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Harmful chemicals of heat not burn product and its induced oxidative stress of macrophages at air-liquid interface: Comparison with ultra-light cigarette

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ABSTRACT

Background: Harmful and potential harmful chemicals (HPHCs) and oxidative stress of macrophages are major factors responsible for smoking-caused chronic respiratory diseases. However, comparisons of HPHCs among heat not burn (HnB) product and ultra-light cigarette and their induced oxidative stress of macrophages have not been investigated.

Aim: The study detected HPHCs deliveries from HnB and ultra-light and measured their induced oxidative stress of macrophages cultured at air-liquid interface (ALI).

Methods: Total particulate matter, tar and 28 chemicals delivered from HnB, ultra-light and 3R4F cigarettes were determined. Mouse mononuclear macrophages at ALI were exposed to the aerosol of three tobacco products. Cell viability was measured by MTT assay. Reduced glutathione was detected by colorimetry method. Reactive oxygen species (ROS) was determined by fluorescence method.

Results: The results showed levels of 26 common HPHCs from both HnB product and ultra-light cigarette were less than that from 3R4F cigarette. HnB product delivered formaldehyde, acetaldehyde, propanal, butyraldehyde and crotonaldehyde more than ultra-light cigarette. The levels of 21 HPHCs were lower in the HnB product compared to the ultra-light cigarette. At the same exposure dose and time, the order of cell viability induced by aerosol of that was HnB > ultra-light > 3R4F, the order of content of intracellular reduced glutathione induced by aerosol of that was HnB > ultra-light > 3R4F. It showed no significant difference of ROS level between ultra-light and HnB in each designed exposure dose. HnB induced more ROS than ultra-light cigarette in each designed exposure time.

Conclusion: Conclusively, most HPHCs from HnB were lower than that from ultra-light, while certain harmful chemicals were higher than ultra-light, e.g., carbonyl compounds. HnB-induced oxidative stress of macrophages is less than ultra-light cigarette.

1. Introduction

Smoking has been demonstrated to be associated with a variety of chronic respiratory diseases including Chronic Obstructive Pulmonary Disease (COPD). COPD is a condition associated with chronic pulmonary inflammation, characterized by macrophage activation, neutrophil recruitment, and cell injury (Nam and Tseng, 2014). Many substances contained in cigarette smoke, including reactive oxygen species (ROS), have been proposed to be responsible for the inflammatory process of COPD. In normal physiological state, there is a balance between oxidation and antioxidation in our body (Cristovao et al., 2013). Cigarette smoke contains a variety of free radicals and oxidative compounds, which can stimulate pulmonary cells, lead to the increase of intracellular ROS levels, and keep cells in the state of oxidative stress (Boukhenouna et al., 2018). Macrophages play an important role in regulating the immune response of the lower respiratory system. They not only phagocytize the debris of tissue cells and pathogens, but also secrete a variety of inflammatory factors, participate in the inflammatory process of damaged tissues, and lead to the occurrence and development of chronic diseases if occurrence of consecutive inflammation invasion (Pappas et al., 2013). Further, the normal function of macrophages are affected by cellular oxidative

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stress. If macrophages are in the state of oxidative stress, it will secrete of inflammatory factors and erode tissue cells. The damaged tissue cells will in turn further secrete inflammatory factors, eventually leading to repeated inflammation of the respiratory system, thereby leading to or promoting the occurrence and development of chronic respiratory diseases such as COPD (Rahman, 2005). Therefore, oxidative stress is an important biological event between macrophage dysfunction and pulmonary inflammation (de Groot et al., 2019).

In recent years, HnB products have been introduced by tobacco companies as the direction of the company's future products of transformation and development, and try to replace the conventional cigarette. The recent scientific reports confirmed that the yields of harmful and potential harmful components (HPHCs) from HnB product were much lower than that of conventional cigarette under the Health Canada intense (HCI) smoking regime (Forster et al., 2018; Jaccard et al., 2017; Schaller et al., 2016). At present time, there is no well-recognized smoking regimes for HnB tobacco product reflecting human puffing behavior conditions or standard machine-testing conditions proposed for HnB tobacco product. Laboratories are currently using diverse testing parameters for quantification of HnB emissions, such as HCI smoking regime (Forster et al., 2018; Jaccard et al., 2017; Schaller et al., 2016), International Organization for Standardization (ISO) smoking regime (Li et al., 2019b; Zenzen et al., 2012) and other alternative puffing conditions based on human puffing behavior (Schaller et al., 2016; Zenzen et al., 2012). There are no widely accepted standards for the laboratory testing of these HnB products. Therefore, the emissions of HnB product should be monitored comprehensively under different regimes, including but not limited to HCI.

