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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)

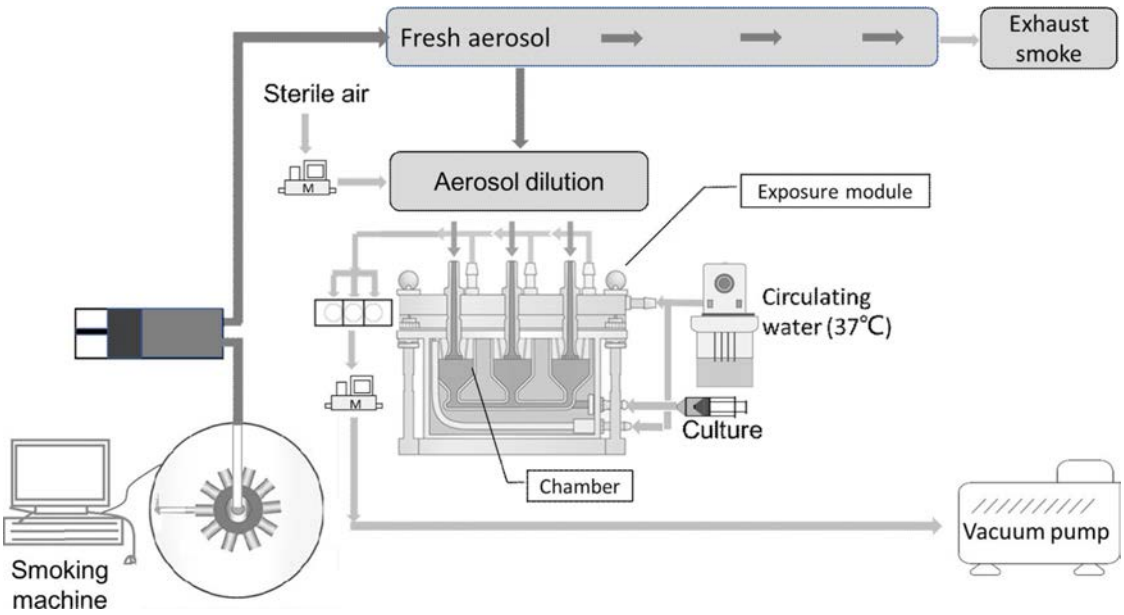

Fig. 1. The schematic diagram of automatic exposure system of aerosol to macrophages at ALI.

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°C. 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

centrifugated at 8000 *g* for10 min. 20 μL of supernatant was piptted to the transparent 96-well plate, followed by 140 μL buffer and 40 μL DTNB. After shaking for 2 min, the absorbance of the mixed solution in each well was read at 420 nm by Microplate Reader (Bio-tek, USA). The standard curve was obtained according to the absorbance and concentration (μg/mL) of GSH standard. Then the concentration of the sample was calculated according to the standard curve. The final intracellular GSH content was expressed as μg/10^6 cells by the calculation of GSH concentration (μg/mL) devided by cell density (cells/mL).

2.7. Detection of intracellular ROS

Modified from Li et al. (Li et al., 2008), intracellular ROS content was determined by fluorescence method. For the dose-response test, the exposure doses were set as 10 %, 20 %, 30 %, 40 % and 50 %, and the 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 were set as 10 min, 20 min, 30 min, 60 min and 90 min, and the exposure dose was 30 %. 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 loaded with cells were immediately taken away from the exposed chamber and added by 1 mL of precooled (4℃) PBS. The adhered cells in the porous membrane were gently forced off by pipetting and transferred to 1.5 mL EP tube and centrifuged at 600 *g* for 10 min. The washed cells were dyed by 1 mL of DCF-DA (10 μM) in serum-free culture medium and incubated for 30 min at 37℃. Following washing twice by pre-cooled (4℃) PBS, the fluorescence of suspended cells were determined by Flow Cytometry (BD Bioscience, USA) at 448 nm excitation and 525 nm emission. The ROS value of each sample were expressed by the percent of the number of cells entering the intensity gate to the number of all collected cells. The statistical result for treated groups were expressed by the ratio of the average percent of the samples of treated group to that of control group.

