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Shaorui Ke

Henan University of Chinese Medicine

Qi Liu

Fudan University

Xinlian Zhang

Fudan University

Yuhan Yao

Fudan University

Xudong Yang

Tsinghua University

Guodong Sui (✉ gsui@fudan.edu.cn)

Fudan University

Research

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**Cytotoxicity analysis of biomass combustion particles in human pulmonary
alveolar epithelial cells on an air-liquid interface/dynamic culture platform**

**Shaorui Ke^{1,2}, Qi Liu², Xinlian Zhang², Yuhan Yao², Xudong Yang^{*,3}, Guodong
Sui^{*,2,4}**

¹ Co-construction Collaborative Innovation Center for Chinese Medicine and
Respiratory Diseases by Henan & Education Ministry of P.R. China, Academy of
Chinese Medical Sciences, Henan University of Chinese Medicine, Zhengzhou
450046, China.

² Shanghai Key Laboratory of Atmospheric Particle Pollution Prevention (LAP3),
Department of Environmental Science & Engineering, Fudan University, Shanghai
200433, P.R. China.

³ Department of Building Science, Tsinghua University, Beijing 100084, P.R. China.

⁴ Jiangsu Collaborative Innovation Center of Atmospheric Environment and
Equipment Technology (CICAEET), Nanjing University of Information Science &
Technology, Nanjing 210044, P.R. China.

E-mail: Shaorui Ke: 15110740019@fudan.edu.cn;

Qi Liu: 13110740002@fudan.edu.cn;

Xinlian Zhang: 15210740031@fudan.edu.cn;

Yuhan Yao: 16110740002@fudan.edu.cn;

Xudong Yang: xyang@tsinghua.edu.cn;

Guodong Sui: gsui@fudan.edu.cn.

Abstract

Background: Exposure to indoor air pollution from solid fuels combustion is associated with lung diseases and cancer. This study aimed to explore the cytotoxicity and molecular mechanisms of biomass combustion-derived particles in human pulmonary alveolar epithelial cells (HPAEPiC) using a platform that combines air-liquid-interface (ALI) and dynamic culture (DC) systems.

Methods: HPAEPiC were cultured on the surface of polycarbonate (PC) membranes in ALI-DC platform. The top surface of cells was sprayed with a solution of biomass combustion soluble constituents (BCSCs) and simultaneously nourished with medium flowing from below PC membranes. BCSC particles' morphology and dosages deposited on the chip was determined for particle characterization. A flow cytometer, laser scanning confocal microscopy (LSCM), and transmission electron microscope (TEM) were used to investigate the apoptosis of HPAEPiC and the changes in the ultrastructure of HPAEPiC induced by BCSCs. Additionally, the underlying apoptotic pathway was studied through the determination of the protein expression levels by western blotting.

Results: Scanning electron microscope (SEM) results demonstrated that the sample processing and delivering approach were very suitable for pollutant exposure of the platform. The decline in cell viability and increase in apoptosis rate after exposure to similar doses of BCSCs were more under ALI-DC conditions than under submerged conditions. This indicated that the ALI-DC platform is a superior system for investigating cytotoxicity of indoor air pollutants in lung cells. The morphology and

ultrastructure of the cells were damaged after exposure to BCSCs. Our results demonstrated that the p53 pathway was activated after exposure of cells cultured using this platform to BCSCs. This further decreased the Bcl-2/Bax ratio thereby activating the expression of caspase-9 and caspase-3, which in turn induced apoptosis in HPAEpiC. Additionally, antioxidants (N-acetyl-cysteine; NAC) could significantly alleviate the cytotoxicity induced by BCSCs.

Conclusions: A novel ALI-DC platform was successfully presented to study cytotoxicity of air pollution on lung cells. Based on the platform, the BCSCs was demonstrated that it could damage mitochondria, produce ROS, and activate p53 in HPAEpiC. Furthermore, BCSCs decreased the Bcl-2/Bax ratio and efficiently upregulated the expression of cleaved caspases-9 and -3, ultimately inducing apoptosis.

Keywords: Biomass combustion, Particles, HPAEpiC, Air-liquid interface, Dynamic culture, Cytotoxicity

Background

Indoor air pollution is the fourth leading cause of deaths worldwide after hypertension, tobacco smoking, and alcohol consumption [1]. It has become a growing worldwide concern; solid fuel combustion is a major source of indoor air pollution [2, 3]. According to a World Health Organization report, approximately 3 billion people still depend on solid fuels (i.e., wood, charcoal or other traditional biomass) to meet the daily energy needs of their family [4]. Moreover, over 4 million premature deaths from diseases attributable to indoor air pollution from solid fuel combustion are

recorded annually [5]. Solid fuel combustion can produce high levels of health-damaging indoor air pollutants, including respirable particulate matter, noxious gases, and many other toxic chemicals [6]. Airborne particulates with large surface areas and strong adsorption capacities carry harmful bacteria, viruses, organic compounds, and heavy metals. These aggravate the severity of human health effects, especially on the cardiovascular and respiratory systems [7]. Globally, approximately 6.5 million people die annually from air pollution-related cardiovascular and respiratory diseases, out of which 3.5 million deaths were associated with indoor air pollution [8, 9].

