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Original Article

Comparative analysis of the toxic effects on the mouse lung of 4 weeks exposure to the heated tobacco product Ploom TECH+ and 3R4F reference cigarettes

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Abstract: Pulmonary emphysema is primarily attributable to prolonged exposure to cigarette smoke. Novel tobacco substitutes, such as heated tobacco products, have emerged as healthier alternatives to cigarettes. The effects of short-term inhalation of a heated tobacco product, Ploom TECH+ (PT+), on the lungs of mice were compared with those of 3R4F reference cigarettes. Male 10-week-old C57BL mice were exposed to clean air (control), 3R4F, or PT+ for 1 h/d, 5 d/week for two or four weeks. After four weeks of exposure, the number of inflammatory cells and proportion of neutrophils and lymphocytes in the bronchoalveolar lavage fluid and the number of macrophages in the lung tissue increased significantly in mice exposed to 3R4F but not in those exposed to PT+. Changes in the expression of genes related to inflammation-related factors were observed in the lung tissues of mice exposed to 3R4F for two and four weeks. Chemokine (C-C motif) ligand 17, resistin-like alpha, and lipocalin 2 were among the upregulated genes. In our previous short-term tobacco inhalation study, these genes were identified as useful markers of emphysema effects induced by exposure to cigarette smoke from Peace cigarettes, detectable before pulmonary histological changes appeared. These effects were not observed in the PT+-exposed mice. These data suggest that PT+ caused less damage to the lungs of mice than 3R4F, particularly regarding the induction of emphysema. (DOI: 10.1293/tox.2024-0069; J Toxicol Pathol 2025; 38: 147–154)

Key words: cigarette smoke, lung, mouse, heated tobacco product, Ploom TECH+, toxicity

Introduction

The World Health Organization released data indicating that the global prevalence of smoking among individuals aged 15 years and older was 17.5% in 2019. An estimated 847 million adult males, 153 million adult females, and 24 million young people aged 13–15 years are current smokers[1]. Epidemiological studies have demonstrated that cigarette smoking increases the risk of developing several types of cancers, such as lung cancer[2, 3] and chronic obstructive pulmonary disease (COPD), which includes emphysema and chronic bronchitis[4].

Heated tobacco products (HTPs) are electronic devices that typically heat processed tobacco leaves to temperatures below the combustion temperature, thereby delivering nicotine-containing aerosols that may be less harmful than traditional tobacco smoke. Peer-reviewed evidence indicates that heat-not-burn tobacco products are effective nicotine delivery devices that expose users and bystanders to substantially fewer harmful and potentially harmful compounds than smoke from burning cigarettes[5]. However, another systematic review indicated that the damage produced by HTPs is associated with pathways related to pulmonary diseases, mechanisms of damage reported to be induced by conventional cigarettes, and new mechanisms of damage specific to these devices[6]. In addition, the prevalence of HTPs use increased in the European and Western Pacific regions between 2015 and 2020, with nearly 5% of the population having tried HTPs and 1.5% identified as current users during the study period[7]. Therefore, it is important to verify the safety of HTPs.

In previous studies, we developed a mouse model of emphysema by exposing C57BL/6J mice to tobacco smoke[8, 9]. We used this model to evaluate the effects of short-term inhalation exposure to tobacco smoke from Peace non-filter cigarettes on mouse lungs. In addition to identifying inflammatory changes in the lungs, we identi-

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fied marker genes that respond to tobacco smoke[10]. These data suggest that the effects of emphysema can be identified after short-term exposure to tobacco smoke. The objective of this study was to evaluate the effects (regarding the risk of emphysema) of short-term inhalation exposure to smoke from an HTP (Ploom TECH+; PT+), compared with exposure to 3R4F reference cigarette smoke in mice.

Materials and Methods

Animal experiments

All animal studies were approved by the Institutional Animal Care and Use Committee of Osaka Metropolitan University Graduate School of Medicine and conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). The Laboratory Animal Center of the Osaka Metropolitan University Graduate School of Medicine is accredited by the Japan Health Sciences Foundation's Center for Accreditation of Laboratory Animal Care and Use.

