Nicotine & Tobacco Research, 2021, 2145–2152
doi:10.1093/ntr/ntab123
Original Investigation
Received August 28, 2020; Editorial Decision June 3, 2021; Accepted June 8, 2021

Original Investigation

A Newly Developed Aerosol Exposure Apparatus for Heated Tobacco Products for In Vivo Experiments Can Deliver Both Particles and Gas Phase With High Recovery and Depicts the Time-Dependent Variation in Nicotine Metabolites in Mouse Urine

Marie Sawa BPharm[1], Akira Ushiyama PhD[2,*], Yohei Inaba PhD[2], Shigehisa Uchiyama PhD[2], Kenji Hattori PhD[1], Yuki Ogasawara PhD[3], Kazuyuki Ishii PhD[1]

[1]Department of Hygienic Chemistry, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan; [2]Department of Environmental Health, National Institute of Public Health, Wako, Saitama, Japan; [3]Department of Analytical Biochemistry, Meiji Pharmaceutical University, Kiyose, Tokyo, Japan

Corresponding Author: Akira Ushiyama, PhD, Department of Environmental Health, National Institute of Public Health, 2-3-6 Minami, Wako, Saitama 351-0197, Japan. Telephone: 81-48-458-6254; Fax: 81-48-458-6270; E-mail: ushiyama.a.aa@niph.go.jp

Abstract

Introduction: There is no standardized aerosol exposure apparatus to deliver heated tobacco products (HTPs) for in vivo experiments. Therefore, we developed a novel HTPs aerosol exposure apparatus for mice and demonstrated that nicotine and other chemicals in HTPs aerosol generated by the apparatus can be delivered to mice which replicate human smoke.

Aims and Methods: The amounts of nicotine, tar, and carbon monoxide (CO) in IQOS (Marlboro Regular HeatSticks) aerosol generated by two types of apparatuses were determined. C57BL/6N mice were exposed to IQOS aerosol, followed by determination of the urinary nicotine metabolites. Further, the skin surface temperature of mice was monitored to confirm the vasoconstriction action of nicotine.

Results: The amount of chemicals in IQOS aerosol by the novel air push-in inhalation apparatus for HTPs (APIA) was equivalent to that of the analytical vaping machine (LM4E) (1.60 ± 0.08 [APIA] vs. 1.46 ± 0.07 mg/stick [LM4E] in nicotine and 0.55 ± 0.04 [APIA] vs. 0.45 ± 0.01 mg/stick [LM4E] in CO). After mice were exposed to IQOS aerosol by APIA, the urinary nicotine metabolite levels were determined; peak values in cotinine and 3-hydroxycotinine (3-HC) were 6.82 μg/mg creatinine at 1 hour after exposure and 32.9 μg/mg creatinine at 2 hours after exposure, respectively. The skin surface temperature decreased and was lower (33.5°C ± 0.5°C) at 30 minutes than before exposure (37.6°C ± 0.8°C).

Conclusions: The new apparatus for HTPs aerosol exposure to mice showed good performances in terms of both chemical analysis of collected aerosol and fluctuations in the urinary nicotine metabolites.

Implications: The APIA reported in this study can expose small animals to HTPs aerosol, including nicotine and other chemical substances as same amounts as LM4E and replicate actual human smoking process by in vivo experiments. Therefore, the experiments using APIA can provide evidence to assess the health risks of HTPs use.

Introduction

Cigarettes contain approximately 600 ingredients, and when burned, result in the release of more than 7000 chemicals.[1] At least 69 of these chemicals are known to cause cancer.[2–5] Cigarette smoking accounts for approximately one-third of cancer-related deaths[6,7] and is associated with various pulmonary and cardiovascular diseases, including chronic obstructive pulmonary disease[8] and atherosclerosis.[9] To reduce health problems caused by cigarette smoking, the tobacco industry released new cigarette-like products, defined as heated tobacco products (HTPs).[10] IQOS, an HTP manufactured by Philip Morris International (New York, NY), was initially sold in 2014 only in Japan and Italy[11]; according to Philip Morris International, IQOS is currently available in 57 countries.[12] Notably, the use of HTPs has recently increased in Japan; for example, HTPs users accounted for 11.3% of the general population aged 15–69 years, and among the current smokers in 2019, more than 30% used HTPs.[13] Although the tobacco industry claims that HTPs are less harmful, few evaluations by public research institutions did not receive funding from the tobacco industry. Based on this, the World Health Organization has declared that at present there is no evidence that suggests that reduced exposure to these chemicals translates to decreased risk in humans.[14]

