*	E_{max} (%)	pEC_{50}
Control (Krebs solution without HDPE)	9	211.70 ± 15.62	6.45 ± 0.07
0.3	8	260.30 ± 33.15	6.50 ± 0.06
1	6	242.60 ± 23.35	6.65 ± 0.09
3	7	317.20 ± 38.66*	6.48 ± 0.11
5	5	376.00 ± 44.71**	5.54 ± 0.24***
10	5	275.40 ± 23.87*	6.57 ± 0.05

DISCUSSION

As polyethylene is one of the most abundant types of microplastics in the environment and the second highest contributor of atmospheric non-fibrous microplastics (Wright et al., 2020), it was chosen as the material to investigate in the present study. In order to obtain a consistent and controlled supply of microplastic particles, industrial grade high density polyethylene pellets were ground and milled using a centrifugal mill. The size range of the particles produced in our lab was comparable to those identified in atmospheric fallout (50 µm – 5000 µm) (Zhang et al., 2020) where more than two-thirds of the microplastics recorded had a diameter below 200 µm.

After 18 hours of incubation with the polyethylene microplastics, the contractile response of the rat tracheal rings towards carbachol (muscarinic agonist) was exaggerated. This suggests that the exposure to these microplastic particles changes the physiological function of the tracheal tissues, specifically the control of air flow into the lungs. This exaggeration effect corresponds to the overactivation of muscarinic receptors in respiratory conditions such as chronic obstructive pulmonary disease (COPD) and asthma which results in bronchoconstriction and over-secretion of mucus (Buels & Fryer, 2012).

During the experiments, these polyethylene microplastics tended to adhere to the tissues. However, at the highest concentration tested (10 mg/ml), they coalesced and formed tiny clusters in the buffer solution, reducing the adhesion to the tissues. This is one reason that the concentration of the sample was not increased above 10 mg/ml. It also provides a possible explanation for a lower response observed in 10 mg/ml compared to 5 mg/ml. Adhesion of these polyethylene particles to the airway tissues seemingly mirrors the deposition of airborne particulate matter in these tissues.

As the airway epithelium is the principal deposition site for airborne particulate matter (Cooper & Loxham, 2019), we postulated that the exposure to these microplastics damages the integrity of the epithelium. This notion can be supported by the findings of previous studies where removal of the epithelial lining of the airway smooth muscles enhanced acetylcholine- and histamine-induced contractions due to the loss of epithelium-derived relaxant factors such as nitric oxide, prostaglandins and adenosine triphosphate (Ruan et al., 2011). Furthermore, a recent study showed that following exposure to polystyrene microplastics (10 to 1000 µg/cm2), the expression of the tight junction proteins including Zonula Occludens, on the human lung epithelial cells was decreased, indicating a disruption to the lung epithelial lining (Dong et al., 2020).

The findings of this study contribute to evidence of the potential adverse effects of microplastic particles on the respiratory system. Nevertheless, some limitations of this study are acknowledged. Firstly, the primary microplastics (originated from the manufacturer) used may not accurately represent microplastics in the environment which may have interacted with chemical or biological pollutants, altering their properties and effects. However, using virgin polyethylene particle as our experimental sample gives the confident of the integrity of the sample. Secondly, the use of isolated tissues disregards the activation of innate defence mechanisms *in vivo*. An *in vitro* isolated tissue set up was employed because it can provide a direct evidence of changes to the physiological function following exposure to the microplastics. Nonetheless, further experiments involving isolation of microplastics from consumer goods such as face masks are being planned to enhance our understanding on the effect of microplastics. Histological investigation is also required to confirm the possible damaging effect of microplastics deposition on the integrity of the epithelial lining of tracheal tissue.

CONCLUSION

To the best of our knowledge, this is the first functional study to demonstrate the direct effect of microplastics on airway smooth muscle contractility. Our results show that exposure to microplastics significantly enhanced the contractile response of rat isolated airway smooth muscle. One plausible reason for this is damage to the epithelial lining. The results reported herein support various published hypotheses related to adverse effects following exposure to microplastics through inhalation. Further work including mechanistic studies and

histological investigation as aforementioned are in the progress to delineate these initial findings.

AUTHOR CONTRIBUTIONS

Nurshafida Adzlin Shamsul Anuar and Li-Yin Pang performed the experiments and wrote the manuscript; Sivathass Bannir Selvam involved in preparation of the polyethylene microplastics; Christopher Gibbins supported the research and edited the manuscript. Kang-Nee Ting supported, designed, supervised, wrote and reviewed the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL

Ethical approval was obtained from the University of Nottingham's Animal Welfare and Ethics Review Body (AWERB) (Reference: UNMC12).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in this work.

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