Nine-week-old male C57BL/6J mice were purchased from Charles River Laboratories (Atsugi, Japan). They were housed five per cage on wood-chip bedding in an air-conditioned animal room at 22 ± 2°C and 55 ± 10% humidity. All mice were allowed free access to a pelleted diet (CE2; Clea Japan Inc., Tokyo, Japan) and tap water, except during exposure to cigarette smoke or clean air. All mice were acclimatized for one week prior to the start of the experiment.

Experimental reference tobacco cigarettes 3R4F (Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY, USA) and PT+ with a MEVIUS Mild Blend (Japan Tobacco Inc., Tokyo, Japan) were used. In animal experiments, each cigarette type was tested in a separate experiment, with a corresponding control group for each. In Experiment 1, 40 mice were divided into two groups: one exposed to clean air (control group) and the other to mainstream 3R4F smoke for 1 h daily (10–11 am) 5 d/week for two or four weeks. Similarly, in Experiment 2, 40 mice were divided into clean air (control) and mainstream PT+ smoke exposure groups under the same conditions. Ten mice from each group were euthanized at the end of week 2, and the remaining 10 at the end of week 4.

Each mouse in the exposure group was placed in an acrylic holder attached to an inhalation exposure chamber (Sibata Scientific Technology Ltd., Saitama, Japan) such that only the nose was exposed to mainstream smoke. Each mouse in the control group was placed in an acrylic holder and exposed to clean air. In the 3R4F and PT+ groups, cigarette smoke was generated using a cigarette smoke generator (SG-300; Sibata Scientific Technology Ltd.) using 3R4F or PT+ cigarettes. For both 3R4F and PT+ cigarettes, mainstream smoke was generated under the Health Canada Intense regime (55 mL puff volume, 30 s puff interval, 2 s puff duration, and 100% blocking of filter ventilation holes). Wet total particulate matter (WTPM) was measured using a glass fiber filter (Advantec, Tokyo, Japan) and minipump (MP-Σ100HN, Sibata Scientific Technology Ltd.) twice per week. The target value of WTPM was set to 1,000 μg/L for each treatment, which is the highest dose of the previous study[11]. Nicotine, propylene glycol, and glycerin concentrations in the exposure atmosphere were analyzed twice weekly by the LSI Medience Corporation (Tokyo, Japan). Carbon monoxide (CO) was monitored twice weekly using a CO monitor (HT-1300N; HODAKA CO., Ltd., Osaka, Japan).

Twenty-four hours after the last exposure to cigarette smoke, 10 mice per group were euthanized via transection of the inferior vena cava under deep anesthesia by inhalation of an overdose of isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) using a small-animal anesthetizer (MK-A110D, Muromachi Kikai Co., Ltd., Tokyo, Japan) coupled with an anesthetic gas-scavenging system (MK-T 100E, Muromachi Kikai Co., Ltd.).

For the collection of bronchoalveolar lavage fluid (BALF), five mice were tracheotomized and cannulated. Phosphate-buffered saline containing 0.3% bovine serum albumin (Merck KGaA, Darmstadt, Germany) and 0.05 mM EDTA-2K (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was used as the BALF diluent. BALF was collected as follows: 2.5 mL of diluent was injected into the lung at a pressure of 15 cm H_2O, and the BALF was collected at a pressure of –8 cm H_2O. Diluent (1.5 mL) was then injected at a pressure of 15 cm H_2O, and the BALF was collected at a pressure of –8 cm H_2O. This step was repeated four times. The six BALF samples were combined and treated as one BALF specimen for each mouse. After bronchoalveolar lavage, the left lung was excised and submerged in RNAlater™ stabilization solution (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), stored at 4°C for one day, and then stored at –80°C until RNA isolation.

The lungs of the remaining five mice in each group were injected with approximately 2 mL of 10% neutral-buffered formalin using a syringe, fixed by immersion in phosphate-buffered formalin for 48 h, embedded in paraffin, and processed for hematoxylin and eosin staining. The number of infiltrating inflammatory cells (macrophages, neutrophils, and lymphocytes) was counted microscopically in five random areas of each lung. In this study, an inflammatory focus was defined as the accumulation of more than 20 inflammatory cells in lung tissue.

