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A Comparative *In Vitro* Toxicological Screening of a Closed-End Heated Tobacco Product *

by

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SUMMARY

Heated tobacco products (HTPs) are a recent category of tobacco products, with their relative safety compared to cigarette smoking and potential to help smokers to quit being two reasons why regulators may consider their market approval. Designed to heat tobacco rather than to burn in order to produce aerosol, different heating techniques are applied to commercial HTPs, which may result in differing aerosol formation. Therefore, each product requires separate assessment. This work focuses on a closed-end HTP (coded as HTP-A), which is electrically heated and designed to allow puffing air flow to bypass its tobacco section, resulting in reduced oxygen concentration within the tobacco section during heating and aerosol forming. To provide a preliminary aerosol chemistry and *in vitro* toxicological screening, this study assessed HTP-A against a commercial electrically heated HTP (IQOS™, coded as HTP-B) and a 3R4F reference cigarette. Under Health Canada Intense (HCI) smoking regime, the levels of 9 regulatory priority toxicants in the aerosol of HTP-A were either reduced or comparable to those in HTP-B on a per-stick basis. Additionally, both HTPs showed significant reduction (greater than 90%) in comparison to those measured in mainstream smoke of 3R4F cigarette for these toxicants. Using a set of standard *in vitro* toxicological assays (Ames, Micronucleus and Neutral Red Uptake), the two HTPs showed no observable responses while significant toxicity responses were recorded for 3R4F's total particulate matter. Based on these preliminary results, the novel closed-end HTP-A design may provide similar toxicological profiles to the comparator HTP-B. Further toxicological and clinical assessments are warranted to evaluate HTP-A's potential for exposure or disease risk reduction. [Contrib. Tob. Nicotine Res. 32 (2023) 146–156]

KEYWORDS

Heated tobacco product; aerosol; chemistry; *in vitro*; toxicological assessment;

ZUSAMMENFASSUNG

Erhitzte Tabakerzeugnisse (HTP) sind eine neue Kategorie von Tabakptrodukten. Ihre relative Sicherheit im Vergleich zum Zigarettenrauchen und ihr Potenzial, Rauchern bei der Raucherentwöhnung zu helfen, sind zwei Gründe, warum die Regulierungsbehörden ihre Marktzulassung in Betracht ziehen könnten. Sie sind so konzipiert sind, dass der Tabak erhitzt und nicht verbrannt wird, um ein Aerosol zu erzeugen. Es werden bei handelsüblichen HTPs unterschiedliche Erhitzungsverfahren angewandt, die zu einer unterschied-

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licher Aerosolbildung führen können. Daher muss jedes Produkt separat bewertet werden. Diese Arbeit konzentriert sich auf ein HTP mit geschlossenem Ende (HTP-A), welches elektrisch beheizt wird und so konzipiert ist, dass der Luftstrom beim Zugvorgang den Tabakteil umströmt, was zu einer geringeren Sauerstoffkonzentration im Tabakteil während der Erhitzung und Aerosolbildung führt. Um eine vorläufige Aerosolchemie und ein toxikologisches *in vitro*-Screening zu erhalten, wurde in dieser Studie die HTP-A mit einer kommerziellen elektrisch beheizten HTP (IQOS™, codiert als HTP-B) und einer 3R4F-Refererenzzigarette verglichen. Unter dem Health Canada Intense (HCI)-Rauchregime waren die Konzentrationen von 9 regulatorisch priorisierten Schadstoffen im Aerosol von HTP-A entweder reduziert oder vergleichbar mit denen von HTP-B auf einer Pro-Stick-Basis. Darüber hinaus zeigten beide HTPs eine signifikante Verringerung (mehr als 90%) im Vergleich zu den im Hauptstromrauch der 3R4F-Zigarette gemessenen Werten für diese Schadstoffe. Unter Verwendung einer Reihe von toxikologischen *in vitro*-Standardtests (Ames, Micronucleus und Neutralrot) zeigten die beiden HTPs keine beobachtbaren Reaktionen, während für den Gesamtpartikelgehalt von 3R4F signifikante Toxizitätsreaktionen festgestellt wurden. Diese vorläufigen Ergebnisse deuten darauf hin, dass das neuartige geschlossene HTP-A-Design möglicherweise ähnliche toxikologische Profile aufweist, wie das Vergleichsprodukt HTP-B. Weitere toxikologische und klinische Bewertungen sind erforderlich, um das Potenzial von HTP-A zur Verringerung der Exposition oder des Krankheitsrisikos zu bewerten. [Contrib. Tob. Nicotine Res. 32 (2023) 146–156]