The lungs are the most critical barrier against direct exposure to indoor air pollution but also the most severely damaged organ. Long-term exposure to indoor air pollution from solid fuel combustion could increase the occurrence and development of lung diseases including lung cancer [10]. Previous studies have shown that 35.7% of respiratory diseases, 22% of chronic lung diseases, and 15% of bronchitis diseases are associated with indoor air pollution [11]. According to epidemiological statistics, lung cancer incidence rate among women in China (20.4 cases per 10 million women) was higher than those in several European countries with higher prevalence of female smokers [12, 13]. This may be because women in China are more likely to be exposed to airborne particles from solid fuel stoves. Because cooking is unavoidable, people are exposed long-termly have to the smoke emitted from dirty cooking stoves. It is essential to study the effects of indoor air pollution from solid fuel combustion on lung health.

It has been reported that airborne particles with various harmful substances adsorbed on its surface can enter the lungs through the respiratory tract [14]. Ultrafine particles (diameter less than 2 μm) can directly enter the pulmonary alveoli, damaging the respiratory system and suppressing immune function, which ultimately induces diseases such as pneumonia, emphysema, silicosis, and lung cancer [15]. Various heavy metals and organic compounds in airborne particles could participate in the pathogenesis of lung diseases through their ability to generate reactive oxygen species (ROS) and activate related signal transduction pathways [16]. Certain lipophilic and low molecular weight (< 1 kD) compounds in airborne particles can bind to extracellular or membrane proteins, initiating multiple signaling pathways that regulate cell proliferation, differentiation, apoptosis, and other cellular processes. Previous studies have confirmed that particulate matter 2.5 ($\text{PM}_{2.5}$) from outdoor air pollution could produce intracellular ROS to activate p53 signaling pathway and the expression of its downstream target proteins, ultimately inducing inflammatory responses and apoptosis in human umbilical vein endothelial HUVECs and lung epithelial A549 cells [17]. Generation of ROS cause oxidative stress, which can cause irreversible damage at the molecular and cellular levels. Inflammatory responses of lung cells can cause breathing problems, reduced lung function, and lung diseases, severely affecting human health and quality of life [18]. Therefore, it is essential to study the toxicity of airborne particles, generated from solid fuel combustion, at the molecular level in lung cells.

Recent studies have focused on the cytotoxicity and molecular mechanism of

particles, emitted during solid fuel combustion, in lung cells. However, the *in vitro* cytotoxicity analyses of these studies were generally performed using cells cultured under traditional submerged conditions, wherein the particles are directly suspended in the cell culture medium containing submerged cells. Submerged cell culture methods provide an unviable synthetic environment for the culture of lung cells as they are entirely covered with the cell culture medium. The ALI technique allows direct contact between aerosols and target cells that are cultured under a very thin liquid film. This technique prevents the interference of the cell culture medium on the exposure of cells to respirable substances and maximizes the contact area between the aerosols and cells [19, 20]. Consequently, the cytotoxicity test conducted under ALI exposure conditions provides more reliable reference values and has practical implication. Therefore, the ALI technique has been the preferred culture method for *in vitro* toxicity testing of airborne particles with respect to pulmonary health.

Many studies on particle toxicity have adopted the ALI systems that are more representative of pulmonary responses against exposure to toxic particles, to obtain new insights that could not be achieved using traditional culture methods. Some of these studies have reported results that are markedly different from those of studies conducted under submerged conditions. Nickel oxide particles and zinc oxide particles, for instance, exhibited higher toxicities under ALI conditions than under submerged conditions at similar doses [21]. However, silica nanoparticles and silver nanoparticles exerted lower toxicities in lung cells cultured under ALI conditions than in those cultured under submerged conditions [22]. Additionally, several studies have

shown that the results of *in vitro* tests with ALI cultures were closer to *in vivo* data than those of *in vitro* tests with submerged cultures, indicating that *in vitro* tests with ALI cultures can provide more reliable reference values [23].

The cytotoxicity of the tested material is assessed based on the physiological and biochemical changes, including metabolic, functional, and gene expression changes, induced in the treated cells. Additionally, the cell growth microenvironment provided by the DC method is another important factor in toxicity testing. The fluid shear produced by dynamic cell cultures has been demonstrated to play an important role in cellular function, development, differentiation, and metabolite secretion [24]. The dynamic cell culture conditions can, for example, influence epithelial and endothelial cell behavior and significantly promote the activity of surfactant proteins [25]. Moreover, it provides a dynamic flow of nutrients and metabolites leading to improved intracellular metabolism and communication *in vitro* [24]. Thus, combining the DC and ALI technology *in vitro* could provide a more effective method to explore the molecular response of lung cells to toxic airborne particle.