Bronchoalveolar lavage fluid analysis

The collected BALF was centrifuged at 1,200 rpm at 4°C for 5 min, and the supernatant was removed. Pellets were resuspended in 1,000 μL PBS. The resuspended pellets (500 μL) were stained with trypan blue, and the number of cells was counted using a hemocytometer and an automatic cell counter. The remaining 500 μL of the cell suspension was subjected to a cytospin procedure using a Shandon Cytospin 3 centrifuge (Shandon Scientific Co., London, England). The slides were stained with Giemsa, and cell enumeration and differential cell counts (neutrophils, lymphocytes, and macrophages) were performed according to standard hematologic criteria.

Microarray analysis in lungs

Total RNA was isolated from the BALF extracted from the left lung tissue of five mice from each group using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Microarray analysis was performed using a Clariom™ D mouse array (Affymetrix, Inc. Santa Clara, CA, USA) obtained from Cell Innovator Inc. (Fukuoka, Japan). After global median normalization, data cleansing was performed to remove values for which the fluorescence intensity was <100. The criteria for regulated genes were a Z score≥2.0 and a ratio ≥2.0 fold for upregulated genes, and a Z score ≤–2.0 and a ratio ≤0.5 for downregulated genes. The differentially expressed genes were analyzed using the Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc., Mountain View, CA, USA) to investigate the functional significance of the upregulated and downregulated genes in the cigarette smoke-exposed group.

mRNA expression analysis in the lungs

cDNA synthesis was performed with 1 μg of RNA using an Advantage RT-for-PCR kit (Takara Bio, Inc., Otsu, Japan). The mRNA expression levels of chemokine (C-C motif) ligand 17 (*Ccl17*; Mm01244826_g1), lipocalin 2 (*Lcn2*; Mm01324470_m1), and resistin-like alpha (*Retnla*; Mm00445109_m1) were determined using TaqMan real-time quantitative PCR. The PCR reagents, sequence-specific primers, and probes for each gene (TaqMan Gene Expression Assay) were purchased from Thermo Fisher Scientific. mRNA expression assays were performed using a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). 18S ribosomal RNA was used as an internal control. The values for the target genes were normalized to those of 18S rRNA.

Statistics

All values are reported as the mean ± standard deviation. Statistical analyses were performed using Prism 9 software (GraphPad Software, Inc., San Diego, CA, USA). Differences in mean values between the control and cigarette smoke-exposed groups were evaluated using a 2-tailed Student's t-test. Differences in the multiplicities of histopathological lesions between the control and treatment groups were evaluated using a 2-tailed Mann–Whitney test. Statistical significance was set at $p<0.05$.

Results

Body and organ weights, food, and water consumption

On day one of Experiment 2, one mouse in the cigarette-exposed group died because of restraint. Because the death was not due to exposure to cigarette smoke, the mouse was excluded from the study. There was no significant difference in the final body weights between the control and 3R4F groups or between the control and PT+ groups at weeks 2 and 4 (Table 1). There were no significant differences in water and food consumption between the groups over the four-week study period (Table 1).

Smoke constituents

Table 2 lists the smoke constituents in each treatment group. WTPM in the 3R4F (962 μg/L) and PT+ exposure atmosphere (1,045.6 μg/L) was approximately 1,000 μg/L. The nicotine concentration in the 3R4F exposure atmosphere was approximately 10 times higher than in the PT+ exposure atmosphere. Propylene glycol and glycerin concentrations were higher in the PT+ exposure atmosphere

Table 1. Body Weight and Average Food and Water Consumption during the Four-week Exposure Period

Treatment	Body weight (g)		Food consumption (g/g BW/day)	Water consumption (g/g BW/day)
	2 weeks	4 weeks		
Control	25.7 ± 1.2	26.4 ± 1.6	0.12 ± 0.01	0.21 ± 0.03
3R4F	25.2 ± 1.3	25.3 ± 1.6	0.12 ± 0.01	0.20 ± 0.02
Control	25.7 ± 1.6	26.1 ± 2.1	0.12 ± 0.01	0.21 ± 0.04
PT+	25.5 ± 1.4	25.8 ± 1.2	0.11 ± 0.00	0.19 ± 0.02