RESUME

Les produits du tabac chauffés (PTT) constituent une catégorie récente de produits du tabac, leur sécurité relative par rapport à la cigarette et leur capacité à aider les fumeurs à arrêter de fumer étant deux raisons pour lesquelles les autorités réglementaires pourraient envisager leur autorisation de mise sur le marché. Bien qu'ils soient conçus pour chauffer le tabac plutôt que pour le brûler afin de produire des aérosols, différentes techniques de chauffage sont appliquées aux produits du tabac à usage domestique, ce qui peut entraîner des différences dans la formation d'aérosols. Par conséquent, chaque produit doit faire l'objet d'une évaluation distincte. Ce travail se concentre sur une HTP à bout fermé (codée HTP-A), qui est chauffée électriquement et conçue pour permettre au flux d'air de bouffée de contourner la section du tabac, ce qui réduit la concentration d'oxygène dans la section du tabac pendant le chauffage et la formation de l'aérosol. Afin d'obtenir une analyse préliminaire de la chimie des aérosols et un dépistage toxicologique in vitro, cette étude a évalué le HTP-A par rapport à un HTP commercial chauffé électriquement (IQOSTM, codé HTP-B) et à une cigarette 3R4F. Dans le cadre du régime de tabagisme intensif de Santé Canada, les concentrations de neuf substances toxiques prioritaires dans l'aérosol du HTP-A étaient soit réduites, soit comparables à celles du HTP-B, par bâtonnet. En outre, les deux HTP ont montré une réduction significative (supérieure à 90%) par rapport aux niveaux mesurés dans la fumée principale de la cigarette 3R4F pour ces substances toxiques. En utilisant une série de tests toxicologiques in vitro standard (Ames, micronucléus et rouge neutre), les deux HTP n'ont pas montré de réactions observables, tandis que des réactions de toxicité significatives ont été observées pour la teneur totale en particules de 3R4F. Ces résultats préliminaires indiquent que la nouvelle conception fermée du HTP-A pourrait présenter des profils toxicologiques similaires à ceux du HTP-B de comparaison. Des évaluations toxicologiques et cliniques supplémentaires sont nécessaires pour évaluer le potentiel du HTP-A à réduire l'exposition ou le risque de maladie. [Contrib. Tob. Nicotine Res. 32 (2023) 146–156]

1. INTRODUCTION

Smoking is one of the main preventable causes of respiratory and other chronical diseases, including COPD and lung cancers (1). The best cause of action for smokers is to quit. However, despite significant public health efforts and the availability of pharmaceutical nicotine replacement therapy products (NRTs), many smokers remain unwilling or unable to give up smoking. As a harm reduction intervention, validated low-risk tobacco products have been made available to current smokers. In the U.S., under the auspice of the FDA's comprehensive tobacco regulation program, modified risk tobacco products (MRTPs) are an authorized category of alternatives that can be marketed to adult smokers (2).

Heated tobacco products (HTPs) are among the classes of novel tobacco products that are available in some markets. They are designed to heat tobacco through a battery-powered heating system to generate nicotine-containing aerosol for inhalation (3, 4). It has been shown that most of the toxicants found in cigarette smoke are formed as a result of incomplete tobacco combustion (5). In a typical HTP device, its heating temperature program and the aerosol formation can be accurately controlled, resulting in the elimination of tobacco combustion and therefore the aerosol formed is found to contain significantly less toxicants than cigarette smoke (6, 7). Most commercial HTPs heat the tobacco below 350 °C using a varied but controlled temperature profile. This contrasts to a burning cigarette which typically runs between 600 and 900 °C (5). However, commercial HTPs come with different heating technologies such as e.g., IQOS™ and glo™ (3, 4), where the heating energy is either transmitted from inside a tobacco rod outwardly or inwardly. More innovations are coming onto the market in HTP designs, some using resistive while others using radiative/non-contact means for heat transfer. This may lead to different degrees of tobacco heating at different locations, and therefore some differences in the chemicals formed in the aerosol. Hence it is required by regulators that each HTP should be chemically and biologically assessed under rigorous preclinical and clinical programs before it is considered for market assess and potentially reduced risk claims.

In commercial HTPs, aerosol is formed by heating reconstituted tobacco material containing aerosol-forming agents such as vegetable glycerol and propylene glycol (3). During heating, the volatile compounds and the aerosol-

forming agents are vaporized from the tobacco substrate and reach supersaturation; upon cooling by the puffing air flow condense to form the aerosol. In most HTP designs, puffing airflow enters from the open end of a tobacco rod and exits through its mouth end - for comparison purpose, this design is called an open-end HTP. In this work, a novel HTP design that prevents the air flow from entering the tobacco end is described - this is called a closed-end HTP. The key design features and its working principle have been described before (8, 9), more details will be given in the experimental section.

In this work, we aimed to compare the aerosol chemistry, first using a limited number of toxicants measured on this close-ended HTP and a commercial HTP (IQOS™). Afterwards biological properties of the aerosol produced were assessed using the standardized regulatory *in vitro* assays (Ames, Micronucleus and Neutral Red Uptake). The results were discussed and presented as the first step towards building a comprehensive preclinical dataset for this novel HTP before any clinical evaluation.