In addition, most tobacco companies adopted HCI smoking regime to test HnB emissions and compared it with 3R4F reference cigarette and high-tar cigarettes in the market, not referred to ultra-light cigarettes (Forster et al., 2018; Jaccard et al., 2017; Li et al., 2019b; Schaller et al., 2016). Ultra-light cigarettes have prevailed for many years in East Asian countries, since smokers misperceived the low-tar cigarette having reduced harm (Assunta and Chapman, 2008; King et al., 2010). As we known, smoking regime greatly affected the emission levels of ultra-light cigarette (Colard et al., 2014; Purkis et al., 2011). Under HCI smoking regime, some ultra-light cigarettes even delivered much more HPHCs than high-tar cigarette (Endo et al., 2009; Hyodo, 2017). If adopting HCI smoking regime, it is not necessary to compare aerosol emissions of HnB product with ultra-light cigarette. Thus, it's better to adopt ISO smoking regime (excluded HCI) to compare HPHCs and biological effect of HnB product with ultra-light cigarette.

In view of the cytotoxicity of HnB product, most studies used submerged cell culture exposure system to screen the in vitro cytotoxicity (Jaunky et al., 2018; Schaller et al., 2016; Thorne et al., 2018). The submerged conditions resulted in the change of physical and chemical properties of airborn substances (Li, 2016; Thorne and Adamson, 2013). Therefore, a modified culture technique establishing cells at an air–liquid interface (ALI) have been developed successfully and becoming a realistic and efficient tool, which preserves the test substance in its natural state, thus mimicking a more realistic exposure scenario (Li, 2016). In our study, we adopted ISO smoking regime to generate aerosol to be exposed to cell cultured at ALI and compared their induced oxidative stress between combustible cigarettes and HnB product.

The present study measured aerosol HPHCs from HnB product, ultra-light and 3R4F cigarettes under ISO smoking regime, including carbonyl compounds and tobacco specific *N*'-nitrosamines (TSNAs). Oxidative stress was determined for macrophages cultured at ALI induced by HnB product, ultra-light and 3R4F cigarettes, which would provide *in vitro* evidence for HnB product inducing oxidative stress.

2. Materials and methods

2.1. Chemicals and reagents

Mouse mononuclear macrophages RAW 264.7 was purchased from American Type Culture Collection (Cell No. TIB-71). Dulbecco's Modified Eagle Medium high glucose (DMEM-H) medium was purchased from GENVIEW Corp. (Germany). Fetal Bovine Serum (FBS) was purchased from AusGeneX Corp. (Australia). HnB stick (HeatSticks™, full flavor) and ultra-light cigarette (1 mg tar) were purchased from retail store in Japan and China, respectively. 3R4F reference cigarette was purchased from University of Kentucky (USA). The commercial reduced glutathione (GSH) assay kit (Cat No. BC1170.) was purchased from Beijing Solarbio Science & Technology Co., Ltd. (China). 3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide (MTT) (Purity 98.0 %), dimethyl sulfoxide (Purity ≥ 99.0 %) and 2′,7′-dichlorofluorescein-diacetate (DCF-DA) (Purity ≥ 95 %) were from SIGMA-ALDRICH Company (USA).

HnB sticks, ultra-light and 3R4F cigarettes were conditioned for minimum 48 h prior to use at (60 ± 3) % relative humidity and (22 ± 1) ℃. All the test tobacco products were smoked according to ISO 3308:2012 (35 mL puff volume, drawn over 2 s, once every minute with ventilation holes unblocked) (ISO, 2012). Chemistry test items and analytical methods were showed in Table 1.