This study aimed to establish a novel platform that can simultaneously achieve ALI exposure and DC conditions to analyze the cytotoxicity and molecular mechanisms of biomass combustion particles in human pulmonary alveolar epithelial cells (HPAEpiC). In our study, HPAEpiC were cultured on the surface of polycarbonate (PC) membranes with pore size of 3 μm and the apical surface was exposed to aerosol particles. The cells were nourished with medium flowing from below the membranes in the platform combining ALI exposure and DC conditions *in vitro*. The decline in

cell viability and increase in apoptosis rate after exposure to similar doses of BCSCs were more apparent under ALI-DC conditions than under submerged conditions. This suggests that the ALI-DC platform was more appropriate for cytotoxicity analyses. Changes in ultrastructure, decline in cell viability, ROS generation, and apoptosis rate were selected as parameters to evaluate the cytotoxicity of biomass combustion soluble constituents (BCSCs) in HPAEpiC. Western blots were prepared and analyzed to confirm whether the ROS-p53 signaling pathway and downstream effector proteins were activated after treatment with BCSCs. The experimental design for studying the cytotoxicity and molecular mechanism of BCSCs in HPAEpiC under ALI-DC conditions is shown in Fig. 1. Additionally, the antioxidant N-acetyl-cysteine (NAC) was introduced into the treatment process to inhibit the generation of ROS. This was done to confirm whether oxidative stress was the primary mechanism of BCSCs-induced apoptosis in HPAEpiC. The objective of this study was to gain a comprehensive understanding of the cytotoxic effects and potential molecular mechanisms of BCSCs in HPAEpiC and to gather scientific data to assess the potential risks to public health associated with BCSCs.

Fig. 1 Summary of the experimental design for studying the cytotoxicity and molecular mechanism of indoor air pollutants generated from biomass combustion in HPAEpiC on the ALI-DC platform.

Methods and Materials

Collection and treatment of biomass combustion samples

Biomass combustion experiments were carried out at a laboratory of Tsinghua University in Beijing, China, in October 2016. The laboratory was 6.2 m long, 2.4 m wide, and 3 m high (the volume was approximately 44.64 m³). During the experiments, all the windows and doors of the laboratory were closed. The daily biomass combustion process was carried out in a laboratory oven with an internal diameter of 15 cm. Each combustion experiment consumed approximately 3 kg of corn stalk biomass. In the biomass combustion process, fine particles were collected on Teflon membranes using a high-capacity sampler (Thermo Fisher, MA, USA),

placed at a height of 1.2 m and operated at a uniform flow rate of 3 L/min. The experiment was repeated three times a day for seven days of continuous sampling.

All the membranes used for collecting samples were cut into small pieces of approximately $1.5 \times 1.5 \text{ cm}^2$, submerged in 50 ml of double-distilled water, and treated under ultrasonic waves for 30 min. The suspension was ultimately dried using a vacuum freeze dryer. Subsequently, the pieces of membranes were immersed in methanol and treated under ultrasonic waves for 2 h, after which the suspension was dried as described above. Finally, we collected all the powder in a centrifuge tube and stored at -20°C . All powders were re-suspended in DMEM (1000 $\mu\text{g/ml}$) and sterilized by sonication for 30 min prior to use. Prior to the experiment, the stock solution was diluted in DMEM to final concentrations of 25, 50, 100, and 200 $\mu\text{g/ml}$.

Design and fabrication of the ALI-DC platform

The ALI-DC platform was designed and fabricated to achieve simultaneous exposure of HPAEpiC to BCSCs under ALI and DC conditions. The platform contains six repetitive units with a top and bottom part (Fig. 2A). The top part has six inlets and six outlets, which were used for circulation of the medium. It also has six sample loading holes through which the BCSCs solution was injected to the ALI system. Each sample loading hole was covered with a PDMS film, which was permeable, to prevent cell contamination and volatilization of the medium. Each PDMS film was punctured for sample injection, after which the sample loading holes were covered with a new PDMS film. The bottom part has six exposed chambers at a 15-degree inclination to achieve uniform distribution of the particles on the PC membranes,

which were placed at the bottom of the holes and used as the substrate for cell growth (Fig. 2B-c). Under each PC membrane was a chamber. These chambers were filled with culture medium and cells on the surface of the PC membranes were nourished with medium flowing from below the membranes. The PC membranes were hydrophilic and porous. The cells were exposed to air on the apical surface and therefore an ALI was formed. Additionally, each chamber had two channels that could be connected to a circulating pump through the top part. These channels were used to provide DC conditions. After curing at 80 °C for 1 h, the top and bottom parts were glued together. The ALI-DC platform were sterilized at 121°C for 30 min and dried at 60°C prior to use.