Chemical comparison between the mainstream smoke of combustible cigarettes and IQOS shows that both contained similar contents of nicotine when compared with the reference cigarette (1R5F).[15] Moreover, HTPs emitted approximately 50% less tar and 99% less carbon monoxide (CO) than combustible cigarettes.[15] Kaur et al.[16] also reported the presence of nicotine, tar, carbonyl compounds, and nitrosamines in IQOS aerosol. Another report resulted that HTPs aerosols contain lower levels of harmful constituents compared with combustible tobacco smoke.[17] Therefore, HTPs do not eliminate but only reduce exposure to hazardous constituent of tobacco smoke. Recently, Uchiyama et al.[18] developed an improved method for analyzing gaseous and particulate compounds generated by HTPs. They demonstrated significantly reduced amounts of chemicals found in combustible cigarettes but an abundance of chemicals such as glycerol and propylene glycol in HTPs. Overall, no considerable difference is noted between HTPs and combustible cigarettes in terms of the total gaseous and particulate compounds.[18] Although there are several studies on the chemical analysis of HTPs, little is known about their effects on health.

To date, studies on health effects of HTPs are limited, except for a series of publications of Philip Morris International researchers.[19] In recently published systematic reviews of the health effects of HTPs, it was concluded that the study number is too small to assess the health effects.[20–24] Therefore, gathering research results from epidemiological, in vivo, and in vitro studies is necessary. Especially, we believe that an in vivo study should have high priority, since the systemic effects of acute and/or subacute exposure can be clarified. To quantitatively determine the effects of HTPs, it is important to have an apparatus that properly exposes animals to aerosols. However, there is no standardized HTPs aerosol exposure apparatus for in vivo experiments. In this study, we developed such an apparatus for in vivo experiments and demonstrated that the chemicals in HTPs aerosol generated by the apparatus can be efficiently delivered to mice. Additionally, to examine the pharmacological effect of nicotine, which is taken into the body through IQOS aerosol, the skin surface temperature of mice was monitored.

Materials and Methods

IQOS Aerosol Generation and Exposure Apparatus

To expose mice to IQOS aerosol, we designed and built two types of smoking apparatuses. Both apparatuses were equipped with computer-controlled syringe pumps using an electric actuator (EZS3; Oriental Motor Co, Ltd, Tokyo, Japan). The pumps were able to change the distance and speed at which the syringe plunger could be pushed or pulled using a control software (MEXE02; Oriental Motor Co, Ltd). By using these apparatuses, various puff profiles (eg, those of the International Organization for Standardization and Health Canada Intense)[25] could be applied. Puff volume was measured and calibrated using a soap-bubble flow meter before daily use. Functioning of the modified conventional smoking apparatus (MCSA), based on the physical negative pressure, resembles that of the smoking machines for combustible cigarettes, except that it has a thermostat control. IQOS HeatSticks with a holder was inserted into the inlet of MCSA (Figure 1, Supplemental Figure 1, and Supplemental Movie 1). The flow channel was warmed to 45°C during smoking to prevent aerosol condensation. In contrast, the air push-in inhalation apparatus for HTPs (APIA), based on the physical positive pressure, comprises an air-pushing pump and a custom-made HTPs chamber. Aerosol was expelled from IQOS HeatSticks with the holder connected to the designated position inside the chamber (Figure 1, Supplemental Figure 2, and Supplemental Movies 2 and 3). We used IQOS 3 DUO holder and "Marlboro Regular" HeatSticks for all experiments. In this study, the puff profile in all experiments was established by the Health Canada Intense smoking regimen, defined as a puff volume of 55 mL, puff duration of 2 seconds, puff interval of 30 seconds, and 100% blocking of the ventilation holes in filter with Mylar adhesive tape.[25,26]

IQOS Aerosol Analysis

To analyze IQOS aerosol, aerosol was collected by the Cambridge filter pad set downstream of IQOS HeatSticks.[27] Each sampling was performed with three HeatSticks per filter pad. After sampling, the filter was weighed, and these samples were extracted by 2-propanol. The amounts of nicotine, water, and tar in the extraction were determined using the following methods.[28] Nicotine in the extractions was measured by gas chromatography coupled with a flame ionization detector, and water was measured by gas chromatography coupled with a thermal conductivity detector. Gas chromatography-flame ionization detector and gas chromatography-thermal conductivity detector analysis were performed on the GC-2014 system (Shimadzu Co, Kyoto, Japan) with