2. MATERIALS AND METHODS

2.1 The heating devices and tobacco sticks

Three products were tested, two commercially available HTPs and one combustible research reference cigarette 3R4F. The two HTPs were commercially available: Mr. Yeah (the closed-end test product, coded as HTP-A) and IQOS™ (the open-end comparator, coded as HTP-B). Each HTP came with its own heating device and dedicated tobacco sticks. The key features of the heating device and tobacco sticks are listed in Table 1, and further details about the products can be found in the literature (9, 10). The tobacco sticks used in HTP-A had ca. 350 mg of reconstituted Chinese flue-cured blended tobacco, 1.81 mg of nicotine, 49.5 mg of glycerol, and 12.2 mg of propylene glycol.

Prior to the smoking experiments, the tobacco sticks were removed from their packages and conditioned at 22 °C ± 2 °C and a relative humidity of 60% ± 3% in accordance with ISO 3402 (28). The devices were fully charged and cleaned between each smoking session according to their operating instructions.

Table 1. Overview of test product specifications. The heating device of HTP-A is branded as Mr. Yeah and the tobacco sticks carry the brand FARSTAR. HTP-B consists of an IQOS™ branded heating device and TEREA branded tobacco sticks.

Parameter	HTP-A	HTP-B	Combustible cigarette
Commercial product (name)	Mr. Yeah	IQOS™	3R4F
Puffing air flow design	Closed-end	Open-end	Open
Heater design	External heating	Central heating	Combustion
Maximum heating temp. (°C)	260	350	/
Puff n° under HCI regime	8	12	8
Tobacco stick-length (mm)	45	45	84
Tobacco stick-weight (mg)	721.70	664.30	1035.00
Tobacco stick-diameter (mm)	7.36	7.18	7.98

The air flow design of HTP-A and HTP-B is schematically shown in Figure 1 (8, 9). In short, the HTP-A airflow pathway (Figure 1A) was from the perforation on the hollow acetate tube because the tobacco end of the heating device was completely sealed. The HTP-B airflow pathway (Figure 1B) was from the tobacco end (8, 9). The working mechanism behind the aerosol formation and transfer for the close-end HTP-A has been described before (8, 9). In brief: The extraction of formed aerosol in HTP-A is facilitated by a negative pressure created by the puffing air through the airflow pathway relative to the tobacco stick.

2.2 Aerosol collection and chemical analysis

The mainstream aerosol/smoke was generated on a Cerulean smoking machine (Cerulean, Milton Keynes, UK) and captured by Cambridge glass-fiber filter pads (Whatman, Maidstone, UK) or cooled impingers with an appropriate solvent for volatile fractions. After the heating devices were preheated according to their respective operating instructions, 3 replicated products were smoked under the Health Canada Intense (HCI) (29) smoking regime, which yielded 8 puffs/stick for HTP-A and 3R4F cigarette, 12 puffs for HTP-B respectively. For screening purposes, we selected 9 toxicants - recommended by the WHO Study Group on Tobacco Product Regulation (TobReg) for mandated lowering - for HTP aerosol and cigarette smoke comparison: carbon monoxide, formaldehyde, acetaldehyde, 1,3-butadiene, benzene, acrolein, B[*a*]P, NNN and NNK (11). Nicotine in the aerosol/smoke was also analyzed. The procedures for chemical analysis of the captured aerosol are listed in Table 2.

2.3 Regulatory in vitro: the Ames assay

The Ames assay was performed in accordance with the Health Canada Official Method T-501 (18). Mutagenicity of collected total particulate matter (TPM) fraction was evaluated using two bacterial strains: *Salmonella typhimurium* TA98 and TA100, both purchased from Molecular Toxicology, Inc. (Boone, NC, USA) with and without metabolic activation. For positive controls, 2-nitrofluorene (Shanghai Macklin Biochemical Co., Ltd., China) was used for TA98, and 4-nitroquinoline *N*-oxide (Shanghai Macklin Biochemical Co., Ltd., China) for TA100 in the metabolic activation experiments (Table 3). After incubation, the plate was incubated at 37 °C ± 1 °C for 48–72 h, and afterwards the number of revertants per plate was counted. Two independent experiments were conducted with the test samples prepared independently.

2.4 Regulatory in vitro: micronucleus (MN) assay

The MN assay was performed in accordance with Health Canada Official Method T-503 (19). The Chinese Hamster Ovarian (CHO) cell line was purchased from BNCC (Beijing, China) and were maintained in Dulbecco's modified eagle medium (Sigma Aldrich, MO, USA) supplemented with 10% fetal bovine serum (Thermo Fisher, Waltham, MA, USA) in a 5% CO_2 incubator at 37 °C ± 2 °C. Cell suspension (1×10^5 cells/mL) was pre-incubated for 24 h before treatment.