Aerosolisation efficiency and deposited doses on the ALI-DC platform

Each PC membrane was measured using an ultramicro electronic balance (Maitre Toledo Electronic Scale, XPR6U) before assembling it onto the ALI-DC platform, named m_0 . Then all the membranes were sandwiched into the designated PDMS parts (not bonded at a high temperature to facilitate membrane weighting) to achieve an efficient deposited dose by the help of the chambers with 15-degree inclination. The BCSCs solution was atomized and sprayed into the chambers using a high-pressure syringe (Penn-Century, America). Aliquots of 10 µl of BCSCs solutions of each concentration were atomized for six times in 20-min intervals (the total sample delivery volume was 60 µl). To meet the detection limit of the ultramicro electronic balance, sample delivery was conducted 10 times to obtain enough deposited dose on the membrane. Next, the membranes were carefully taken out from the platform, and

dried completely in a vacuum freeze dryer. Finally, all the membranes carrying deposited particles were weighed again, named m_{total} . The average deposited dose ($\bar{D}$) and the aerosolization efficiency ($\bar{E}$) were calculated using the following formula:

$$\bar{m}_d = \frac{1}{3} \sum (m_{total,i} - m_{0,i}) / 10 \quad (i=1, 2, \text{ or } 3; \text{ equ1});$$

$$\bar{D} = \bar{m}_d / S \quad (\text{equ2}); \quad \bar{E} = \bar{m}_d / m_a \quad (\text{equ3})$$

Among them, $\bar{m}_d$ represents the average mass of deposited particles on the PC membrane, S is the cell growth area in each chamber of the ALI-DC platform ($S=0.28 \text{ cm}^2$), m_a is the calculated value of total mass of atomized particles, which calculated as BCSCs concentrations $\times$ atomization volume ($600 \text{ }\mu\text{l}$).

Cell culture and exposure BCSCs on the ALI-DC system

The PC membranes were modified with 0.01% poly-L-lysine (PLL) solution to facilitate the cell adherence and growth. Prior to cell seeding on the platform, the medium was slowly injected into the chambers beneath the membrane via the inlets of the bottom parts. Subsequently, $100 \text{ }\mu\text{l}$ of the cell solution ($1 \times 10^6 \text{ cells/ml}$) was seeded on the upper surface of the membranes. After static cultivation for 2 h, the medium above the membrane was removed. This led to the formation of an ALI at the upper surface of the PC membrane. For dynamic cultures in thermostatic incubator, the bottom part of chip was covered with the top part, and each sample loading hole was covered with a PDMS film. Two circulating pumps (Hamilton, PSD/4 syringe pump) was introduced to supply the medium flowing on the chip.

Since the cells cultured on the membrane cannot be observed directly under bright-field microscopy, the growth density of cells was measured using the cell

counting. When the number of cells reached 1.0×10^5 – 1.3×10^5 , the BCSCs samples of 0, 25, 50, 100, and 200 $\mu\text{g/ml}$ were sprayed to each chamber using the high-pressure syringe. In addition, to compare with the cell culture on the platform, HPAEpiC cultured in a 96-well plate using the conventional method (submerged condition) was taken as control. Briefly, 120 μl of the cell solution (1×10^6 cells/ml) was added in each well of the plate. When the number of cells reached 1.2×10^5 – 1.5×10^5 , the cells were exposed to the same BCSCs samples via the same atomization method. Finally, both the ALI-DC platform and the 96-well plate were placed in a humidified incubator under 5% CO_2 , at 37 °C for 24 h.

Scanning electron microscopy

In order to observe deposition and morphology of particles, scanning electron microscope (SEM) was used to compare the BCSC particles on sampling membranes and the ALI-DC platform. All the membrane specimens were fixed on the sample stage of SEM, respectively, and treated by spray-gold prior to observation. Samples were then imaged with a Mira3 LM (Tescan, Czech Republic) SEM, using a secondary electron detector.

Transmission electron microscopy

The cells were treated with BCSCs at concentrations of 200 $\mu\text{g/ml}$ for 24 h and harvested into EP tubes. After centrifugation, the cells were fixed with 2.5% glutaraldehyde at 4°C for 24 h. Subsequently, the cells were washed with PBS solution three times and post-fixed with 1% osmium tetroxide for 2 h at 25°C. The cells were dehydrated in graded ethanol solutions, infiltrated with epoxide resin, and

cured at 60°C for 48 h. Finally, the cells were cut into ultrathin sections (40-70 nm) using a FC7-UC7 (Leica, Germany), stained with 1% uranyl acetate and 0.1% lead citrate for 30 min each, and observed on a Tecnai G220 TWIN transmission electron microscope (TEM; FEI, America).

Statistical analyses

All the experiments were performed in triplicate and data were presented as mean $\pm$ SD. Statistical analyses were performed using SPSS software (SPSS Inc., USA). Multiple comparisons of data were made using one-way ANOVA and mean differences between two groups were tested by independent student's t test. A p-value greater than 0.05 is considered statistically significant.

Results

ALI-DC platform fabrication and cell cultivation

The ALI-DC platform was fabricated to achieve simultaneous ALI exposure and DC condition for cytotoxicity study of air pollution on lung cells. It is a hexagonal (side length: 27 mm, height: 12 mm) microfluidic chip consisting of six repetitive units, each containing a circular PC membrane approximately 9 mm in diameter (Fig. 2B-a). The PC membrane is placed on top of a 6-mm-wide circular hole connecting with two thin channels (0.6 mm wide, 0.1 mm high). The top of the PC membrane has a tapered chamber with a 6-mm base diameter and a 15-degree inclination (Fig. 2B-b and c).