Table 2. Average Smoke Constituents of 3R4F and PT+ treatments during the Four-week Exposure Period

Treatment	No. of samples	WTPM (μg/L)	Nicotine (μg/L)	Propylene glycol (μg/L)	Glycerin (μg/L)	CO concentration (ppm)
Experiment 1						
Control	-	-	-	-	-	-
3R4F	8	963 ± 29.0	61.5 ± 3.3	N.D.	101.8 ± 3.5	1,138 ± 20.7
Experiment 2						
Control	-	-	-	-	-	-
PT+	8	1,046 ± 199.4	6.6 ± 1.3	295.7 ± 52.5	525.1 ± 69.9	0 ± 0.0

WTPM: Wet total particulate matter; CO: carbon monoxide; 3R4F: reference tobacco cigarettes 3R4F; PT: Ploom TECH+; N.D.: not detected.

than in the 3R4F exposure atmosphere. CO was detected only in the 3R4F exposure atmosphere.

Inflammatory cells in bronchoalveolar lavage fluid

Representative microscopic images of the BALF are shown in Fig. 1A. Some dust cells and neutrophils were present in the 3R4F group but not in the control or PT+ groups. The total number of inflammatory cells in the BALF of the group exposed to 3R4F for four weeks was significantly higher than that in the control group, whereas this was not so after two weeks (Table 3). The proportion of neutrophils and lymphocytes in the 3R4F group was significantly higher than that in the control group at two and four weeks (Table 3). There were significantly more macrophages in the BALF of 3R4F mice ($5.9 \pm 1.1 \times 10^5$ cells) than in the control group ($3.6 \pm 0.2 \times 10^5$ cells; $p<0.05$) at four weeks but not at two weeks (Table 3). Macrophages in the BALF of 3R4F mice were larger than usual, which may be attributed to the phagocytosis of dust particles. There were no significant differences in the number of inflammatory cells in the BALF of the PT+ group compared to the control group (Table 3).

Histological analyses of the lung

Representative microscopic images of the lung tissues are presented in Fig. 1B. There was no expansion of the alveolar spaces, destruction of the alveolar walls, or fibrotic lesions in the lung tissue in any of the groups (Fig. 1B). Examination of the inflammatory cells within the alveolar space revealed that most were macrophages, with a few neutrophils and lymphocytes (Table 4). The number of macrophages per unit area was significantly higher in the 3R4F group but not in the PT+ group than in their respective control groups at four weeks (Table 4). There was a small accumulation of inflammatory cells on the vessel wall and

Table 3. Inflammatory Cells in the Balf

Treatment	No. of mice	Total cells ($\times 10^5$ cells)	Macrophages (%)	Neutrophils (%)	Lymphocytes (%)
Experiment 1					
2 weeks					
Control	5	5.2 ± 0.2	99.3 ± 0.2	0.0 ± 0.1	0.7 ± 0.2
3R4F	5	5.9 ± 1.9	95.7 ± 1.5**	3.0 ± 1.3**	1.3 ± 0.4*
4 weeks					
Control	5	3.7 ± 0.2	99.2 ± 0.5	0.1 ± 0.1	0.7 ± 0.5
3R4F	5	6.4 ± 1.2***	91.8 ± 1.7***	6.2 ± 1.4***	2.0 ± 0.5**
Experiment 2					
2 weeks					
Control	5	5.5 ± 1.4	99.4 ± 0.2	0.2 ± 0.3	0.4 ± 0.1
PT+	5	5.2 ± 1.2	99.6 ± 0.1	0.0 ± 0.1	0.3 ± 0.1
4 weeks					
Control	5	5.2 ± 1.2	99.6 ± 0.1	0.2 ± 0.0	0.2 ± 0.2
PT+	5	4.9 ± 0.8	99.4 ± 0.4	0.1 ± 0.1	0.4 ± 0.3

*p<0.05, **p<0.01, ***p<0.001: Significantly different from Control group. BALF: bronchoalveolar lavage fluid; 3R4F: reference tobacco cigarettes 3R4F; PT: Ploom TECH+.

