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The use of a 0.20 μm particulate matter filter decreases cytotoxicity in lung epithelial cells following air-liquid interface exposure to motorcycle exhaust☆

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ABSTRACT

This study was designed to investigate whether the use of a 0.20 μm particulate matter (PM) filter reduced the cytotoxicity induced by motorcycle exhaust (ME), a mixture of gases and particles, in lung epithelial cells cultured in air-liquid interface (ALI) inserts. The concentrations of PM, carbon monoxide, carbon dioxide, total hydrocarbons (THC), total volatile organic compounds, and nitrogen oxides in both filtered ME (fME) by a 0.20 μm filter and non-filtered ME (non-fME) were measured. Lung epithelial cells were exposed to clean air, fME, or non-fME in the ALI chamber. Cell relative viabilities (CRV) and the reactive oxygen species (ROS) generation were determined. Our results revealed that $PM_{2.5}$ was the main compound of PM in ME. After filtration, PM and THC levels were significantly reduced, as compared with non-fME. When compared with the clean air exposed group, the CRV in both fME and non-fME-exposed group was significantly reduced ($p < 0.001$), while their ROS generation were markedly increased ($p < 0.001$). When compared with non-fME-exposed group, the CRV and ROS generation were significantly improved following fME exposure ($p < 0.05$). As a result, of PM and THC concentrations were decreased approximately 90% and 22.71%, respectively, the CRV was improved from 40.4% (non-fME) to 55.7% (fME), and the increased ROS generation by non-fME was decreased about 51.6%. When BEAS-2B cells were exposed to fME, a time-dependent reduction in CRV was observed. In conclusion, our findings suggest that ME-exposure in the ALI system induces cytotoxicity and oxidative stress responses. The addition of a 0.20 μm PM filter significantly modifies the particulate composition in PM and the concentration of THC, and shows protective effects by improving the survival of exposed lung epithelial cells and reducing the ROS generation. Therefore, emission factors such as different size of PM and THC from motorcycles may play a role in ME-induced toxicity.

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1. Introduction

Motorcycle is an economical transportation in the developing world. China has the largest number of motorcycles in the world. Currently, there are more than 224 million motor vehicles and almost half of them are motorcycles in the mountain villages (Li et al., 2015). Therefore, motorcycle exhaust (ME) is considered as a major source of air pollution and a potential health hazard in urban areas. As the use of motorcycles increases, the risk for human exposure to ME is also significantly increased. Thus, it is necessary to study the impact of ME on the environment and their biological effects.

Like other air pollution exposure, people are constantly exposed to ME mainly *via* the lung (inhalation), but also potentially *via* the skin or even the eyes. The lung is exposed to a large volume of inhaled air that comprises of numerous particles and gases, and airway epithelial cells are the first cells to come into contact with inhaled toxic compounds. Toxicology studies in animals have showed that major effects of short-term ME exposure includes lung and systemic inflammation (Chen et al., 2013; Lee et al., 2004). Significantly increased IL-8 production was detected in human

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Abbreviations

ALI	Air liquid interface
ANOVA	Analysis Of Variance
CI	Confidence interval
CO	Carbon monoxide
CO_2	Carbon dioxide
CCK-8	Cell Counting Kit-8
CRV	Cell relative viability
DCFH-DA	dichloro-dihydro-fluorescein diacetate
DE	diesel exhausts
fME	Filtered motorcycle exhaust
LDH	Lactic dehydrogenase
ME	Motorcycle exhaust
MEP	Motorcycle exhaust particulates
non-fME	Non-filtered motorcycle exhaust
ND	Not detectable
NOx	Nitrogen oxides
PAHs	polycyclic aromatic hydrocarbons
PM	Particulate matter
$PM_{2.5}$	particulate matter with an aerodynamic diameter of 2.5 μm or smaller
PBS	Phosphate buffered saline
SD	Standard deviation
THC	Total hydrocarbons
TVOCs	Total volatile organic compounds
TWCC	Three-way catalytic converter

airway epithelial cells following motorcycle exhaust particles (MEP) (Lee et al., 2005). Since most of MEP toxicity studies are cell-based *in vitro* (Kuo et al., 1998; Ueng et al., 2000, 2005; Wang et al., 2002), the evaluation of pulmonary toxicity induced by ME as a mixture of gases and particles is limited.