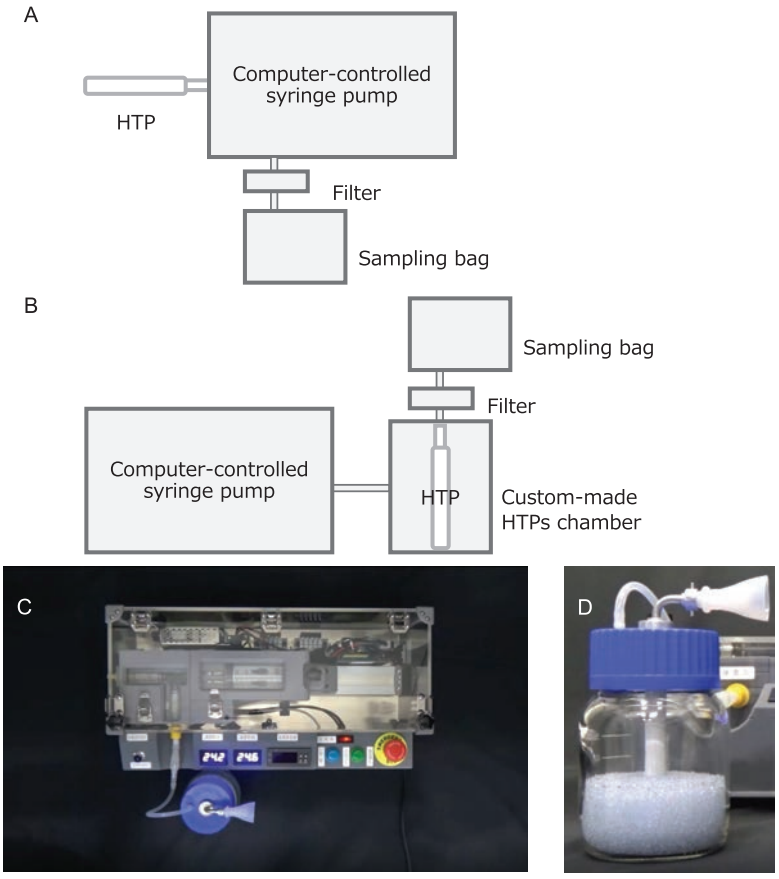

Figure 1. Heated tobacco product (HTP) aerosol exposure apparatus for in vivo experiments. (A and B) Schematic of modified conventional smoking apparatus (MCSA) (A) and air push-in inhalation apparatus for HTPs (APIA) (B). (C) Bird-eye view of APIA. (D) Custom-made HTPs chamber of APIA.

analytical column (HP-INNOWax: 30 m × 0.250 mm × 0.25 μm, Agilent Technologies Inc, Santa Clara, CA). The amount of tar was calculated by subtracting the amounts of nicotine and water from the total particulate matter (TPM). IQOS gas phase was collected in the sampling bag (Figure 1A and B), then the concentration of CO was determined by an off-line analysis using a nondispersive infrared (IR) analyzer (IR200, Yokogawa Electric Co, Tokyo, Japan).

To compare the amounts of chemicals in IQOS aerosol, we used a commercially available vaping machine (LM4E; Borgwaldt, Hamburg, Germany), which we usually used to identify chemicals in the aerosol.

Animals

Male C57BL/6N mice aged 10–12 weeks were purchased from Japan SLC, Inc (Shizuoka, Japan). The mice were kept in individually ventilated cage systems (Super Mouse 1400; Lab Products Inc, Seaford, DE) in a 12-hour light and dark cycle with free access to water and standard chow (FR-2; Funabashi Farm, Chiba, Japan). Room temperature and humidity were kept at 23°C ± 1°C and 50% ± 10%, respectively.

All experimental protocols were approved by the Committee for Animal Experiments at the National Institute of Public Health (protocol number 31-006) and complied with all the guidelines and laws for animal experiments in Japan.

IQOS Aerosol Exposure for Mice

To prepare for the monitoring of skin surface temperature by IR camera, the hair of mice was shaved on the day before exposure. For instance, mice were anesthetized by intramuscular injection of a mixture of ketamine (90 mg/kg body weight) and xylazine (10 mg/kg body weight), and hair around the lower back to buttock area were shaved using animal shaver and hair removal cream.

Urine and skin surface temperature were analyzed using the following procedures. Before exposure of IQOS aerosol, urine was collected, and the temperature was measured as the timepoint of pre-exposure. Each mouse was then placed in a tube-shaped retainer such that their nose was as close as possible to the outlet of APIA. The mouse was then exposed to aerosol from HeatSticks. The retaining time was as short as possible to avoid restraint stress. Skin surface temperature was measured at timepoints of 0, 5, 10, 15, 30, 60, and 120 minutes after exposure. The voluntary urine under conscious condition was sampled at the timepoints of 1, 2, 4, and 6 hours after exposure and stored at −80°C for analysis of nicotine metabolites.

Measurement of Skin Surface Temperature

For measuring skin surface temperature, IR images of mice were captured at an angle from the back using an IR camera (InfReC G100EX, Nippon Avionics Co, Ltd, Tokyo, Japan). To obtain the

reliable temperature, we captured three images at each timepoint. The temperature of the shaved area was calculated from each image, and the average value was obtained from these three images.