Table 4. Inflammatory Cells and Foci in the Lung

Treatment	No. of mice	Number of inflammatory cells (/mm²)			Incidence of inflammatory foci	Multiplicity of inflammatory foci
		Macrophage	Neutrophils	Lymphocytes		
Experiment 1						
2 weeks						
Control	5	127.5 ± 16.5	0.0 ± 0.0	0.9 ± 1.3	0	0.00 ± 0.00
3R4F	4	156.0 ± 28.0	0.4 ± 0.8	2.3 ± 1.9	1	0.25 ± 0.50
4 weeks						
Control	5	118.2 ± 8.5	0.0 ± 0.0	1.8 ± 1.3	1	0.20 ± 0.00
3R4F	5	183.8 ± 20.8***	0.9 ± 1.3	3.6 ± 1.3	2	0.40 ± 0.55
Experiment 2						
2 weeks						
Control	5	119.4 ± 24.7	0.0 ± 0.0	1.2 ± 1.3	1	0.20 ± 0.45
PT+	5	117.9 ± 27.5	0.0 ± 0.0	0.6 ± 0.8	1	0.20 ± 0.45
4 weeks						
Control	5	112.5 ± 18.2	0.0 ± 0.0	0.9 ± 1.3	1	0.20 ± 0.45
PT+	5	119.4 ± 12.6	0.0 ± 0.0	0.3 ± 0.7	1	0.20 ± 0.45