As the integration of studies is required by regulatory authorities to reduce or replace *in vivo* animal experiments, *in vitro* cell culture-based studies are still the major approach. To date, there are two major culture models, submerged culture and air-liquid interface (ALI), to cultivate and expose lung cells to air particles. Submerged cell culture is a classic model to study ME exposure-induced toxicity, however, the major drawback of this exposure model is that cells are not directly exposed to the whole complex and dynamic mixture of gases, such as nitrogen oxides (NOx), carbon monoxide (CO), carbon dioxide (CO_2), volatile and particle compounds consisted in ME. A newly developed ALI exposure model simulates the real life air exposure allowing for studying effects of complex air exposures on airway epithelial cells *in vitro* under near-realistic conditions (Maier et al., 2008). ALI is a powerful tool for studying substance effects directly on human biology without using animals or human volunteers. This exposure system has been adopted to test toxicity of mixtures such as diesel exhaust (DE) (Kooter et al., 2013; Okubo et al., 2015) and gasoline exhaust (Künzi et al., 2015). Direct exposure of cells in the ALI system has the advantage of minimizing adverse effects from culture medium, enhancing the pharmacological and/or toxicological extrapolation from *in vitro* to *in vivo* scenario, and representing a more realistic exposure scenario compared to the exposure system with cells cultured under submerged conditions (Kim et al., 2013; Lenz et al., 2009; Paur et al., 2011). Advanced *in vitro* studies using combinations of cells cultured in the ALI is useful for particle uptake and mechanistic studies, in which cell viability and oxidative stress levels are the most commonly measured parameters (Fröhlich and Salar-Behzadi, 2014; Herzog et al., 2013; Lenz et al., 2009).

As a mixture of particles and gases, ME can induce complex health effects on human, and the impact of particles is more significant. Increasing evidence from animal studies have demonstrated that MEP can cause adverse health effects. MEP is a complex group of air pollutants that vary in size, and PM is classified as coarse particles (Particles between 10 and 2.5 μm in diameter), fine particles (particles between 2.5 and 0.1 μm in diameter), and ultrafine particles (particles with a diameter less than 0.1 μm) (Kim et al., 2015). Particle properties such as size and surface area may induce different biological responses (Maier et al., 2008). However, it remains to be addressed which fraction of MEP responsible for observed health effects and which particle feature plays a role in initiating adverse mechanisms.

Cell viability assays are vital step in determining the cellular response to a toxicant providing information on cell death, survival, and metabolic activities (Lee et al., 2011). Currently, there are several cell viability assays including MTT assay, neutral red assay, Cell Counting Kit-8 (CCK-8) assay, and lactate dehydrogenase (LDH) leakage assay. ME exposure has been shown to induce genotoxicity and cytotoxicity, and influence cell proliferation (Kuo et al., 1998; Lee et al., 2005). CCK-8 and LDH release assays are commonly used to assess the rate of cellular proliferation and quantify cell viability. Damages on cell membrane and mitochondria due to exposure to environmental compounds can cause the release of cholecystokinin or LDH to medium. Therefore, detections of cholecystokinin and LDH in the cell culture media will indicate the occurrence of cell membrane and mitochondrial damages (Vergaro et al., 2016; Wen et al., 2015).

In the present study we chose to employ human alveolar carcinoma cells (A549) and human bronchial normal cells (BEAS-2B), representing the principal cells of respiratory system, as lungs are the main potential exposure target organ (Ursini et al., 2014). Moreover, several recent studies on PM toxicity were performed on alveolar carcinoma A549 cells which maintain many morphological and biochemical characteristics of type II pneumocytes (Durga et al., 2014; Okubo et al., 2015; Xie et al., 2012). Also bronchial epithelial cells (BEAS-2B) are targets of inhalation and play an important role in the maintenance of mucosal integrity as mechanical barriers against various PM (Takizawa, 2004).

To investigate the pulmonary toxicity of ME, the ALI exposure system was adopted in the current *in vitro* study. Before ME exposure experiments, we analyzed the characteristics of the exhaust emission from motorcycle using gasoline fuels with three-way catalytic converters (TWCC). Human lung cells (A549 and BEAS-2B) were then cultured on the insert membrane in the ALI exposure chamber and exposed to the characterized ME. Furthermore, we examined whether the addition of 0.20 μm PM filter would result in different cytotoxicity and intracellular ROS generation. Relations between different effects and specific composition of ME as well as the correlation between exposure time and cytotoxicity were also evaluated.

2. Materials and methods

2.1. Sampling of motorcycle exhausts

Sampling exhaust emission was carried out in a two-wheeler

motorcycle. The vehicle equipped with TWCC. The most widely used 93# gasoline in China with octane number of 93 from China Petroleum Company was used as the fuel. The motorcycle was conditioned at cold starting and idling before the 2 min' ME sampling using Tedlar bag (Beijing Safelab Technology Ltd, China). All ME samples were collected for the characterization analysis and *in vitro* exposure experiments.

2.2. Characterization of motorcycle exhaust

In order to quantify sizes of particles distributed in ME, we used two aerodynamic particle size spectrometers, *TSI*-3321 (scanning diameter: 0.5–20.0 μm) and *SMPS*-3938 (scanning diameter: 0.011–0.610 μm) to determine the number, surface area and mass concentration of ME particles in all diameters (TSI Corporation, USA). The CO and CO_2 concentrations in the emission were measured using non-dispersive infrared analyzer SM-200 for CO, and GXH-3011 for CO_2 (Beijing Hua Yun Analytical Instrument Research Institute, China). The concentration of total volatile organic compounds (TVOCs) was determined using volatile toxic gas analyzer TVA-1000(Thermo Fisher, USA). The total hydrocarbon (THC) level was analyzed by gas chromatography GC-2010 (SHIMADZU, Japan) using methods for determination of mixed hydrocarbons in the air of workplace (GBZ/T160.40–2004, No.3 in China). The NOx concentration was determined using N-(1-naphthyl)-ethylenediamine dihydrochloride spectrophotometric method as described previously (Yu et al., 2014).