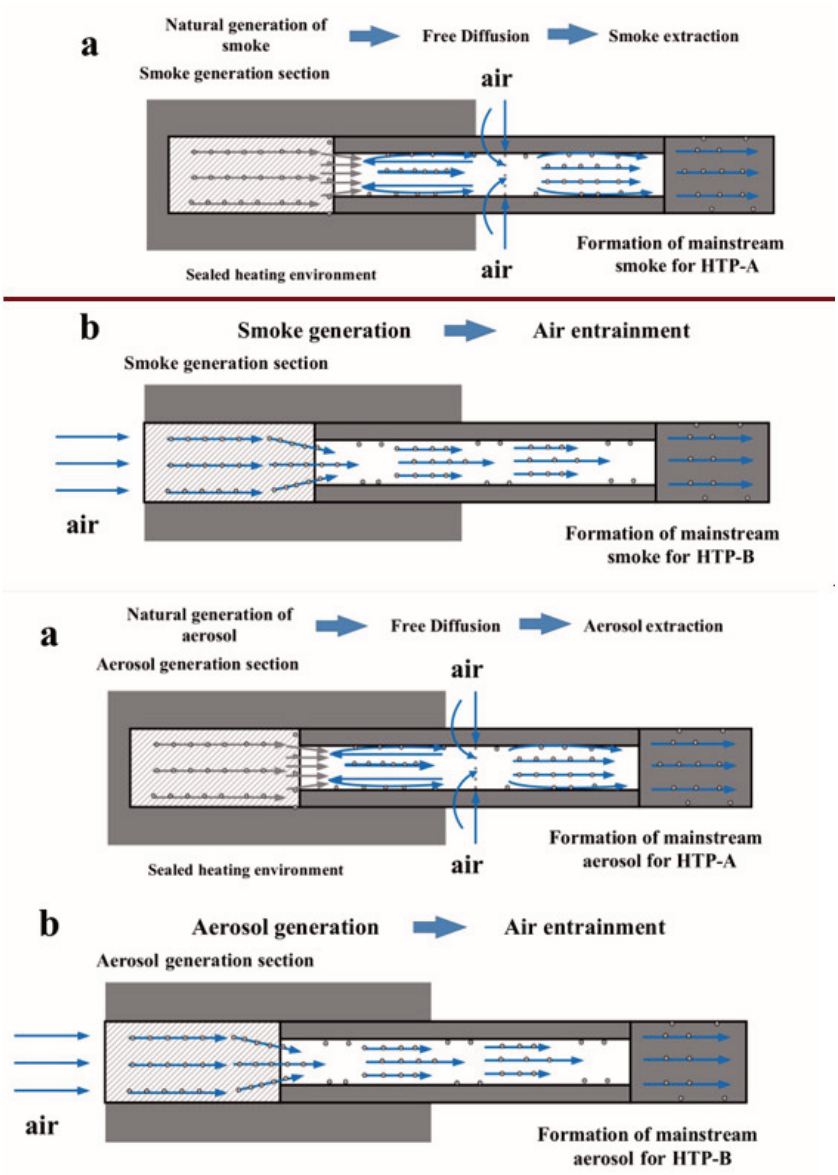

Figure 1. Schematic diagram comparing air flow path for the closed-end HTP-A (a: the test product) and an open-end HTB-B (b: see Ref. 8, 9)

Table 2. Analytical procedures used for HTP aerosol and cigarette smoke chemical analyses.

Analyte	Brief description	Reference
Nicotine	By gas chromatography with flame ionization detection	ISO 10315 (12)
CO	By nondispersive infrared photometry	ISO 8454 (13)
Polycyclic aromatic hydrocarbons	Extracted by hexane and analyzed by GC-MS	CRM 91 (14)
N-nitrosamines	Extracted by acetic acid and analyzed by HPLC-MS/MS	CRM 75 (15)
Volatile organic compounds	By GC-MS using simultaneous trapping of adsorbent cartridges in a Cambridge filter without cryogenic impinger	WHO SOP 09 (16)
Carbonyl compounds	By HPLC, using simultaneous trapping of adsorbent cartridges in a Cambridge filter without cryogenic impinge	WHO SOP 08 (17)

Table 3. *Salmonella typhimurium* strains characteristics, source and positive controls.

Strains	Mutation	Antibiotic resistance	Positive controls	
			Without S9 (μg/plate)	With S9 (μg/plate)
TA98	His D3052	Ampicillin	2-Nitrofluorene (4 μg/plate)	2-Amlinofluorene (10 μg/plate)
TA100	His G46	Ampicillin	4-Nitroquinoline *N*-oxide (0.5 μg/plate)	2-Amlinofluorene (10 μg/plate)

For positive controls, cyclophosphamide A (CPA) (Dalian Meilun Biotechnology Co., Ltd., Dalian, Liaoning Province, China) for ST (short-term)+S9, mitomycin C (MMC) (GlpBio, Montclair, CA, USA) for ST-S9 and mitomycin C for LT (long-term)-S9 were used in the experiments (Table 4). For short-term exposures, the cell culture was treated with serially diluted test samples for 3 h without or with S9-mix containing Aroclor 1254-induced rat liver homogenate (Molecular Toxicology, Inc., Boone, NC, USA). After removal of the test sample, the cells were incubated for 27 h. For LT exposures, the cells were incubated with test sample for 30 h in the absence of the metabolic activation system.