***p<0.001: Significantly different from Control group. 3R4F: reference tobacco cigarettes 3R4F; PT+: Ploom TECH+.

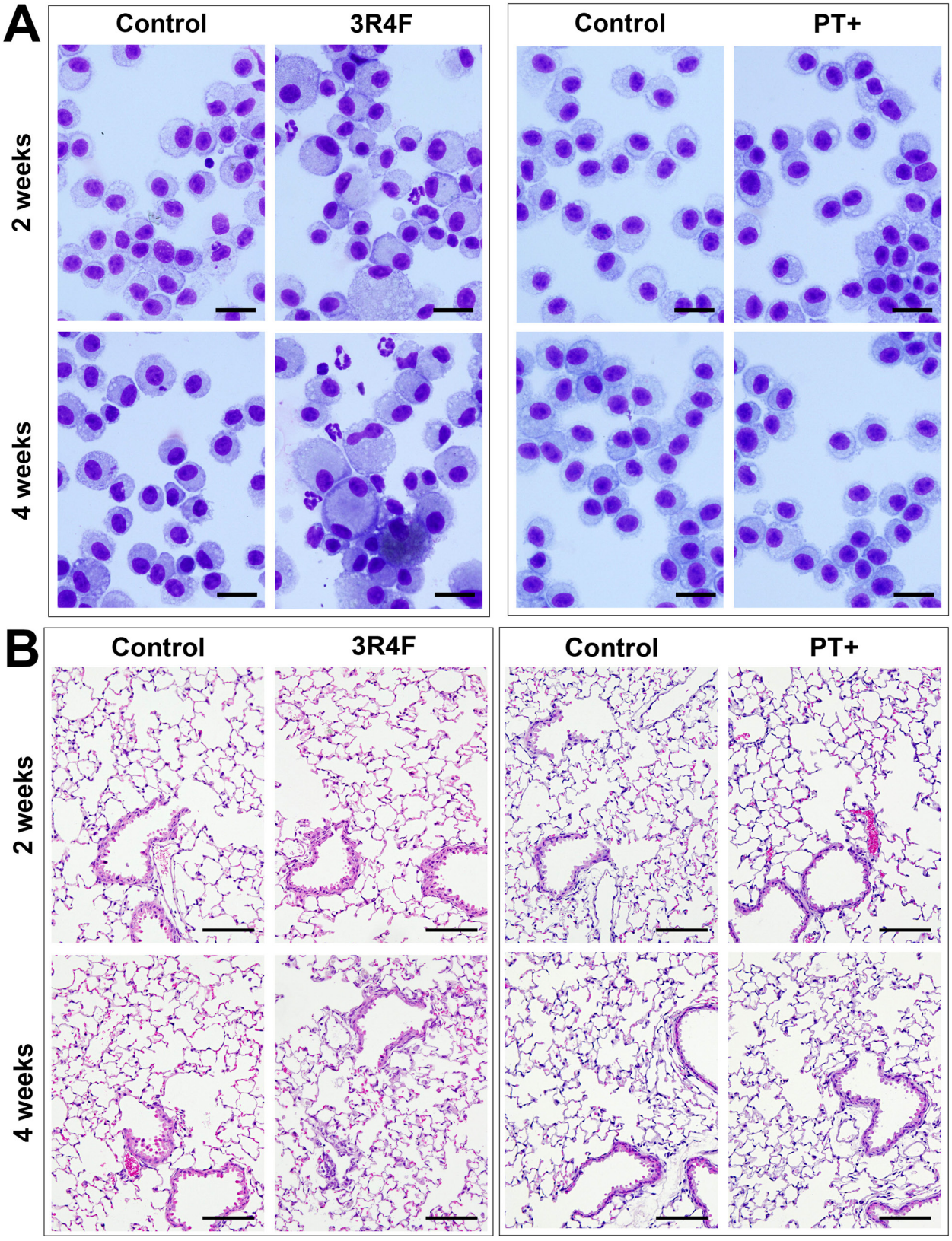

Fig. 1. Histology of mouse bronchoalveolar lavage fluid (BALF) and lungs. (A) BALF from control and 3R4F- or PT+-exposed mice at two and four weeks. (B) Lungs of control and 3R4F- or PT+-exposed mice at two and four weeks. Bar=20 μm (A) and 100 μm (B); 3R4F, reference tobacco cigarettes 3R4F; PT+, Ploom TECH+.

Table 5. Candidate Key Genes in the Response to 3R4F Exposure

Gene_Symbol	Gene_Description	Expression ratio	
		2 weeks	4 weeks
Marco	macrophage receptor with collagenous structure	5.2	7.3
Ccl17	chemokine (C-C motif) ligand 17	5.3	5.9
Lcn2	lipocalin 2	4.9	5.9
Ch25h	cholesterol 25-hydroxylase	3.3	5.1
Cd68	CD68 antigen (Cd68), mRNA.	4.1	5.0
Retnla	resistin like alpha	2.4	3.9
Itgax	integrin alpha X	2.7	3.2
Ly75	lymphocyte antigen 75	2.2	2.7
Saa3	serum amyloid A 3	2.8	2.4
Clec4n	C-type lectin domain family 4, member n	2.5	2.3
Clec7a	C-type lectin domain family 7, member a	2.5	2.2

3R4F: reference tobacco cigarettes 3R4F.

interstitial space in 0–2 of the five mice in each group, but there was no significant difference among the groups (Table 4). Papillary protrusions into the lumen were not observed in the bronchial epithelium, and no significant differences were detected between the groups (Fig. 1B).

Microarray data analysis of the lung

A total of 86 (67 upregulated and 19 downregulated) and 99 (77 upregulated and 22 downregulated) genes were differentially expressed in the lungs of mice exposed to 3R4F smoke for two and four weeks, respectively, compared to the controls. The number of commonly upregulated and downregulated genes after two and four weeks of exposure was 44 and 4, respectively (Supplementary Table 1). The IPA indicated that these genes were associated with the immune response, phagocytosis, and activation of phagocytes in the Diseases and Bio-functions categories (Supplementary Table 2). The top regulator effects network analysis predicted that CSF2, IKBKB, L2HGDH, NFkB, TLR3, TLR9, and MYD88 are the primary upstream regulators (Supplementary Table 3). Eleven genes, listed in Table 5, were predicted as key common genes in the IPA Diseases and Bio-functions categories (Supplementary Table 2) and the top regulator effects network analysis (Supplementary Table 3).

A total of 28 (11 upregulated and 17 downregulated) and 27 (15 upregulated and 22 downregulated) genes were differentially expressed in the lungs of mice exposed to PT+ smoke for two and four weeks, respectively, compared to controls. The numbers of commonly differentially expressed genes after two and four weeks of exposure were 3 and 2, respectively. Of these five, four genes had gene name information: immunoglobulin heavy variable 1 (Ighv1)-63 and Igkv1-110 were upregulated, and Ighv1-50 and Ighv1-76 were downregulated (Supplementary Table 4). Because of the small number of commonly differentially expressed genes associated with PT+ treatment, neither diseases nor bio-functions in the Diseases and Bio-functions categories nor regulators in the top regulator effects network analysis were detected by the IPA.