Cell growth was observed using DAPI in-situ staining to verify that whether the PC membrane was successfully modified and suitable for cell adhesion. From the images shown in Fig. 2C. The cell density gradually increased with the elongation of culture

time; it reached 80% on the day 5 and exceeded 90% on the day 7, indicating that the cells were well proliferating (Fig. 2C).

Fig. 2 (A) A schematic illustration showing the overall concept of the ALI-DC platform and ALI exposure. (B) Photographs of the fabricated ALI-DC platform (a), experimental setup for DC in the platform (b), and basic structure of the platform for cells exposure to tested substances (c). (C) Photographs of the DAPI-stained nuclei of HPAEpiC cultured on the modified PC membrane on the day 1, 3, 5, and 7, respectively. Length unit: mm; Scale bar = 20 μ m.

Particle deposition on the platform

The average deposited doses on the membrane (Fig. 3A) were 2.23 ± 0.93 , 6.21 ± 1.94 , 13.76 ± 3.48 , and $18.19 \pm 4.32 \mu\text{g}/\text{cm}^2$, corresponding to the atomized samples with 25, 50, 100, and 200 $\mu\text{g}/\text{ml}$ BCSCs, respectively. As shown in Fig. 3B and C, the irregular polygonal particles were smaller than 5 μm in diameters, most of which were less than 2.5 μm , belonging to fine particles. Moreover, the images Fig. 3E and F demonstrated that microscopic appearance of the deposited BCSC particles on the PC membrane of the chip were basically consistent with that of the samples on the sampling membrane.

Fig. 3 Deposition of BCSCs sample on the ALI-DC platform. (A) The average deposited dose ($\mu\text{g}/\text{cm}^2$) of each BCSCs samples; (B) The SEM image of Teflon membrane; (C) The SEM image of the particles collected on the Teflon membranes.

(D) The SEM image of PC membrane; (E) and (F) The SEM images of PC membranes exposed to the 100- and 200- μ g/ml BCSCs samples, respectively.

Comparison of the cytotoxic effects on cells cultured on the ALI-DC platform and under submerged conditions

Changes in cell viability and apoptosis rate were analyzed to evaluate the cytotoxic effects induced by BCSCs on the ALI-DC platform and under submerged conditions.

After exposure to BCSCs, cell viability decreased, and apoptosis rate increased in similar dose-dependent manners under two conditions. In the group of cells treated with 0 μ g/ml of BCSCs, cell viability in the ALI-DC platform increased from 90.6% to 96.2% and apoptosis rate decreased from 9.84% to 4.37% (Fig. 4). The cell viabilities under the ALI-DC condition were significantly lower than those under submerged conditions, especially when BCSCs concentration increased to 50 μ g/ml.

With a high BCSCs concentration of 200 μ g/ml, the difference between two conditions became smaller (Fig 4A). Fig. 4B shown that the changes of apoptosis rates matched with the decline in cell viability under both conditions. Cells on the ALI-DC platform got higher apoptosis rates than those under submerged conditions.

Fig. 4 Comparison of the cytotoxic effects in cells cultured in the ALI-DC platform and under submerged conditions. The cell viabilities (A) and apoptosis rates (B) of HPAEpiC after treatment for 24 h with BCSCs at concentrations of 0, 25, 50, 100, and 200 µg/ml. The lines above the bars indicate significant differences between the two groups of cells (*: $P < 0.05$, **: $P < 0.01$).

Changes in the morphology of HPAEpiC

TEM was used to observe changes in the ultrastructure of HPAEpiC after treatment with BCSCs of 200 µg/ml for 24 h. The results showed that the cells in the control group retained their ultrastructure and morphology (Fig. 5A and D). However, in the treated group, the morphology of the cells was severely damaged after exposure to BCSCs (Fig. 5B and C), including increased cytoplasmic vacuolization and mitochondria degeneration. (Fig. 5E and F).

Fig. 5 TEM images of (A) and (D) cells in control group, (B), (C), (E), and (F) cells in experimental group exposed to 200 mg/ml of BCSCs. (N: nucleus; M: mitochondria; Black arrow: vacuoles).

Phosphorylated p53 and decrease in the Bcl-2/Bax ratio

To explore the molecular mechanisms underlying BCSCs-induced cytotoxicity, the expression of phosphorylated p53, Bax, and Bcl-2 proteins was measured. Western blot analyses revealed that the expression of phosphorylated p53 increased (Fig. 6A and B). The results also showed that Bax expression levels significantly increased in a dose-dependent manner from that of the control group levels (Fig. 6 C and E). Conversely, the anti-apoptotic Bcl-2 expression level decreased with increasing doses of BCSCs (Fig. 6 C and D). The Bcl-2/Bax ratio fell more than 60% in the group of 25-μg/ml BCSCs and continually decreased in a dose-dependent manner with increasing doses of BCSCs (Fig. 6F).