2.2. Cell culture

RAW 264.7 cells were obtained from male tumor mice induced by A-MuLV leukemia virus. The adherent growth of RAW 264.7 cells facilitated the ALI exposure study. RAW 264.7 cells is commonly used as the *in vitro* cell model to study the immune response by exogenous compounds (Bi et al., 2018; Zhang et al., 2018). RAW 264.7 cells were cultured at 37℃ and 5% CO_2 in DMEM-H medium supplemented with 10 % FBS, 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin. The apical side of porous membrane of each insert was seeded by 1.0×10^6 cells in 1.5 mL supplemented DMEM-H culture medium. Following the addition of 2 mL supplemented DMEM-H culture medium to the well, each insert loaded with cells was then inserted into each well of 6-well culture plate. The 6-well culture plate was then transferred to incubator and cells were cultured at 37℃ and 5% CO_2 for 24 h.

Table 1
Analytes and Analytical methods.

category	Test items	Methods
Conventional analytes	total particle matter, tar, nicotine, Water and carbon monoxide	(ISO, 2000)
Aromatic amines	1-naphthylamine, 2-naphthylamine, 3-aminobenzene, 4-aminobenzene	(Zhang et al., 2017)
Volatile organic compounds	1,3-butadiene, isoprene, acrylonitrile, benzene, toluene	(CORESTA, 2018e)
Carbonyl compounds	formaldehyde, acetaldehyde, acetaldehyde, butyraldehyde, acetone, acrolein, crotonaldehyde, 2-butanone	(CORESTA, 2018c)
TSNAs	NNN、NNK、NAT、NAB	(CORESTA, 2014)
Ammonia	Ammonia	(CORESTA, 2018a)
Phenolic	Phenolic	(CORESTA, 2018d)
Polycyclic aromatic hydrocarbons	Benzo[a]pyrene	(CORESTA, 2018b)
Hydrogen cyanide	Hydrogen cyanide	(Liu et al., 2014)

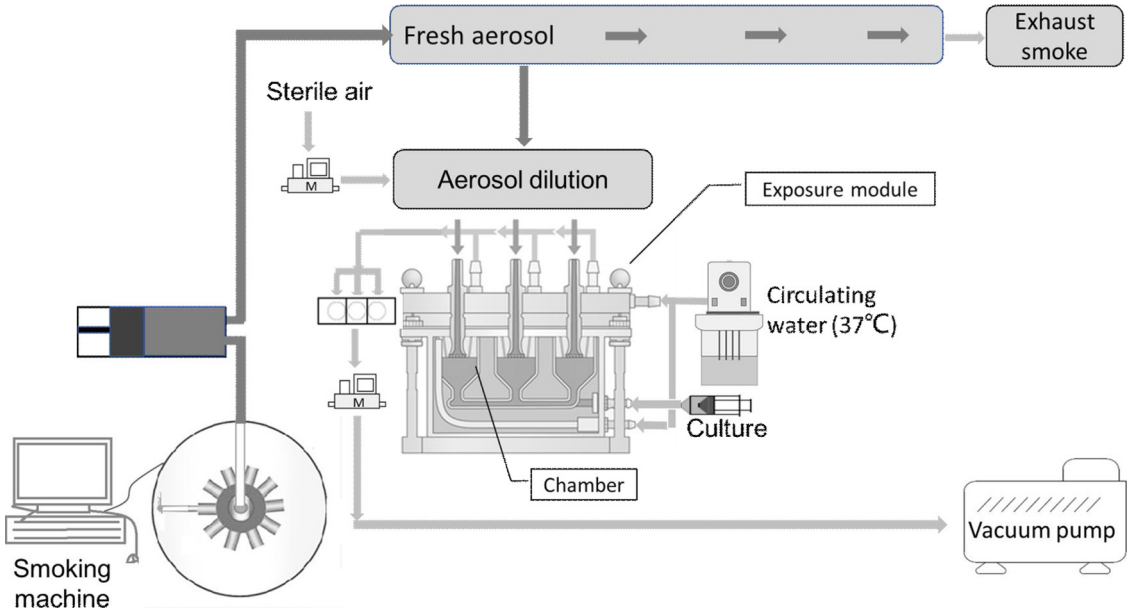

Fig. 1. The schematic diagram of automatic exposure system of aerosol to macrophages at ALI.