2.8. Data statistics

EXCEL 2013 was used for data analysis and diagram. The column values of each index in the figures were the average values of three repeated tests (mean ± S.D.). One-way ANOVA was used to discriminate comparisons among 3R4F cigarette, ultra-light cigarette and HnB product. Values of $p < 0.05$ were considered statistically significant. In the following figures, different letters “a, b, c” on the column represented the mean value of the biological endpoint of the three tobacco products were significantly different ($p < 0.05$) in the same *X* axis point (time or dose) and the same letters represented no significant difference.

3. Results

3.1. Analytes in the aerosol of three tobacco products

Five common analytes, four aromatic amines, five volatile organic compounds, eight carbonyl compounds, four TSNAs, ammonia, phenol, benzo[*a*]pyrene and hydrogen cyanide werer measured from the aerosol of three tobacco products in the study. As for 3R4F cigarette, the data of those chemicals were from the well-recognized study of Roemer et al’s (Roemer et al., 2012). As can be seen from Table 2, yields of 27 common analytes from both HnB and ultra-light were less than that from 3R4F. Yields of 22 HPHCs from HnB and 23 HPHCs from ultra-light were reduced by more than 70 % relative to 3R4F. Yields of 18 HPHCs from HnB and 18 HPHCs from ultra-light were reduced by more than 80 % relative to 3R4F. Yields of 17 HPHCs from HnB and 6 HPHCs from ultra-light were reduced by more than 90 % relative to 3R4F. In addition, Table 2 showed the levels of some carbonyl compounds from HnB were higher that from ultra-light cigarette, including formaldehyde, acetaldehyde, propionic aldehyde, butyral and crotonaldehyde. Although combustion pyrolysis products (e.g., polycyclic aromatic hydrocarbons) were few, the tobacco specific carcinogens including NNN, NNK, NAT and NAB were obviously detected in the aerosol from HnB product, which delivered 6.2, 3.4, 7.2 and 3.1 ng, respectively, in a stick.

3.2. Cell viability

Fig. 2 showed the cell viability decreased with the exposure dose increasing. Significant differences ($p < 0.05$) were present among different tobacco products at respective exposure dose (40 %, 60 %, 80 %), while there was no significant difference among three tobacco products at the exposure doses (10 %, 20 %). The order of cell survival of three tobacco products at the testing exposure doses was HnB > ultra-light > 3R4F. As for time-response, with increase of exposure time, cell viability gradually decreased. As shown in Fig. 3, there were significant differences among different tobacco products at respective exposure time (60 min, 90 min, 120 min, 150 min). The order of cell survival of three tobacco product at the testing exposure time was HnB > ultra-light > 3R4F.

3.3. Changes of intracellular GSH

As shown in Fig. 4, with increase of exposure dose, intracellular GSH content gradually decreased for all the three tobacco products. The reduction of GSH induced by 3R4F was more than HnB product. Except the 20 % exposure dose, each testing exposure dose presented significant differences ($p < 0.05$) of intracellular GSH content among the three tobacco products. And only more than 20 % of exposure dose, HnB showed a decreased trend for intracellular GSH. As shown in Fig. 5, the intracellular GSH content decreased rapidly for all three tobacco products at the designed exposure time (0 min ~ 120 min). The decreased rate was 3R4F > ultra-light > HnB. Comparing the decreasing trend of the figures between dose-response and time-response, it indicated that the exposure time was more sensitive to GSH of RAW 264.7 cells at ALI culture condition.

3.4. Changes of intracellular ROS

Fig. 6 showed the intracellular ROS changes with the exposure dose of the three tobacco products. With the exposure dose increased, ROS induced by 3R4F greatly increased, while ROS induced by ultra-light and HnB slightly increased. At the same exposure dose, ROS level induced by 3R4F was significantly ($p < 0.05$) higher than that by both ultra-light and HnB, except 10 % exposure dose. While, there was no significant difference of intracellular ROS level between ultra-light and HnB. Simlilar results (Fig. 7) were obtained for time-response of ROS induced by the three tobacco products. It showed ROS levels increased with exposure time and 3R4F induced more ROS than both HnB and ultra-light cigarette in the exposure time of 20 min, 30 min, 60 min, and 90 min.