Identification of early-response biomarker genes for 3R4F exposure

In a previous study, *Ccl17*, *Retnla*, and *Lcn2* were concluded to be useful response biomarker genes for the toxic effects of Peace cigarette smoke exposure for as short a time as two or four weeks[10]. In this study, these were also detected as key genes in the lungs of the 3R4F group mice (Table 5). The expression levels of these genes were confirmed using quantitative RT-PCR. Similar to the outcomes of the microarray analysis, the expression of these genes in the lungs of 3R4F-exposed mice was markedly higher than that in control mice at both two and four weeks (Fig. 2). In contrast, there were no significant differences in the expression of these genes between the PT+ and control groups at two and four weeks (Fig. 2).

Discussion

HTPs are a novel category of smoking devices that contain fewer deleterious substances than traditional cigarettes. However, the effects of these devices remain unclear. In this study, we focused on PT+, an HTP, and compared its effects on the lungs of mice exposed to PT+ smoke and 3R4F reference cigarette smoke for two and four weeks. We found that 3R4F elicited a spectrum of effects in mouse lungs, whereas PT+ demonstrated minimal impact within the study's timeframe.

COPD is a prevalent chronic respiratory disease in humans characterized by irreversible airflow limitation. Emphysema is the principal pathological feature of COPD, which results in a high mortality rate and substantial annual healthcare expenditure. Exposure to cigarette smoke has been regarded as a traditional method for long-term modeling of emphysema, and mice have been identified as a suitable experimental model to identify the mechanisms of emphysema[12]. This short-term study was conducted using our mouse model of emphysema induced by exposure to Peace non-filter cigarettes[8, 9] and was consistent with our previous short-term study[10]. The present results indicate that PT+ smoke exposure has a diminished impact on the lungs