Determination of Nicotine Metabolites in Urine

The collected urine sample (10 µL) was mixed with 80 µL 100 mM sodium acetate buffer (pH 4.5), and 30 µL water, 2.6 µL sodium hydroxide, and 1 µL internal standard were added to each sample. The ENVI-Carb columns were conditioned with 2 mL acetonitrile and 2 mL water. Each sample was loaded onto the ENVI-Carb column and washed with 1 mL water and 1.5 mL 20% methanol. Columns were dried for 1 minute; then, each sample was eluted with 1.5 mL acetonitrile. Then, the elution was dried under a stream of nitrogen gas. The extracted residues were redissolved in 100 µL acetonitrile. Liquid chromatography-tandem mass spectrometry analysis was performed on Micromass Quattro LC (Xevo TQ-S, Waters Co, Milford, MA). Samples (5 µL) were injected onto a CORTECS UPLC HILIC column (2.1 mm × 100 mm, 1.6 µm) (Waters Co) at 40°C. The mobile phase, 100 mM ammonium formate/0.03% formic acid (10:90, vol/vol) (A) and acetonitrile (B), was delivered at a flow rate of 0.3 mL/min. The creatinine concentration was quantified using LabAssay Creatinine Kit (FUJIFILM Wako Pure Chemical Co, Osaka, Japan) following the manufacturer's protocol. Concentrations of nicotine metabolites were standardized by creatinine concentration.

Statistical Analysis

The results of skin surface temperatures were expressed as mean ± standard deviation (SD). For comparison of the two groups, statistical difference was determined by unpaired, two-tailed Student's *t* test.

Results

Comparison of Chemicals in IQOS Aerosol Generated by Novel Exposure Apparatus for In Vivo and Analytical Vaping Machine (LM4E)

Before these apparatuses were used for in vivo experiments, the amounts of chemicals in IQOS aerosol were analyzed. As reference, obtained data were compared with those of IQOS aerosol collected by LM4E. Using these apparatuses, we collected IQOS aerosol exhausted from the outlet. Table 1 shows the amounts of chemicals in IQOS aerosol generated by MCSA, APIA, and LM4E. When we compared the amount of chemicals collected by the exposure apparatuses with that of LM4E, the ratios for MCSA were 54.9% (TPM), 46.9% (nicotine), 44.8% (water), 90.7% (tar), and 115.4% (CO). In contrast, the ratios for APIA were 97.2% (TPM), 109.6% (nicotine), 93.3% (water), 108.7% (tar), and 121.4% (CO) (Table 1). In addition, the color of Cambridge filter pads appeared to be different in accordance with the amount of tar (Supplemental Figure 3). These data show that APIA might perform better than MCSA in terms of IQOS inhalation in mice. Therefore, we decided to use APIA for the following in vivo experiments.

Measurements of Nicotine Metabolites in Urine After IQOS Exposure

In this experiment, to demonstrate whether nicotine in IQOS aerosol generated by APIA was effectively delivered to mice, the levels of nicotine metabolites in the urine at designated timepoints after exposure were quantified using liquid chromatography-tandem mass spectrometry. Cotinine levels in the urine, adjusted by creatinine concentration, reached the highest value (6.82 µg/mg creatinine) at 1 hour after exposure and then rapidly decreased to almost half the value (3.32 µg/mg creatinine) at 2 hours (Figure 2A). In contrast, 3-HC, another nicotine metabolite, increased more slowly than cotinine. The highest value (32.9 µg/mg creatinine) of 3-HC was recorded at 2 hours after exposure (Figure 2B). This difference is theoretically reasonable because 3-HC is one of the major metabolites of cotinine.[29] These results suggest that IQOS aerosol generated by APIA was effectively inhaled through the nose and that nicotine in IQOS aerosol was delivered to the rest of the body via the nasal mucosa, trachea, and lungs.[30]

Measurement of Skin Surface Temperature

To clarify the pharmacological action of absorbed nicotine into the blood, the skin surface temperature after IQOS aerosol exposure was monitored using the IR camera (Figure 3A). The skin surface temperature at pre-exposure was 37.6°C ± 0.8°C (mean ± SD; $n = 7$). After exposure, the temperature was gradually decreased and was the lowest (33.5°C ± 0.5°C) at 30 minutes, approximately 4°C decrease compared with that at pre-exposure. Then, the temperature slowly recovered to the original level at 120 minutes (Figure 3B). Although not quantitative, the activity of mice also recovered to the pre-exposure status. During exposure, mice were restrained in the tube-shaped retainer; thus, it may cause restraint stress and affect the body temperature. To deny this possibility, the skin surface temperature of IQOS-exposed mice was compared with that of sham-exposed mice (exposed to only room air exhausted from APIA according to the same protocol with IQOS exposed). The temperature of the IQOS-exposed group showed significant decrease