4. Discussion

With the rise of consumers' health awareness and implement of “Framework Convention on Tobacco Control” (Wipfli and Huang, 2011), cigarette smoking is getting less and less in many parts of the world. In the past decades, tobacco companies have repeatedly attempted to research and develop alternative tobacco product, including ‘low-tar’ cigarettes, electronic cigarettes and heated tobacco products (Moazed et al., 2018). In recent years, HnB products, claimed as the risk-reduced product, have been developed and introduced to many global markets (Ludicke et al., 2019, 2018b; Smith et al., 2016; St Helen

Table 2
Yields of analytes delivered from HnB stick, ultra-light and 3R4F cigarettes under ISO smoking regime.

Category	Analytes	Unit (per cigarette or stick)	3R4F Mean (SE)	HnB Mean (SD)	Reduction (%)[a]	Ultra-light Mean (SD)	Reduction (%)[b]
Conventional analytes	Total particle matter	mg	9.77 (0.04)	24.87 (3.23)	−153.3	1.47 (0.03)	85
	Tar	mg	7.98 (0.03)	7.68 (0.4)	3.6	1.25 (0.05)	84.3
	Nicotine	mg	0.707 (0.005)	0.55 (0.01)	24.7	0.12 (0.01)	83.6
	Water	mg	1.08 (0.04)	16.64 (1.2)	−1515.5	0.11 (0.005)	89.3
	Carbon monoxide	mg	11.2 (0.1)	0.21 (0.005)	98.1	2.25 (0.02)	80.1
Aromatic amine	1-naphthylamine	ng	10.95 (0.4)	NQ	-	1.08 (0.02)	90.1
	2-naphthylamine	ng	5.69 (0.007)	NQ	-	0.45 (0.01)	92.8
	3-Aminobiphenyl	ng	1.81 (0.03)	NQ	-	0.35 (0.01)	80.7
	4-Aminobiphenyl	ng	1.01 (0.01)	NQ	-	0.26 (0.01)	70.8
Volatile Organic Compounds	1,3-butadiene	μg	38.5 (1.2)	NQ	-	10.2 (0.4)	74.6
	Isoprene	μg	395 (11)	0.46 (0.01)	99.9	46.2 (3.6)	88
	Acrylonitrile	μg	26.4 (0.7)	NQ	-	0.7 (0.02)	97.2
	Benzene	μg	45.7 (0.9)	0.17 (0.005)	99.7	4.9 (0.1)	90.1
	Toluene	μg	73.6 (1.4)	0.87 (0.03)	98.8	4.12 (0.2)	94.6
Carbonyl compounds	Formaldehyde	μg	20 (0.7)	8.8 (0.3)	58.3	4.2 (0.1)	80.1
	Acetaldehyde	μg	567 (10)	130.2 (4.6)	77.6	100.6 (4.5)	82.7
	Propionic aldehyde	μg	48.4 (0.9)	10.2 (0.4)	79.3	7.9 (0.32)	83.9
	Butyral	μg	26.4 (0.8)	15.6 (0.6)	40.9	6 (0.26)	77.3
	Acetone	μg	201.3 (13.2)	20.3 (0.7)	89.9	36.2 (1.26)	82
	Acrolein	μg	56.7 (1.4)	4.3 (0.2)	91.8	8.5 (0.3)	83.7
	Crotonaldehyde	μg	10.1 (0.3)	2.4 (0.07)	74.7	1.8 (0.06)	81.1
	2-butanone	μg	10.3 (0.4)	4.3 (0.1)	58.3	8.4 (0.4)	18.4
TNNAs	NNN	ng	92.1 (1.5)	6.2 (0.2)	93.4	66.1 (3.1)	29.2
	NNK	ng	85.5 (1.8)	3.4 (0.18)	95.8	7.3 (0.3)	90.9
	NAT	ng	92.9 (4.4)	7.2 (0.3)	92	34.4 (1.5)	61.9
	NAB	ng	9.6 (0.46)	3.1 (0.09)	69.9	3.5 (0.1)	66
Ammonia	Ammonia	μg	11.1 (0.2)	2.5 (0.1)	75.5	2.9 (0.07)	71.6
Phenols	Phenol	μg	7.04 (01)	NQ	-	1.3 (0.05)	81.7
Polycyclic aromatic hydrocarbons	Benzo[a]pyrene	ng	6.73 (0.11)	NQ	-	1.7 (0.03)	75.7
Hydrogen cyanide	Hydrogen cyanide	μg	70.9 (1.9)	NQ	-	9.8 (0.2)	85.8