Table 4. *In vitro* mammalian cell micronucleus test.

Positive controls	Ultimate density
Cyclophosphamide A (short-term + S9)	0.2 μg/mL
Mitomycin C (short-term – S9)	1.0 μg/mL
Mitomycin C (long-term – S9)	0.5 μg/mL

2.5 Regulatory in vitro: neutral red uptake (NRU) assay

The NRU assay was performed using CHO cell line in accordance with Health Canada Official Method T-502 (20). The CHO cell suspension (5×10^4 cells/mL) was pre-cultured for 24 h in 96-well microtiter plates. The cells were treated with TPM fractions for 24 h. Sodium lauryl sulfate was used as the positive control. Dimethyl sulfoxide (DMSO) alone was used as solvent control. The treated cells were washed with PBS, and then incubated with a medium containing 50 μg/mL neutral red dye for 3 h. The cells were then fixed with 1% formalin solution for 1–2 min. After removal of the fixative, the neutral red dye taken up by the viable cells was extracted by adding 50% ethanol containing 1% (*v/v*) acetic acid, and the absorbance at 540 nm was measured using a microplate reader. The experiments were conducted in triplicates.

2.6 Data treatment

For aerosol and smoke chemistry, each product was analyzed in triplicates. The findings were reported as mean ± standard deviation (SD). For analytes that were systematically below the level of quantification (LOQ), not quantified (NQ) was assigned. For the biological testing data, SPSS 24.0 software (IBM, New York, NY, USA) was used for statistical analysis. All data were analyzed by ANOVA, $P < 0.01$ were considered as extremely significant.

3. RESULTS

3.1 Toxicant levels in HTP aerosol and 3R4F smoke

The TPM results and selected toxicant levels are shown in Table 5, on both per-stick and per-mg of nicotine basis. The TPM levels of HTP-A and HTP-B were slightly higher than those of 3R4F cigarettes when compared on a per-stick basis. The CO yields for both HTPs were below LOD whereas those from 3R4F were 24.69 mg/stick - the reduction of CO for the HTPs was a clear confirmation that the tobacco in each system did not experience a significant thermal breakdown or combustion. Formaldehyde, acetaldehyde and acrolein were detected in HTP-A with quantifiable levels, with HTP-A's formaldehyde yields at 76%, acetaldehyde at 28%, and acrolein at 56% lower than those of HTP-B, respectively. These levels were also 96% (formaldehyde), 83% (acetaldehyde) and 93% (acrolein) lower than those in 3R4F cigarette smoke, respectively. In addition, 1,3-butadiene was below LOQ for both HTPs whereas in 3R4F it was at 8.13 μg/stick. The yields of benzene and benzo(*a*)-pyrene in the two HTPs were significantly lower than those of 3R4F cigarette smoke. The yields of NNK and NNN were 98% and 99% lower than those of 3R4F cigarette.

3.2 Ames assay responses

To assess potential mutagenicity effect, the TPM fractions of aerosols were tested in the Ames assay. The assay was performed using two tester strains, TA98 and TA100, in either presence or absence of exogenous metabolic activation system. The results are shown in Tables 6 and 7. In the presence of metabolic activation, the 3R4F TPM gave reproducible positive responses up to 500 μg TPM per plate (Table 6). In contrast, the TPM fraction of the two HTPs aerosol did not induce significant increase in the number of revertants up to 2000 μg TPM/plate under any of the assay conditions (Table 7).

3.3 MN assay responses

To assess the genotoxicity of the TPM fractions for the HTP aerosol, the MN assay was performed under three conditions, a 3-h treatment with and without metabolic activation and a 24-h treatment without metabolic activation. The results are shown in Table 8. The 3R4F TPM displayed clear increases in MN frequency under all three conditions. For the TPM fractions of the two HTP aerosols, there were no statistically significant increases in MN frequency up to 600 μg TPM/mL treatment.

Table 5. Selected toxicant yields from two HTPs and 3R4F.