Table 1. Chemical Analysis of IQOS Aerosol Generated by LM4E, MCSA, and APIA

			Aerosol exposure system	
		LM4E ($n = 5$)	MCSA ($n = 7$)	APIA ($n = 5$)
Parameters	Unit	Mean ± SD	Mean ± SD	Mean ± SD
TPM	(mg/stick)	40.6 ± 0.63	22.2 ± 2.28	39.4 ± 1.31
Nicotine	(mg/stick)	1.46 ± 0.07	0.68 ± 0.11	1.60 ± 0.08
Water	(mg/stick)	30.3 ± 0.98	13.5 ± 1.29	28.2 ± 0.71
Tar	(mg/stick)	8.85 ± 1.13	8.02 ± 1.06	9.62 ± 0.64
CO	(mg/stick)	0.45 ± 0.01	0.52 ± 0.01	0.55 ± 0.04

APIA = air push-in inhalation apparatus for HTPs, CO = carbon monoxide, MCSA = modified conventional smoking apparatus, and TPM = total particulate matter. LM4E is a vaping machine (Borgwaldt).

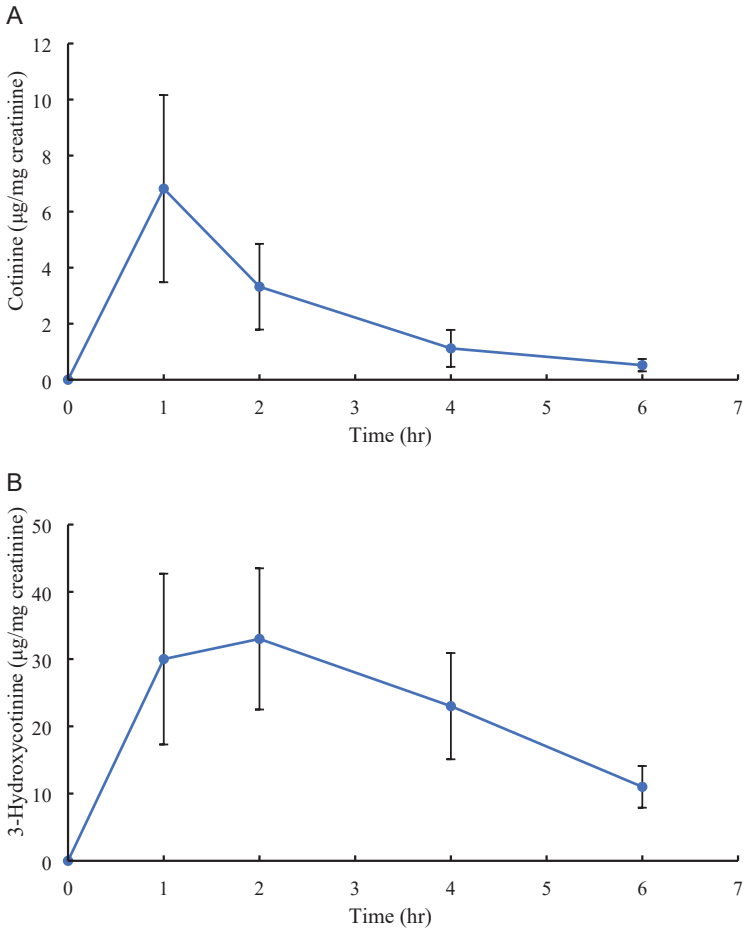

Figure 2. Time course of urinary levels of nicotine metabolite after IQOS exposure, adjusted by creatinine concentration. Each mouse was exposed to one IQOS HeatSticks through the air push-in inhalation apparatus for HTPs (APIA). (A) Cotinine levels (mean ± SD, $n = 7$) and (B) 3-HC levels (mean ± SD, $n = 7$).

immediately after exposure and 5–60 minutes (Figure 3B) compared to that in the sham-exposed group.

Discussion

In this study, we developed an apparatus to expose mice to HTPs aerosol, which has not been reported until now, and that resolves the defects of loss of chemicals because of aerosol condensation. To study the biological and health effects of HTPs in mice, this apparatus can replicate actual human smoking process, so it is beneficial in terms of understanding the effects of HTPs aerosol.

Regarding health effects, currently, there is no evidence demonstrating that HTPs, including IQOS, are less harmful than combustible cigarettes.[14,17] It is noteworthy that HTPs contain chemicals that are not found in conventional cigarette smoke, which may have associated health effects; however, such effects have not been studied in in vivo and in vitro experiments.[17,18] In addition, there is insufficient evidence to support the claim that HTPs are less harmful than conventional cigarettes because HTPs emit fine particles (<2 μm) that can easily access the lung and potentially damage the lung tissue.[31] Therefore, to determine the health effects of HTPs as an alternative smoking method, further investigations are needed.