Note: (1) "NQ" means the analyte has not been quantitatively detected;
(2) "a" means the reduction rate of HnB stick relative to 3R4F cigarettes;
(3) "b" means the reduction rate of ultra-light cigarette relative to 3R4F cigarette.
(4) "-" means that the reduction rate of 3R4F cigarette is infinitely close to 100 % under the present analytical conditions.
(5) “SE” means “standard error” and “SD” means “standard deviation”.

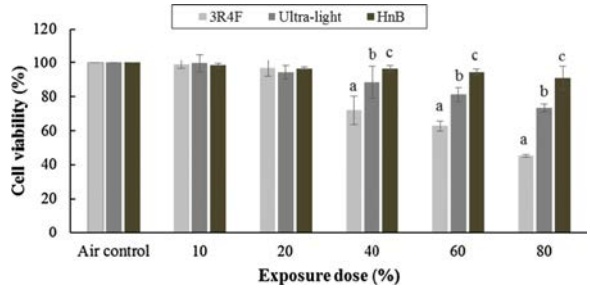

Fig. 2. Dose-repose of cell viability induced by the three tobacco products. "a, b, c" represents the difference of cell viability induced by different tobacco product at the same exposure dose. Different letters mean the significant difference ($p < 0.05$) amomg tobacco products and the same letters mean no significant difference.

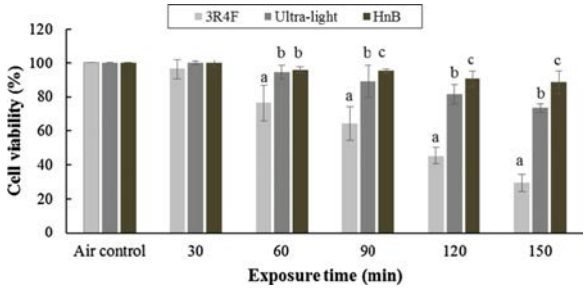

Fig. 3. Time-repose of cell viability induced by the three tobacco products. "a, b, c" represents the difference of cell viability induced by different tobacco product at the same exposure time. Different letters mean the significant difference ($p < 0.05$) among tobacco products and the same letters mean no significant difference.

et al., 2018). For no combustion, those HnB products avoid high HPHCs emission. Tobacco companies have argued these products with the potential to reduce the risk of harm in smokers and represent a harm reduction alternative that could aid conventional cigarette smokers (Ludicke et al., 2019, 2018a, b). A comparison of aerosol toxicant profiles between the HnB stick and 3R4F cigarette revealed that, while containing rather lower toxicant levels (reduced by more than 90 %) than 3R4F smoke, the HnB stick emissions contained significant high levels of volatile organic compounds, including known toxicants such as acrolein, acetaldehyde and formaldehyde (Forster et al., 2018).

Unlike conventional cigarettes, the aerosol of HnB product is generated by heating tobacco stick. The formation process of HPHCs is quite different from that of conventional cigarette. Compared to 3R4F cigarette, 73 % of tested HPHCs from HnB product are reduced by more than 90 % under HCI (Health Canada Intense) smoking regime (Jaccard et al., 2017; Jaunky et al., 2018), while, Li et al. reported that only 62 % of tested HPHCs under ISO smoking regime and 69 % of HPHCs under HCI smoking regime are reduced by more than 90 % (Li et al., 2019b). At present, HCI smoking regime are mostly adopted in the measurement of HPHCs from HnB product. Our study showed that under ISO smoking regime 61.5 % of tested HPHCs from HnB product are reduced by more than 90 % compared to 3R4F cigarette. From those reports employing ISO or HCI smoking regimes, it can be seen that most of HPHCs from HnB cigarette are much lower than conventional cigarette. Although in our study compared to 3R4F, the number of the greatly reduced HPHCs (> 90 %) from HnB stick are more than ultra-light cigarette, the