Analyte	Unit	HTP-A	HTP-B	3R4F
Nicotine	mg/stick	0.47 ± 0.01	0.98 ± 0.05	1.05 ± 0.02
Total particulate matter	mg/stick	29.06 ± 1.36	30.15 ± 1.28	24.23 ± 1.64
	mg/mg nicotine	61.83 ± 2.90	30.77 ± 1.30	23.08 ± 1.56
Carbon monoxide	mg/stick	0.19 ± 0.01	0.25 ± 0.01	24.69 ± 0.01
	mg/mg nicotine	0.40 ± 0.02	0.26 ± 0.01	23.52 ± 0.01
Formaldehyde	µg/stick	1.08 ± 0.18	4.46 ± 0.56	26.60 ± 2.14
	µg/mg nicotine	2.30 ± 0.38	4.55 ± 0.57	25.33 ± 2.04
Acetaldehyde	µg/stick	123.5 ± 19.53	172.42 ± 6.10	722.47 ± 36.11
	µg/mg nicotine	262.77 ± 41.55	175.94 ± 6.22	688.07 ± 34.39
Acrolein	µg/stick	4.78 ± 0.91	10.90 ± 0.18	71.92 ± 2.84
	µg/mg nicotine	1.02 ± 1.94	11.12 ± 0.18	68.50 ± 2.70
1,3-Butadiene	µg/stick	NQ	NQ	8.13 ± 0.17
	µg/mg nicotine	NQ	NQ	7.74 ± 0.16
Benzene	µg/stick	0.17 ± 0.00	0.36 ± 0.05	49.95 ± 1.54
	µg/mg nicotine	0.36 ± 0.00	0.37 ± 0.05	47.57 ± 1.47
Benzo(*a*)pyrene	ng/stick	NQ	NQ	24.00 ± 0.64
	ng/mg nicotine	NQ	NQ	22.86 ± 0.61
NNK	ng/stick	6.61 ± 0.86	5.32 ± 0.89	281.00 ± 16
	ng/mg nicotine	14.06 ± 0.83	5.43 ± 0.91	267.62 ± 15
NNN	ng/stick	1.79 ± 0.26	8.37 ± 0.55	163.43 ± 11.19
	ng/mg nicotine	3.81 ± 0.55	8.54 ± 0.56	155.65 ± 10.66

NQ: not quantified
Machine-smoking regime: 55 mL puff volume, 2 s puff duration, 30 s puff interval. Results are expressed as means ± standard deviation (± SD). Replicate n = 3.

Table 6. Revertant colonies of *Salmonella typhimurium* obtained following exposure to different doses of TPM from 3R4F.

Dose (µg/plate)	TA98		TA100	
	−S9	+S9	−S9	+S9
NC	38.0 ± 2.4	60.0 ± 5.9	115.3 ± 3.8	156.3 ± 2.9
0	25.7 ± 4.0	51.0 ± 5.1	121.7 ± 6.0	107.0 ± 6.4
50	38.3 ± 8.2	87.7 ± 5.2	125.7 ± 8.5	105.0 ± 3.7
100	28.3 ± 7.5	124.3 ± 5.4*	127.3 ± 7.6	115.3 ± 7.5
250	32.7 ± 6.1	237.7 ± 11.8*	143.3 ± 8.3	133.0 ± 4.1
500	34.7 ± 2.1	233.7 ± 33.0*	137.7 ± 11.1	171.7 ± 13.0
PC	899.0 ± 24.0	2437.3 ± 106.0	2719.7 ± 55.5	781.7 ± 95.7

NC: negative control, with no solvent (DMSO). PC: positive control, 2-aminoflourene was used for both TA98 and TA100 with metabolic activation (S9 mix), 2-nitrofluorene for TA98 without S9, 4-nitroquinoline *N*-oxide for TA100 without S9.
*: the number of revertant colonies was more than twice of NC. Results are expressed as means ± standard deviation (± SD).
Replicate n = 3.

Table 7. Revertant colonies of *Salmonella typhimurium* obtained following exposure to different doses of TPM from HTP-A and HTP-B.

Dose (µg/plate)		TA98		TA100	
		HTP-A	HTP-B	HTP-A	HTP-B
-S9	NC	38.0 ± 2.4	38.0 ± 2.4	115.3 ± 3.8	115.3 ± 3.8
	0	25.7 ± 4.0	25.7 ± 4.0	121.7 ± 6.0	121.7 ± 6.0
	50	24.0 ± 3.7	30.3 ± 4.0	124.0 ± 4.1	108.0 ± 9.9
	500	35.0 ± 3.7	28.7 ± 4.1	120.3 ± 8.7	105.0 ± 8.3
	1000	30.7 ± 2.4	33.3 ± 5.7	114.3 ± 6.2	129.3 ± 5.3
	2000	30.7 ± 3.8	32.7 ± 4.5	120.7 ± 7.9	125.3 ± 3.1
	PC	899.0 ± 24.0	899.0 ± 24.0	2719.7 ± 55.5	2719.7 ± 55.5
+S9	NC	60.0 ± 5.9	60.0 ± 5.9	156.3 ± 2.9	156.3 ± 2.9
	0	51.0 ± 5.1	51.0 ± 5.1	107.0 ± 6.4	107.0 ± 6.4
	50	48.3 ± 7.6	48.3 ± 3.1	116.0 ± 19.0	100.0 ± 4.3
	500	48.7 ± 7.4	56.0 ± 4.3	112.0 ± 10.7	100.7 ± 5.4
	1000	49.0 ± 7.1	40.3 ± 5.2	113.7 ± 12.4	100.7 ± 9.2
	2000	51.0 ± 2.8	41.7 ± 7.0	94.0 ± 16.8	105.3 ± 1.2
	PC	2437.3 ± 106.0	2437.3 ± 106.0	781.7 ± 95.7	781.7 ± 95.7

NC: negative control, with no solvent (DMSO); PC: positive control, 2-aminoflourene was used for both TA98 and TA100 with metabolic activation (S9 mix), 2-nitrofluorene for TA98 without S9, 4-nitroquinoline *N*-oxide for TA100 without S9. Results are expressed as means ± standard deviation (± SD). Replicate n = 3.