Fig. 6 Effect of BCSCs on the phosphorylation of p53 and the expression of Bcl-2 and Bax in HPAEpiC. Representative Western blot analysis of p-p53 (A), Bax (C), and Bcl-2 (C), with β-actin as a loading control. The relative gray value of p-p53 (B), Bcl-2 (C), Bax (D), and Bcl-2/Bax (E) in HPAEpiC. * $P < 0.05$, ** $p < 0.01$.

Discussion

The ALI-DC platform design and sample exposures

HPAEpiC were cultured on the surface of PC membranes. They were exposed to BCSCs on the apical surface and nourished with medium flowing from below the membranes. The medium was circulated by two microliter circulating pumps through the inlets and outlets. An ALI-DC platform was successfully built for the first time in this way. In contrast to conventional methods, the ALI-DC platform provided a simulation of inhaled air for lung cells cultured on PC membranes and offered the cells an appropriate shear stress using dynamic culture (Fig. S1). Cell viability under the ALI-DC conditions increased from 90.9% to 95.6% while the apoptosis rate reduced from 9.84% to 4.37% (Fig. 3). Similar promoting effects were also found in a

dynamic bioreactor for cultivation of human aortic endothelial cells (HAECs), and in microfluidic devices for differentiation of mouse embryonic stem cells [26, 27]. The result demonstrated the medium flowing beneath the membrane could facilitate metabolism of cells. Therefore, the ALI-DC platform could provide a suitable surface and an active nourishment for the growth of HPAEpiC.

For the sample exposure on the ALI, the tapered chamber was introduced to the platform for aerosol delivery. The traditional vertical cylindrical chamber above ALI usually brings a dead end on the edge of the cell substrate to prevent particles' distribution on the ALI [28, 29]. As a result, the cells growing in the dead end probably not contact with particles, thereby affecting the experimental results. Herein, according to the study by Siriri et al, we designed the chamber with a tilt angle of 15 degrees to inhibit the formation of exposed dead ends thus solving the above-mentioned problem effectively [30]. With the combination of spray-type delivery and the tapered chamber, the aerosolization efficiency of sample could be between ~41.62% and ~64.21%, and the deposited dose displayed a positive correlation with the BCSCs concentrations (Fig. 3A). These results suggested a stable performance of the sample delivery approach on the platform. Moreover, the SEM images exhibited that no obvious effects of sample processing were found on the BCSC particles' state (Fig. 3B and C); endotoxin (ET) toxicity tests showed that bacterial ET residual in the BCSCs samples would not effect on the HPAEpiC growth (Fig. S2), demonstrating that the sample collection, extraction, and atomization methods used in our study were feasible. We believe this method is more effective

than conventional methods for exploring the molecular response of lung cells to toxic substances, particularly for research on air pollution and drugs for pulmonary administration.

Evaluation of the ALI-DC platform for BCSCs toxicity tests

The alveoli are where the crucial gas exchange takes place and there is a layer of fluid on each alveolar surface, forming a liquid-air interface [31]. The ALI exposure has been considered to be a more practical, effective way than traditional submerged exposure [23, 32, 33]. To validate the applicability of the ALI-DC platform, we compared the cytotoxic effects of BCSCs on cells cultured on the ALI-DC platform and under submerged conditions. Results shown in Fig. 4 suggested that the BCSCs exerted higher cytotoxicity to HPAEpiC on the platform than that of control. Similarly, in the assessment of zinc oxide nanoparticle toxicity, lung epithelial A549 cells suffered more severe damages when exposed to (ZnO) NPs on the ALI than in the submerged conditions [34]. Moreover, same type of vehicle exhaust pollutants also showed different cytotoxicity on lung epithelial BEAS-2B cells between using the two methods [35]. These differences benefitted from the thin liquid film of medium covering the cells; the platform could provide a higher contact efficiency between the particles and the cells than that of the submerged culture [36-38]. Therefore, the ALI-DC platform is able to be used as more effective approach than conventional methods for exploring the molecular response of lung cells to toxic substances, particularly for research on air pollution and drugs for pulmonary administration.

Cytotoxic effects of BCSCs on the HPAEpiC

Particle size is an important factor associated with biotoxicity of air pollution. SEM images displayed that the BCSCs particles belonged to fine particles (smaller than 2.5 micrometers, PM_{2.5}) (Fig. 3C). It indicated that BCSCs particles in indoor air would escape the protection of the villi in the nose and trachea, then go directly to the alveoli of the lungs and cause severe damage [39, 40]. Many studies have proved that PM_{2.5} can cause cytotoxicity in different kinds of human cells [41, 42]. In our previous study, we demonstrated that BCSCs could induce inflammatory responses and apoptosis in human keratinocytes. Similar toxicity of BCSCs was also found in the HPAEpiC, showing a significant dosage effect (Fig. 4). It attributed to the BCSCs contained a variety of toxic substances, such as polycyclic aromatic hydrocarbons, volatile organic compounds, heavy metals, etc [43, 44]. These substances can cause cellular structural damages, functional or metabolic disorders, and induce apoptosis [45]. Clinical studies have shown that lung cell apoptosis is implicated in a wide range of pulmonary diseases, including pulmonary edema, acute lung injury, pulmonary fibrosis, and pneumonia [46, 47]. Indeed, even very low concentration indoor-air pollution from solid fuel combustion can increase the incidence of lung diseases.