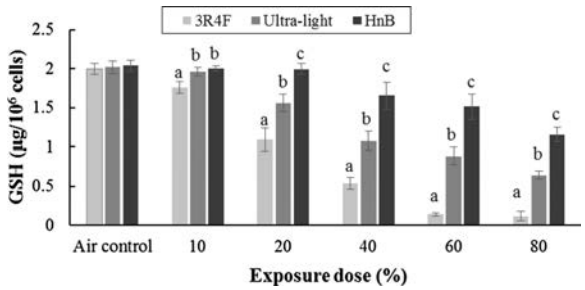

Fig. 4. Dose-response of intracellular GSH induced by the three tobacco products. "a, b, c" represents the difference of intracellular GSH induced by different tobacco product at the same exposure dose. Different letters mean the significant difference ($p < 0.05$) among tobacco products and the same letters mean no significant difference.

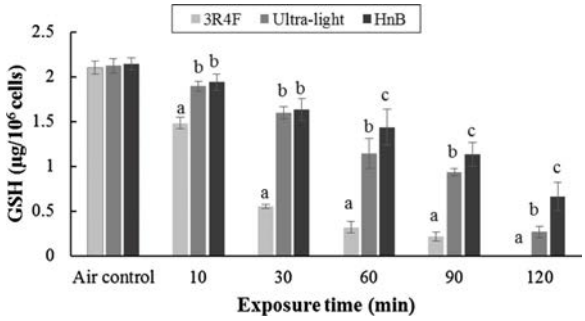

Fig. 5. Time-response of intracellular GSH induced by the three tobacco products. "a, b, c" represents the difference of intracellular GSH induced by different tobacco product at the same exposure time. Different letters mean the significant difference ($p < 0.05$) among tobacco products and the same letters mean no significant difference.

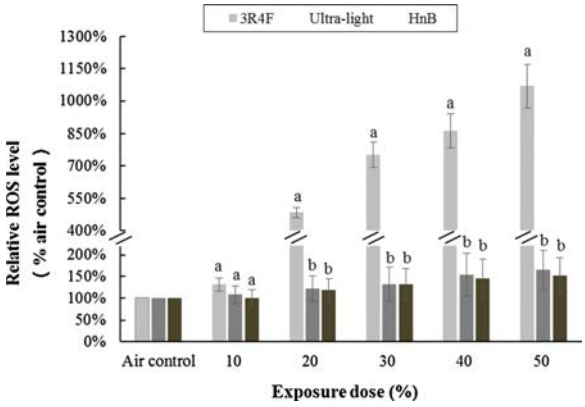

Fig. 6. Dose-response of intracellular ROS induced by three tobacco products. "a, b, c" represents the difference of intracellular ROS induced by different tobacco products at the same exposure dose. Different letters mean the significant difference ($p < 0.05$) among tobacco products and the same letters mean no significant difference.

number of the moderately reduced HPHCs (≤90 %) was less than ultra-light cigarette. Moreover, HnB stick delivered more levels of carbonyl compounds (formaldehyde, acetaldehyde, propanal, butyraldehyde and crotonaldehyde) than ultra-light cigarette. As we known, formaldehyde is well known as the carcinogen, listed as class I by International Agency for Research Cancer (IARC, 2006). Crotonaldehyde, one member of α,β-unsaturated aldehyde family, was well demonstrated to be the pro-inflammatory stimulant for macrophages and the major mediators of cigarette smoke–induced macrophage oxidative stress, a condition that characterizes patients with COPD (Facchinetti et al., 2007). In addition, substantial reductions of NNN and NNK (93.4 % and