Table 8. Cell genotoxicity of different TPM fractions of HTP-A and HTP-B, and 3R4F.

Sample	Concentration (µg/mL)	MN frequencies (%)		
		3h + 21h -S9	3h + 21h +S9	24h + 0h -S9
Vehicle	11	1.49 ± 0.23	1.52 ± 0.54	1.08 ± 0.08
HTP-A	75	1.37 ± 0.33	1.28 ± 0.04	1.29 ± 0.08
	150	1.08 ± 0.37	1.20 ± 0.07	1.19 ± 0.04
	300	1.05 ± 0.07	1.21 ± 0.01	1.40 ± 0.08
	600	1.15 ± 0.17	1.10 ± 0.10	1.30 ± 0.01
HTP-B	75	1.11 ± 0.02	1.20 ± 0.14	1.26 ± 0.16
	150	1.33 ± 0.22	1.07 ± 0.18	1.28 ± 0.11
	300	1.36 ± 0.30	1.28 ± 0.01	1.33 ± 0.13
	600	1.69 ± 0.30	1.29 ± 0.21	1.32 ± 0.11
3R4F	14.3	2.03 ± 0.21	2.16 ± 0.92	2.53 ± 0.05
	28.5	3.21 ± 1.02	2.46 ± 0.98	2.85 ± 0.20
	57.0	3.93 ± 1.34	2.31 ± 1.29	2.65 ± 0.05
	114.0	4.49 ± 1.58	2.67 ± 1.49	3.03 ± 0.30
PC	-	5.68 ± 0.29	5.08 ± 0.55	6.09 ± 0.37
NC	-	1.40 ± 0.30	1.32 ± 0.30	1.20 ± 0.11

NC: negative control, with no solvent (DMSO); PC: Positive Control; Mitomycin C (MMC) was used for 3h/24h treatment without S9. Cyclophosphamide A (CPA) was used for 3h treatment with S9. Results are expressed as means ± standard deviation (± SD). Replicate n = 3.

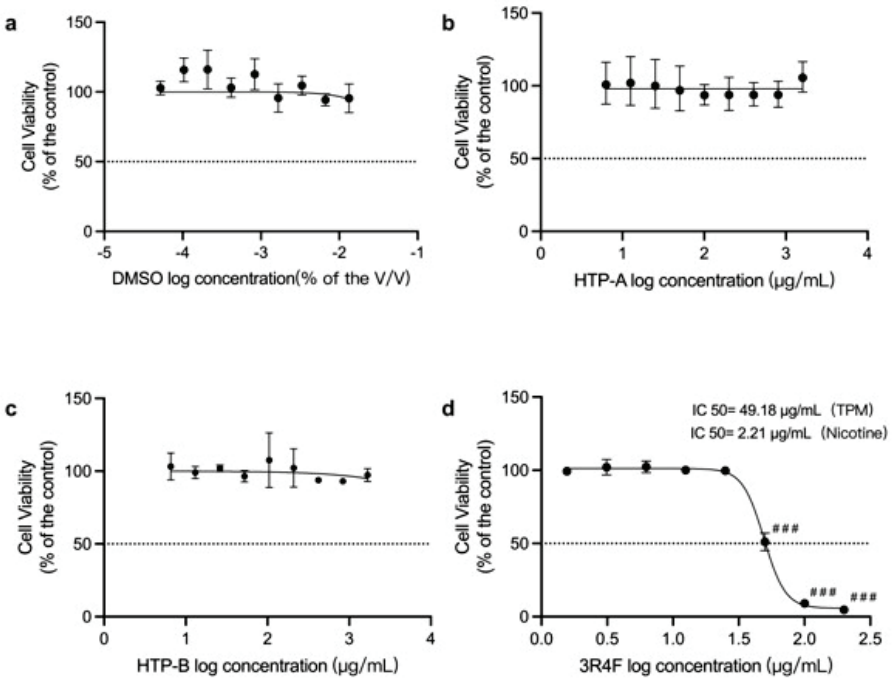

Figure 2. Cell viability in NRU assay of HTP-A and HTP-B aerosol, and 3R4F smoke. Cell viability of CHO cells was determined by NRU assay following 24 h exposures of DMSO (a) or HTP-A (b), HTP-B (c) and 3R4F (d). The data represent the mean ± SD (n = 3) ($^{###}P < 0.01$).

3.4 The neutral red uptake (NRU) assay responses

The NRU assay protocol followed the one described in the literature (21). The CHO cells showed significantly reduced viability after exposure to 3R4F smoke compared with that after exposure to the two HTP aerosols (Figure 2). The IC_{50} of HTPs could not be calculated. In contrast, the mean IC_{50} values of 3R4F was 49.18 μg/mL for TPM, and 2.21 μg/mL for nicotine.