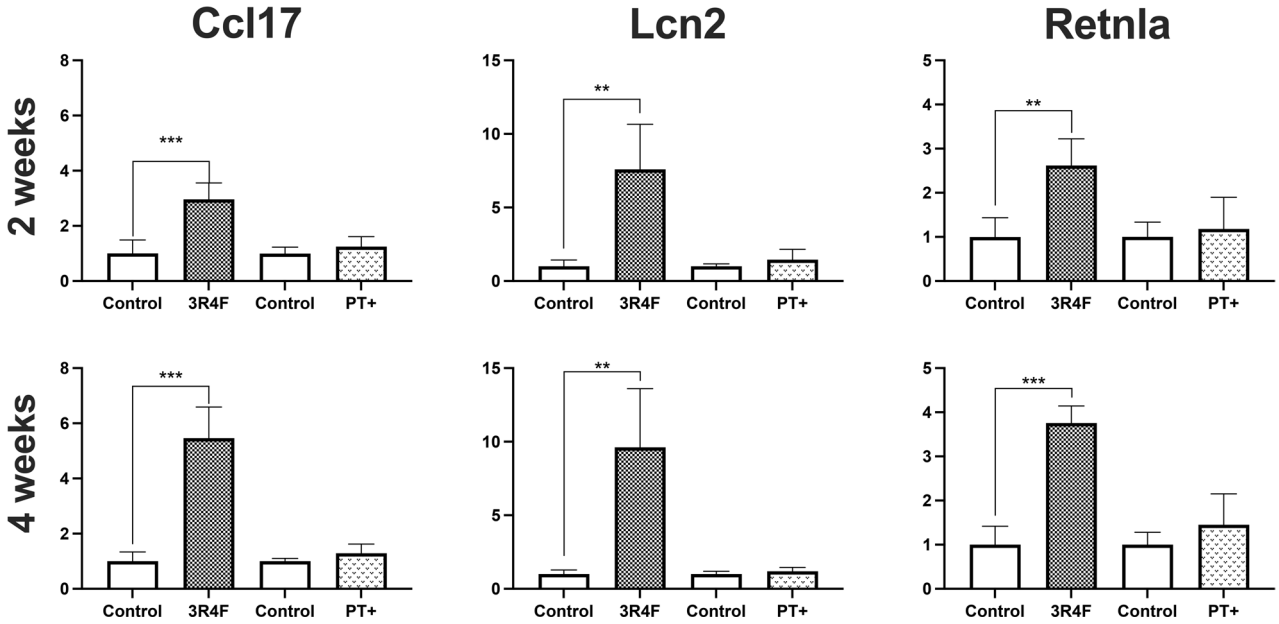

Fig. 2. RNA expression of *Ccl17*, *Retnla*, and *Lcn2* in the lungs of control and 3R4F- or PT+-exposed mice at two and four weeks. Significant differences between the treatments and their respective control groups at **p<0.01; ***p<0.001. 3R4F: reference tobacco cigarettes 3R4F; PT+: Ploom TECH+.

compared to conventional cigarette 3R4F smoke with regard to the induction of emphysema.

In contrast to our previous study, which exposed mice continuously to cigarette smoke diluted with air[10], this study employed the Health Canada Intense analytical machine puffing regime, mainly because it provides the most relevant comparative basis for evaluating the composition of heated tobacco product aerosols in cigarette smoke[13]. In addition, this study utilized 3R4F cigarettes rather than the commercially marketed Peace non-filter cigarettes employed in our previous study. Despite these differences, the effects of exposure to burning tobacco smoke in this study were similar to those observed in our previous study, including the presence of inflammatory cells in the BALF and changes in RNA expression in the lungs[10]. Notably, the expression of *Ccl17*, *Retnla*, and *Lcn2* was elevated in the lungs of mice exposed to Peace non-filter cigarette smoke in our previous study and to 3R4F cigarette smoke in this study. This finding supports our hypothesis that *Ccl17*, *Retnla*, and *Lcn2* may serve as valuable response biomarkers for assessing cigarette smoke-related toxicity in short-term studies[10].

Our investigations revealed no significant effects of PT+ smoke. PT+ smoke did not induce a significant influx of inflammatory cells into the BALF, nor did it cause any discernible changes in the lung tissue. Furthermore, less significant changes were observed in the lung gene expression profiles than those caused by exposure to burning cigarette smoke. Similar results in the lungs and BALF of mice have been reported in a study using a tobacco heating system (THS) 2.2 (also an HTP)[14]. In that study, mice were exposed to 3R4F or THS 2.2 smoke for 18 months. They reported that increased pulmonary inflammation, altered lung function, and emphysematous changes were observed in 3R4F-exposed mice but not in THS 2.2-exposed mice. However, other studies using a different HTP (IQOS) have reported that HTP induces inflammatory cell infiltration and cytokine expression in the lungs and BALF to the same extent as exposure to traditional burning tobacco smoke[15, 16]. These data suggest that the effects of HTPs may depend on the type of device used. Our results indicated that PT+ induced significantly less damage to the mouse lungs than burning cigarette smoke.

Since this short-term study used the tobacco smoke emphysema model[8–10], the data from this experiment may only reflect emphysema-associated effects. Carcinogenesis is one of the most significant effects of tobacco smoking[2]. In the experiments described by Wong *et al.*[14], exposure to 3R4F smoke for 18 months induced lung tumors in A/J mice, but THS 2.2 smoke did not, suggesting that PT+ carcinogenicity may also be low. However, the present study was based on a mouse emphysema model[8, 9], which was not designed to examine the impact of long-term exposure to smoke on lung carcinogenesis. Therefore, further research is required to confirm the carcinogenic potential of PT+ smoke in the lungs.

In conclusion, this short-term study demonstrated that PT+ causes less damage to the lungs of mice than the burning smoke from 3R4F, a traditional cigarette. It should be noted that our study has limitations. It was conducted on mice rather than humans, and the effects were observed for a maximum of four weeks. Although the results are limited, it is probable that PT+ cigarettes cause less damage to the lungs than traditional cigarettes. As noted above, toxic effects may vary with the HTPs used. Our results indicated

that the methods used in the present study can be used to compare the lung toxicities of different HTPs.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflict of interest.

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