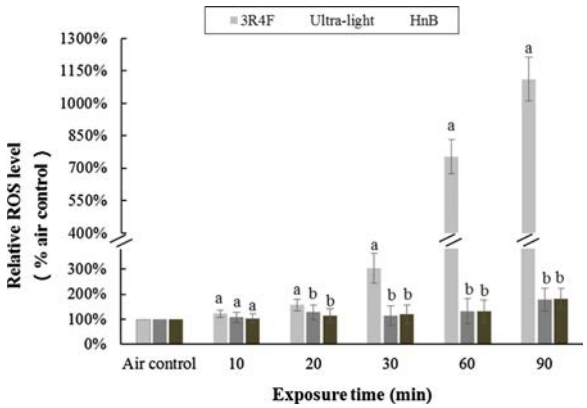

Fig. 7. Time-response of intracellular ROS induced by three tobacco products. "a, b, c" represents the difference of intracellular ROS induced by different tobacco products at the same exposure time. Different letters mean the significant difference ($p < 0.05$) among tobacco products and the same letters mean no significant difference.

95.8 %) were achieved for HnB product compared to the 3R4F. Despite the > 90 % reduction in total TSNAs relative to 3R4F, the HnB product still have potential carcinogenicity because theoretically there is no threshold of carcinogenic effect.

Successive oxidative stress can directly damage pulmonary epithelial and immune cells and promote inflammatory response (Rahman, 2005). Macrophages and epithelial cells can secrete a variety of inflammatory chemokines, such as IL-8 and MCP-1, which induce the transfer of neutrophils, monocytes and lymphocytes into the lung. When activated, inflammatory cells release inflammatory mediators. If keeping in inflammatory condition, those inflammatory factors will affect lung structure and further promote inflammatory response (Barnes, 2014; de Groot et al., 2019). Thus, during the occurrence and development of COPD, oxidative stress and inflammation interact with and promote each other (Rahman and MacNee, 2000). In the present study, we chose the representative cellular molecular (GSH and ROS) to study whether HnB aerosol induced oxidative stress of macrophages. In addition, macrophage is an important object in the study of cell phagocytosis, cellular immunity and molecular immunology. Past studies have confirmed that many chemical components (ROS, acrolein, crotonaldehyde, etc.) in cigarette smoke lead to a series of cellular chain reactions, including oxidative stress, apoptosis, autophagy, secretion of inflammatory factors (Oviedo et al., 2016). Our results of the study showed that both HnB product and combustible cigarettes resulted in decrease of GSH and increase of ROS, indicating that HnB product could induce the oxidative stress of macrophages. Wong et al. found that during 90 days of inhalation toxicity test in rats, the number of macrophages, lymphocytes, neutrophils and eosinophils in bronchial alveolar lavage fluid caused by 3R4F cigarette smoke was significantly higher than that of air control group and HnB product group, and there was no significant difference between HnB product and air control group, indicating that HnB product could not induce the accumulation of immune cells in respiratory system (Wong et al., 2016).While, Taylor et al. used high-throughput submerged cell culture exposure system to detect cellular oxidative stress and found that HnB product had little effect on the change of GSH and ROS (Taylor et al., 2018). One difference between our and Taylor et al's study was the cell culture exposure system, considering the direct or undirect exposure pathway of cells to aerosol. For better maintaining physical and chemical properties of aerosol, ALI cell culture exposure system was widely accepted and considered to be a appropriate tool to mimic the realistic exposure scenario. In the study, macrophages cultured at ALI showed GSH decrease and ROS increase when exposed to the aerosol of HnB product, which indicated HnB product induced oxidative stress in immune cells.

5. Conclusion

The study firstly compared aerosol HPHCs of HnB product with that of ultra-light cigarette. It was demonstrated that most of HPHCs from HnB product were less than that from ultra-light cigarette, except some carbonyl compounds. Using ALI cell culture exposure system, GSH and ROS in macrophages were determined exposed to aerosol of HnB product and ultra-light cigarette. It was confirmed that HnB product induced oxidative stress in macrophages in the dose- and time-response ways, although less than that of ultra-light cigarette to some extent.

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Declaration of Competing Interest

None.

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