4. DISCUSSION

In our previous studies, the aerosol release behaviors of the main TPM components of these two HTP systems were compared, such as glycerol, nicotine and water, on a paired closed-end *vs*. open-end product comparison. On an identical tobacco stick and device design (apart from the open *vs*. closed air flow) basis, closed-end HTP-A was found to be more efficient in releasing these agents (8). The reason was that the HTP-A system removed the convective heat transfer of the tobacco bed better when compared with existing open-ended HTP-B. And the HTP-A's mainstream aerosol was formed without the repeated interruption of incoming air and its cooling effects (8, 9). The formation process and mechanism of the two aerosols also directly determined the differences in the content of various components in aerosols, especially for those harmful and potentially harmful constituents.

In this work, the two HTPs under investigation were not matched in their design characteristics, and therefore it was difficult to deduce the deeper mechanistic reasons behind the toxicant yields observed in Table 5. These have also been reflected by the differences in puff numbers and released nicotine levels for the two HTPs: HTP-A produced less nicotine and most of the measurable toxicants as compared to those in HTA-B on a per-stick basis, but when the yields were normalized to per-nicotine basis, the differences were marginal or reversed.

Generally speaking, the TobReg 9 toxicants cover the four main categories of releasing behavior (11, 22):

- carbon monoxide being biomass-pyrolysis- and combustion-driven;
- formaldehyde and acetaldehyde being generated by low-temperature decomposition of sugars;
- 1,3-butadiene/benzene/acrolein being the products of carbohydrate pyrolysis;
- B[*a*]P and NNN/NNK due to thermal distillated or evaporative release of those already present in the stick.

Significant reductions across these four categories are a reasonable indication that this novel closed-end HTP (HTP-A) was maintaining the key emission performance that is typically found in the leading commercial HTP products (such as HTP-B), and therefore it was worthwhile to be investigated for its toxicological effects. The relative increases or decreases in these toxicants were most likely caused by the differences in tobacco blends, heating mechanisms, and not just by the fact that HTP-A had a reduced availability of oxygen during puffing (23).

In previous toxicological studies of differently designed HTPs (18, 24–26), standard regulatory genotoxicity and cytotoxicity assays were used as the first steps in screening in addition to toxicant emission analyses. Under the test conditions, it was impossible to determine the IC_{50} for the two HTPs up to 3000 μg/mL of TPM. This contrasted with the IC_{50} of 3R4F smoke which was determined to be

49.18 μg/mL of TPM (Figure 2). The exposure of the cells to TPMs from the two HTPs at 2000 μg TPM/plate failed to elicit cell viability responses (Table 7). These results are in agreement with the fact that the two HTPs emitted significantly lower toxicant levels as compared to those found in 3R4F smoke (Table 5). This is due to their mainly heated aerosol formation mechanism, and glycerol and water making up the main components of TPMs (6, 7). The Ames and MN results showed that the TPMs of the two HTPs were not genotoxic under these regulatory toxicological assays. These assays were not sensitive to distinguish any difference between the two HTPs, whether or not the comparisons were made on a per-unit mass of TPM and/or per-unit mass of nicotine in the aerosol. These findings are also in line with those published previously for the other HTP products (24–26). It appears that despite all the design differences in heating technology and tobacco blend compositions (U.S. blended *vs.* Chinese blended), the general aerosol emission chemistry using a subset of representative analytes, such as the WHO's TobReg 9, as used in this study, was reasonably robust to predict the qualitative, if not quantitative, toxicological outcomes. More sensitive cell lines such as 3D cell lines that represent the epithelial functions of respiratory systems and/in combination with system toxicology may be required to delineate some of the residual toxicological effects. As previous studies on HTPs have pointed out, inhalation exposures to HTP aerosols are not without risks. Further preclinical and clinical assessments should be carried out before a novel HTP could be considered a reduced-exposure- or reduced-risk tobacco product (27). As a preliminary screening evaluation, this study is not without its limitations, the main one being only based on a subset of aerosol chemistry and standard regulatory toxicological assays. Numerous studies on different HTP designs have been performed and are available in the literature. Our study used a comparative approach to set the basis for further evaluations. Any conclusions or observations should be viewed as such.

5. CONCLUSIONS

This work described a preliminary aerosol chemistry and regulatory *in vitro* screening on a novel closed-end HTP, with comparison to a commercial open-end HTP and 3R4F research cigarette. The effects of the HTP product design differences on aerosol chemistry were mainly reflected in the number of puffs available by the two systems. The resultant nicotine emissions were not matched on a per tobacco stick basis. Comparisons were therefore made on both per nicotine mass and per tobacco stick basis in order to set the ground for *in vitro* toxicological comparison. Using a set of regulatory toxicological assays (Ames, MN and NRU), the two HTPs behaved indistinguishably under the conditions tested and showed no observable responses, in contrast to the clear toxicity induced by TPM from 3R4F mainstream smoke and coincided with their significant reduced levels of WHO TobReg 9 toxicants. Further preclinical and clinical evaluations are needed before the novel HTP could be considered as a reduced-exposure or reduced-risk tobacco product.

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AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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