The objective of our research project is to clarify the biological effects of HTPs aerosol exposure on mice; however, there were technical difficulties because HTPs aerosol has much more moisture than combustible cigarette smoke. Concretely speaking, the major problem is that aerosol condensation occurs in the channel of the syringe-based smoking apparatus, which induces losses of chemicals, including nicotine.

Actually, in our preliminary experiments prior to this study, we found that nicotine generated by IQOS HeatSticks was detected in water formed by dew condensation inside the channel of the syringe-based smoking apparatus used in combustible cigarettes. In the past, when animals were exposed to smoke from combustible cigarettes, researchers did not need to pay attention to condensation because the smoke did not have enough humidity to result in condensation, even when the generated smoke cooled down. Based on the findings of our study, we believe that it is very important to prevent condensation in the case of HTPs. Therefore, we realized that the exposure apparatus, which prevents water condensation, should have high priority to achieve our goals. In this study, we developed two exposure apparatuses, MCSA and APIA, which can be applied to mice and any other small animal. First, chemicals recovered from the apparatuses were quantified and compared with the data obtained with

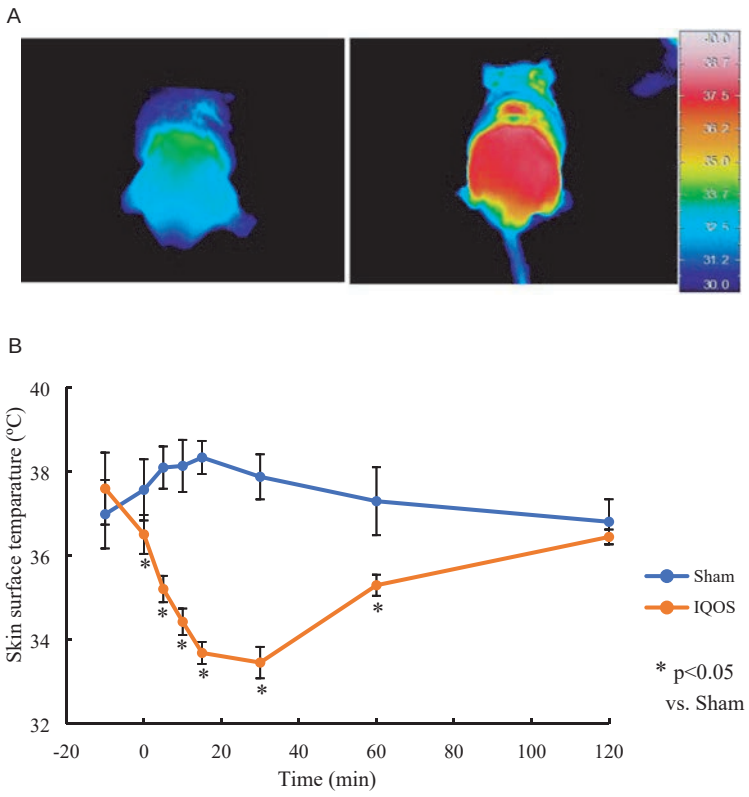

Figure 3. Time-dependent change in skin surface temperature after IQOS aerosol exposure. Each mouse was exposed to one IQOS HeatSticks through the air push-in inhalation apparatus for HTPs (APIA). (A) A typical infrared image of the mouse skin. These images were at timepoint of 30 minutes after IQOS aerosol (left) and sham exposure (right). (B) Skin surface temperature measured by infrared camera at pre-exposure and 0–120 minutes after exposure of IQOS aerosol and sham exposure (IQOS, $n = 7$; sham, $n = 5$).

LM4E. The results showed that the amounts of chemicals recovered in MCSA were approximately half of those in LM4E supposedly because of water condensation or direct absorption of chemicals in the channel. Although we did not analyze the nicotine that remained in the channel, it is reasonable if we consider that the chemical characteristics of nicotine allow it to dissolve easily with water. If MCSA recovers half of all the components constantly, the experiment might be possible by giving the mice twice as much to smoke. However, the amount of each recovered component differs; for instance, in case of CO and tar, the amounts recovered by MCSA were nearly the same as that recovered by LM4E (Table 1). Using MCSA for the in vivo experiments makes the interpretation of results difficult. Therefore, we assumed that MCSA has difficulty in replicating actual human exposure and attempted to develop another type of apparatus named APIA to prevent condensation. The notable point of APIA is that the HTP stick inserted into its holder penetrates the hole of the custom-made HTPs chamber. When a particular amount of air is pushed into the chamber, an equal amount of HTPs aerosol is expelled from the outlet of the chamber. In addition to this, the channel was kept as short as possible to avoid condensation. This novel apparatus enabled us to collect particles and gas phase in almost the same amounts as that collected using LM4E. On the basis of this result, we have concluded that APIA was able to effectively expose mice to HTPs aerosol. By connecting a custom-made face mask to the outlet of the chamber, mice can be directly exposed to HTPs aerosol. To the best of our knowledge, such an apparatus has never been reported previously, and this is the first study on HTPs aerosol exposure apparatus for in vivo experiments.