2.3. Aerosol generation

Fully automatic smoking machine (Beijing Huironghe Technology Corp., China) was employed to generate aerosol under ISO smoking regime. The smoking machine was designed to generate and transfer aerosol consecutively. When one puff was finished in 2 s by the piston of smoking machine, the channel of the puff port was closed and channel of the exposure module was simultaneously opened. The inhaled aerosol in the piston chamber was pushed slowly in the following 58 s into the opening channel tube of exposure module. After then, the channel of puff port was re-opened and the channel of the exposure module was simultaneously closed, which means another cycle of aerosol generation and tranferration took place. Each puff of fresh aerosol was envenly mixed with sterile air and averagely divided into three parts corresponding to the triplicated chambers of a exposure module. The divided aerosol was then inhaled into the chamber of exposure module under the weak negative pressure by the downstream vacuum pump (Fig. 1).

2.4. Aerosol exposure to macrophages at ALI

24 h after cells seeding, the media in the insert was removed. Then, the inserts was transferred into the chamber of exposure module. In the study, fresh aerosol without dilution was considered to be 100 % of dose. The various dose of diluted aerosol was set as the percentage of the volume of fresh aerosol to the total diluted aerosol (sum of fresh aerosol and sterile air). The vacuum pump was placed in the downstream of exposure module and vacuum flow rate was maintained at 10.0 mL/min for each chamber. The distance between the nozzle of trumpet to porous membrane of the insert was set as 1.5 mm. The aerosol was evenly impregnated onto monolayer cells. Ttriplicate inserts, housed in a stainless steel exposure module, were set as one group. Meanwhile, sterile air exposure to cells was set for each experiment as the control group (Fig. 1).

2.5. Cell viability assay

Referring to Mosmann (Mosmann, 1983), the cell viability was measured by MTT assay. For dose-response test, the exposure dose was set as 10 %, 20 %, 40 %, 60 % and 80 %, and exposure duration was 1 h. The sterile air treatment in 1 h exposure duration was set as the control and results of other treatment groups were expressed as the percentage of the treatment to the control. In the time-response test, the exposure duration was set as 30 min, 60 min, 90 min, 120 min and 150 min, and the exposure dose was 40 %. At each exposure duration, the sterile air treatment was performed accordingly to make treatments groups results be expressed as the percentage of the treatment to the control. Following exposure, the inserts with cells were immediately removed from exposure chamber and transferred to the 6-well culture plate. 1 mL of 0.5 mg/mL MTT was added to the inserts and incubated for 3 h at 5% CO_2 and 37℃. After incubation, the 6-well culture plate loaded with inserts was centrifugated at 4000 rpm for 10 min to make he superfluous MTT solution penetrate through porous membrane of the insert. Then, 1.5 mL of dimethyl sulfoxide was added to the insert to resolve the purple crystal formazan. The formazan resolution was gently shaked for 10 min. 100 μL of formazan solution was pipetted to the transparent 96-well plate and the absorbance was detected at 490 nm by Microplate Reader (Bio-tek, USA). The cell viability was calculated as follows: cell viability (%) = [(absorbance of treatment group - absorbance of blank group) / (absorbance of sterile air group - absorbance of blank group)] × 100 %, where absorbance of blank group is the absorbance of the 100 μL dimethyl sulfoxide.

2.6. Detection of intracellular GSH content

Referring to Li etal (Li et al., 2019a), GSH was detected according to the instruction of of assay kit provided by Beijing Solarbio Science & Technology Co., Ltd. For dose-response test, the exposure doses were set as 10 %, 20 %, 40 %, 60 % and 80 %, and the exposure duration was 1 h. For the time-response test, the exposure duration were set as 10 min, 30 min, 60 min, 90 min and 120 min, and the exposure dose was 40 %. After exposure, the inserts loaded with cells were immediately taken away from the exposed chamber and added by 1 mL of precooled (4°C) phosphate buffered saline (PBS). The adhered cells on the porous membrane were gently forced off by pipetting. Then, 50 μL of the cell suspension was used to measure cell density (cells/mL) by Automatic Cell Counting Instrument (ThermoFish Scientific, USA). For the remaining cell suspension, 900 μL was transferred to 1.5 mL EP tube and centrifuged at 600 *g* for 10 min. The centrifuged cells were then washed twice by precooled (4°C) PBS and lysed in 30 μL of protein precipitator by repeated process of freezing in liquid nitrogen and thawing at 37°C water bath. The lysed cell suspension was then