2.3. Cell culture

The human lung epithelial carcinoma cell line A549 was purchased from the Institute of Basic Medical Sciences, Chinese Academy of medical sciences (No. 3111C0001CCC 000002) and maintained as described in previous study (Yu et al., 2014). The human bronchial normal cell line BEAS-2B was purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, People's Republic of China) and maintenance was described previously (Bai et al., 2016). For exposure experiments, cells were cultured on 24 mm diameter of 6-well Corning Transwell® permeable culture inserts with 0.40 μm pore size (Cat.No.3450, Corning, NY, USA). The inserts were not coated or treated prior to seeding. Before seeding cells, inserts were covered with 1 ml of media inner chamber (apical surface) and 2 ml of media in the outer chamber (the basolateral side) for 1 h. After removing the media from the inner chamber, 1 ml cell suspension was added at a density of 4.0×10^5 per insert and cultured for 24 h in an incubator (5% CO_2, 37 °C and maximal relative humidity).

2.4. ME air liquid interface exposure conditions

Before exposure, media was removed from the inner chamber, and both inner and basolateral side of inserts were washed twice with phosphate buffered saline (PBS, Gibco). The Transwell inserts were then transferred into the ALI exposure device (HRH-CES1332, Beijing Huironghe Technology CO., LtD., China). As show in Fig. 1, cells were exposed to either clean air consisted of 21% O_2 and 79% N_2 (Beijing Oriental Medical Gas Co., Ltd., China); or non-filtered ME (marked as non-filtered ME exposed group, non-fME) and filtered ME using the Acro® 50 Vent Device with a 0.20 μm PTFE Membrane (PN4251, Pall Corporation, USA) (marked as filtered ME exposed group, fME). The ALI exposure system consisting of three glass medium-containing wells was temperature controlled by circulating 37 °C water. The ME or clean air was drawn into exposure wells through upper chambers at a flow rate of 25 ml/min/well for 1 h to A549 cells, and 15 ml/min/well for 30, 60, or 90min to BEAS-2B cells.

2.5. Determination of cell viability using Cell Counting Kit (CCK-8) assay and lactate dehydrogenase (LDH) released assay

In order to determine the cellular toxicity following ME exposure, both CCK-8 assay (purchased from Dojindo Laboratories, Japan) and LDH released assay (purchased from Beyotime Bioengineering Institute, China) were applied in A549 cells, and CCK-8 assay was used in BEAS-2B cells. Both assays were processed according to manufacturers' instructions. Results were expressed as cell relative viability percentage (CRV %).

2.6. Analysis of intracellular reactive oxygen species (ROS) by flow cytometry

Intracellular ROS generation was detected by flow cytometry using a peroxide-sensitive fluorescent probe 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA, Nanjing Jiancheng Bioengineering Institute, China) as described by Zhang et al. (2015) with slight modification. In brief, cells cultured on the Corning Transwell inserts were washed twice with PBS after the ALI exposure and

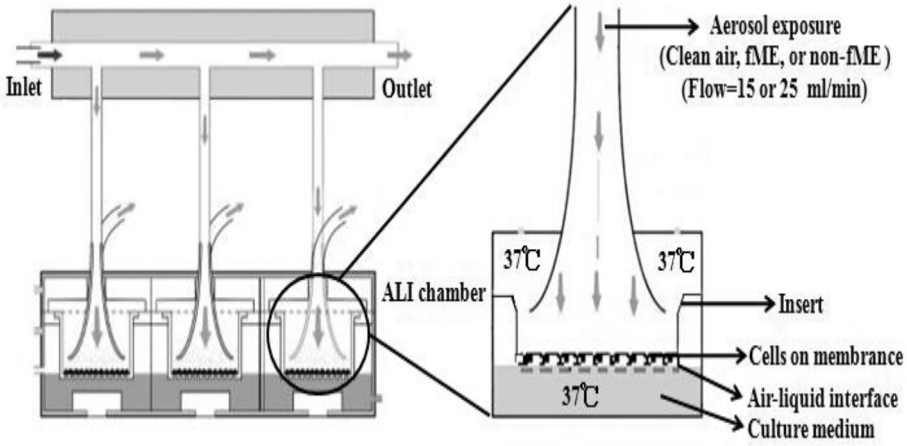

Fig. 1. A schematic of the air-liquid interface (ALI) exposure system. The motorcycle exhaust (ME) or clean air was delivered to cells grown on the membrane, and 37 °C medium below the membrane provides nutrition for cells. Cells were exposed directly to the ME or clean air inside the ALI chamber.