In this study, mice were exposed to IQOS aerosol with the Health Canada Intense smoking regimen via APIA. To determine if mice were effectively exposed, the urinary levels of nicotine metabolites were analyzed after IQOS exposure. As a result, both cotinine and 3-HC were quantitatively detected in the collected urine on a time-dependent manner after exposure. The levels of cotinine and 3-HC in the urine (creatinine correction value) showed at maximum of 1 and 2 hours after exposure, respectively. This result indicates that nicotine in HTPs aerosol entered the blood circulation via the respiratory system and that the metabolism of nicotine in mice is faster than that in humans. The analyzed data of nicotine metabolites in the collected urine showed a large variation at 1 and 2 hours after exposure, which was considered to be because of the urination frequency of mice.

It is hypothesized that IQOS aerosol exposure induces adverse health effects via circulatory system.[27] Because nicotine absorbed in the blood causes a pharmacological action in the form of vasoconstriction, which results in an impaired peripheral blood flow, oxidative stress is generated in excess. In this study, we observed a decline in the body surface temperature immediately after IQOS aerosol exposure, reaching the lowest level at 30 minutes after exposure. This suggests that the vasoconstrictor effect of nicotine is maintained for

a relatively long time along with the possibility to trigger any other effects.

The design concept of MCSA in this study is similar to that of a combustible cigarette smoking machine. However, it was revealed that there is a possibility of underestimation against the health effects of HTPs aerosol if we use MCSA for in vivo experiments. In contrast, APIA in this study allows mice to be effectively exposed to HTP aerosol. It is noteworthy that APIA can deliver both particles and gas phase with high recovery. Therefore, APIA is strongly recommended as a standard HTPs exposure apparatus for in vivo experiments.

In conclusion, we have developed an experimental apparatus and established a suitable procedure for HTPs aerosol exposure in mice without loss of chemicals in HTPs aerosol. The results of urine analysis and body surface temperature measurements after exposure showed that the animals were fully exposed to IQOS aerosol. Considering these observations, we conclude that APIA can be used for in vivo experiments involving HTPs aerosol exposure.

Supplementary Material

A Contributorship Form detailing each author's specific involvement with this content, as well as any supplementary data, are available online at https://academic.oup.com/ntr.

Funding

This work was supported by JSPS KAKENHI grant number JP19H04141 to AU and partially supported by the Health and Labour Science Research grants from the Ministry of Health, Labour and Welfare of the Japanese Government to YI.

Declaration of Interests

None declared.

Acknowledgments

The authors would like to thank Enago (www.enago.jp) for the English language review. The authors would also like to thank Mrs Masako Oosawa, Mrs Kasumi Yamanaka, Mrs Kumi Tanaka, and Mrs Yasuko Hara for their assistance in the experiments and Dr Kazuhiko Nakadate and Dr Akane Shinohara for their expert advice.