In the results of TEM, the abnormal morphologies of mitochondria and other signs of cellular injury further demonstrated toxic stress of BCSCs to HPAEpiC (Fig. 5), matching with the results of viability and apoptosis. Notably, the damage of mitochondria is often a clue to reactive oxygen species (ROS) stress induced by PM_{2.5}, which may results in severe adverse effects on normal cellular metabolisms. For example, ROS generation triggered by PM_{2.5} could cause significant decreases in

viability of human endothelial cells EA.hy926; PM_{2.5}-induced oxidative stress activated autophagy in A549 cells, etc [48, 49]. Thus, these results suggested that studying the molecular mechanisms of BCSCs-mediated cytotoxicity is essential because it will provide an experimental basis for the prevention of lung injury and diseases.

The molecular mechanisms underlying BCSCs-induced cytotoxicity in HPAEpiC

Fine particles can attack cell via oxidizing intercellular macromolecules (e.g. ROS) to disorder gene expressions and signaling pathways of apoptosis [50]. In our study, BCSCs significantly increased ROS production in HPAEpiC (Fig. S3), meanwhile, caused swellings and damages of mitochondria-the primary producer of ROS (Fig. 5). It is well known that increased ROS production can induce the phosphorylated p53 and activate the expression of downstream proteins [51]. These target proteins in the apoptosis-related pathway are cancer-causing biological factors [52]. The western blot analysis revealed that the expression of phosphorylated p53 was enhanced after exposure to BCSCs (Fig. 6A). Additionally, we noticed that simultaneous exposure of HPAEpiC to NAC and BCSCs markedly decreased ROS generation and phosphorylated p53. These findings indicate that the ROS-mediated p53 pathway may be the primary pathway involved in BCSCs-induced apoptosis of HPAEpiC.

Due to the activation of p53 can regulate Bcl-2 protein family related mitochondrial pathways, we then investigated expression of anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein [53]. The ratio between these two proteins determines susceptibility of cells to apoptosis [54]. Therefore, the decreased Bcl-2/Bax ratio of

the treated HPAEpiC further suggested that the p53 pathway participated in apoptosis induced by BCSCs. (Fig. 6F) Furthermore, we found that the expression of downstream protein caspase-9 was activated, which ultimately activated caspase-3 (Fig. S4). In sum, our study demonstrated, for the first time, that BCSCs can damage mitochondria, produce ROS, and activate the p53-Bcl-caspase pathway to ultimately induce the apoptosis in HPAEpiC (Fig. 7).

However, it is noteworthy that after adding NAC, the apoptosis rates of the treatment groups were still significantly higher than that of the control group (Fig. S5). It indicates that BCSCs activated other apoptosis-related signaling pathway except the p53 pathway. Therefore, more investigations on the molecular mechanisms underlying the cytotoxicity of BCSCs in lung cells are still urgent needed.

Fig. 7 The molecular mechanism underlying BCSCs-induced cytotoxicity in HPAEpiC. The p53 signaling pathway was activated after exposure of HPAEpiC to

BCSCs, significantly enhancing the expression of Bax, cleaved caspase-9, and cleaved caspase-3, and inhibiting the expression of Bcl-2.

Conclusion

In this study, we fabricated a novel ALI-DC platform to investigate the cytotoxicity of BCSCs in HPAEpiC. This platform allowed direct exposure of the cells to the aerosols and provided circulating DC conditions for the cells. It is a more effective approach than submerged exposure for investigating the cytotoxicity of harmful substances to lung cells, particularly for studies on air pollution and drugs for pulmonary administration. Our study demonstrates, for the first time, that BCSCs induced apoptosis in HPAEpiC through the activation of the ROS-p53-caspase pathway. These findings offer theoretical evidence of biomass combustion-induced cytotoxicity in lung cells, lay a foundation for future studies on this topic, and may contribute to the prevention and treatment of lung diseases induced by indoor air pollutants from solid fuel combustion.

Additional files

Five results are discussed in the Supplementary Material.

Abbreviations

Table 1: The list of abbreviations in this manuscript.

Abbreviation	Definition
HPAEpiC	Human pulmonary alveolar epithelial cells
ALI	Air-liquid-interface
DC	Dynamic culture

BCSCs	Biomass combustion soluble constituents
TEM	Transmission electron microscope
SEM	Scanning electron microscope
PC	Polycarbonate
ROS	Reactive oxygen species
NAC	N-acetyl-cysteine
PM _{2.5}	Particulate matter 2.5
PDMS	Polydimethylsiloxane
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle's Medium
PBS	Phosphate buffered saline
FITC	Fluorescein isothiocyanate
PI	Propidium iodide
DAPI	4',6-Diamidino-2-phenylindole-2-dihydrochloride
PVDF	Polyvinylidene difluoride
BSA	Bovine serum albumin
PMSF	Phenylmethanesulfonyl fluoride
TBST	Tris-buffered saline, 0.1% Tween-20
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Corresponding authors

* E-mail: xyang@tsinghua.edu.cn; gsui@fudan.edu.cn. Tel.: +86-021-55664504. Fax: +86-21-65642080.