References

1. *How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General*. Publications and Reports of the Surgeon General; 2010.
2. Hecht SS. Research opportunities related to establishing standards for tobacco products under the Family Smoking Prevention and Tobacco Control Act. *Nicotine Tob Res*. 2012;14(1):18–28.
3. Hoffmann D, Hoffmann I, El-Bayoumy K. The less harmful cigarette: a controversial issue. A tribute to Ernst L. Wynder. *Chem Res Toxicol*. 2001;14(7):767–790.
4. IARC. Tobacco smoke and involuntary smoking. IARC Monograph: Evaluation of Carcinogenic Risks to Humans. 2004;83:1–1438.
5. IARC. Some non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. IARC Monograph: Evaluation of Carcinogenic Risks to Human. 2010;92:1–853.
6. Inoue M, Sawada N, Matsuda T, et al. Attributable causes of cancer in Japan in 2005—systematic assessment to estimate current burden of cancer attributable to known preventable risk factors in Japan. *Ann Oncol*. 2012;23(5):1362–1369.
7. Park S, Jee SH, Shin HR, et al. Attributable fraction of tobacco smoking on cancer using population-based nationwide cancer incidence and mortality data in Korea. *BMC Cancer*. 2014;14:406.
8. Forey BA, Thornton AJ, Lee PN. Systematic review with meta-analysis of the epidemiological evidence relating smoking to COPD, chronic bronchitis and emphysema. *BMC Pulm Med*. 2011;11:36.
9. Fowkes FG, Rudan D, Rudan I, et al. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. *Lancet*. 2013;382(9901):1329–1340.
10. Farsalinos KE, Yannovits N, Sarri T, Voudris V, Poulas K. Nicotine delivery to the aerosol of a heat-not-burn tobacco product: comparison with a tobacco cigarette and e-cigarettes. *Nicotine Tob Res*. 2018;20(8):1004–1009.
11. Tabuchi T, Gallus S, Shinozaki T, Nakaya T, Kunugita N, Colwell B. Heat-not-burn tobacco product use in Japan: its prevalence, predictors and perceived symptoms from exposure to secondhand heat-not-burn tobacco aerosol. *Tob Control*. 2018;27(e1):e25–e33.
12. *In how many countries do you sell your cigarette brands?* 2020. https://www.pmi.com/faq-section/faq/in-how-many-countries-do-you-sell-your-cigarette-brands. Accessed August 13, 2020.
13. Hori A, Tabuchi T, Kunugita N. Rapid increase in heated tobacco product (HTP) use from 2015 to 2019: from the Japan 'Society and New Tobacco' Internet Survey (JASTIS). *Tob Control*. 2021;30(4):474–475.
14. World Health Organization (WHO). *Heated Tobacco Products: Information Sheet*. 2nd ed. 2020. https://www.who.int/publications/i/item/WHO-HEP-HPR-2020.2. Accessed August 13, 2020.
15. Bekki K, Inaba Y, Uchiyama S, Kunugita N. Comparison of chemicals in mainstream smoke in heat-not-burn tobacco and combustion cigarettes. *J UOEH*. 2017;39(3):201–207.
16. Kaur G, Muthumalage T, Rahman I. Mechanisms of toxicity and biomarkers of flavoring and flavor enhancing chemicals in emerging tobacco and non-tobacco products. *Toxicol Lett*. 2018;288:143–155.
17. St Helen G, Jacob Iii P, Nardone N, Benowitz NL. IQOS: examination of Philip Morris International's claim of reduced exposure. *Tob Control*. 2018;27(suppl 1):s30–s36.
18. Uchiyama S, Noguchi M, Takagi N, et al. Simple determination of gaseous and particulate compounds generated from heated tobacco products. *Chem Res Toxicol*. 2018;31(7):585–593.
19. Smith MR, Clark B, Lüdicke F, et al. Evaluation of the tobacco heating system 2.2. Part 1: description of the system and the scientific assessment program. *Regul Toxicol Pharmacol*. 2016;81(suppl 2):S17–S26.
20. Ratajczak A, Jankowski P, Strus P, Feleszko W. Heat not burn tobacco product—a new global trend: impact of heat-not-burn tobacco products on public health, a systematic review. *Int J Environ Res Public Health*. 2020;17(2):409.
21. Drovandi A, Salem S, Barker D, Booth D, Kairuz T. Human biomarker exposure from cigarettes versus novel heat-not-burn devices: a systematic review and meta-analysis. *Nicotine Tob Res*. 2020;22(7):1077–1085.
22. Kopa PN, Pawliczak R. IQOS—a heat-not-burn (HnB) tobacco product—chemical composition and possible impact on oxidative stress and inflammatory response. A systematic review. *Toxicol Mech Methods*. 2020;30(2):81–87.
23. Başaran R, Güven NM, Eke BC. An overview of iQOS® as a new heat-not-burn tobacco product and its potential effects on human health and the environment. *Turk J Pharm Sci*. 2019;16(3):371–374.
24. Simonavicius E, McNeill A, Shahab L, Brose LS. Heat-not-burn tobacco products: a systematic literature review. *Tob Control*. 2019;28(5):582–594.
25. Health Canada. *Official Method T-115, Documentation of "Tar", Nicotine and Carbon Monooxide in Mainstream Tobacco Smoke*. 1999:1–6.
26. *WHO TobLabNet SOP 1—Standard Operating Procedure for Intense Smoking of Cigarettes*. Geneva, Switzerland: World Health Organization; 2012.
27. Nabavizadeh P, Liu J, Havel CM, et al. Vascular endothelial function is impaired by aerosol from a single IQOS HeatStick to the same extent as by cigarette smoke. *Tob Control*. 2018;27(suppl 1):s13–s19.

28. *WHO TobLabNet SOP 10—Standard Operating Procedure for Determination of Nicotine and Carbon Monoxide in Mainstream Cigarette Smoke Under Intense Smoking Conditions*. Geneva, Switzerland: World Health Organization; 2016.
29. Benowitz NL, Jacob P 3rd, Fong I, Gupta S. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. *J Pharmacol Exp Ther.* 1994;268(1):296–303.
30. Martin EM, Clapp PW, Rebuli ME, et al. E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke. *Am J Physiol Lung Cell Mol Physiol.* 2016;311(1):L135–L144.
31. U.S. EPA. *Integrated Science Assessment (ISA) for Particulate Matter (Final Report, Dec 2019)*. Washington, DC: U.S. Environmental Protection Agency; 2019.