Availability of data and materials

All data and materials are specifically referenced within the manuscript. These are all openly available.

Authors' contributions

The authors collaboratively prepared the manuscript. Shaorui Ke and Qi Liu designed the ALI-DC platform performed experimental work. Shaorui Ke drafted the manuscript. Xinlian Zhang and Yuhan Yao made substantial contributions to the analysis and interpretation of the data. Xudong Yang collected the biomass combustion samples, involved in the planning and critically revising the manuscript. Guodong Sui was the project leader, he provided funding and reviewed of the manuscript. All authors have confirmed approval of the final version of the submitted manuscript.

Competing interests

The authors have no competing financial interest to declare.

Consent for publication

All authors have read and approved the manuscript for publication. There are no outstanding issues or requirements.

Ethics approval and consent to participate

There were no requirements to obtain 'consent to participate', because it was not applicable for our study.

Authors ' information

¹ Co-construction Collaborative Innovation Center for Chinese Medicine and Respiratory Diseases by Henan & Education Ministry of P.R. China, Academy of Chinese Medical Sciences, Henan University of Chinese Medicine, Zhengzhou 450046, China.

² Shanghai Key Laboratory of Atmospheric Particle Pollution Prevention (LAP3), Department of Environmental Science & Engineering, Fudan University, Shanghai 200433, P.R. China.

³ Department of Building Science, Tsinghua University, Beijing 100084, P.R. China.

⁴ Jiangsu Collaborative Innovation Center of Atmospheric Environment and Equipment Technology (CICAEET), Nanjing University of Information Science & Technology, Nanjing 210044, P.R. China.

E-mail: Shaorui Ke: 15110740019@fudan.edu.cn; Qi Liu: 13110740002@fudan.edu.cn; Xinlian Zhang: 15210740031@fudan.edu.cn; Yuhao Yao: 16110740002@fudan.edu.cn; Xudong Yang: xyang@tsinghua.edu.cn; Guodong Sui: gsui@fudan.edu.cn.

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Figures

Figure 1

Summary of the experimental design for studying the cytotoxicity and molecular mechanism of indoor air pollutants generated from biomass combustion in HPAEpiC on the ALI-DC platform.

Figure 2

(A) A schematic illustration showing the overall concept of the ALI-DC platform and ALI exposure. (B) Photographs of the fabricated ALI-DC platform (a), experimental setup for DC in the platform (b), and basic structure of the platform for cells exposure to tested substances (c). (C) Photographs of the DAPI-stained nuclei of HPAEpiC cultured on the modified PC membrane on the day 1, 3, 5, and 7, respectively. Length unit: mm; Scale bar = 20 μ m.

Figure 3

Deposition of BCSCs sample on the ALI-DC platform. (A) The average deposited dose ($\mu\text{g}/\text{cm}^2$) of each BCSCs samples; (B) The SEM image of Teflon membrane; (C) The SEM image of the particles collected on the Teflon membranes. (D) The SEM image of PC membrane; (E) and (F) The SEM images of PC membranes exposed to the 100- and 200- $\mu\text{g}/\text{ml}$ BCSCs samples, respectively.

Figure 4

Comparison of the cytotoxic effects in cells cultured in the ALI-DC platform and under submerged conditions. The cell viabilities (A) and apoptosis rates (B) of HPAEpiC after treatment for 24 h with BCSCs at concentrations of 0, 25, 50, 100, and 200 µg/ml. The lines above the bars indicate significant differences between the two groups of cells (*: $P < 0.05$, **: $P < 0.01$).

Figure 5

TEM images of (A) and (D) cells in control group, (B), (C), (E), and (F) cells in experimental group exposed to 200 mg/ml of BCSCs. (N: nucleus; M: mitochondria; Black arrow: vacuoles).

Figure 6

Effect of BCSCs on the phosphorylation of p53 and the expression of Bcl-2 and Bax in HPAEpiC. Representative Western blot analysis of p-p53 (A), Bax (C), and Bcl-2 (C), with β -actin as a loading control. The relative gray value of p-p53 (B), Bcl-2 (C), Bax (D), and Bcl-2/Bax (E) in HPAEpiC. * $P < 0.05$, ** $p < 0.01$.

Figure 7

The molecular mechanism underlying BCSCs-induced cytotoxicity in HPAEpiC. The p53 signaling pathway was activated after exposure of HPAEpiC to BCSCs, significantly enhancing the expression of Bax, cleaved caspase-9, and cleaved caspase-3, and inhibiting the expression of Bcl-2.

Supplementary Files

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