A Standardized Method for the Preparation of a Gas Phase Extract of Cigarette Smoke

Tsunehito Higashi,* Yosuke Mai, Yuichi Mazaki, Takahiro Horinouchi, and Soichi Miwa

Department of Cellular Pharmacology, Graduate School of Medicine, Hokkaido University; West 7, North 15, Kita-ku, Sapporo 060–8638, Japan.

Received January 20, 2016

The gas phase of cigarette smoke is important from the viewpoint of human health, because it can pass through alveolar epithelium and enter the circulation. There is no standard method for the preparation of a gas phase extract of cigarette smoke (CSE), although CSE is widely used for research instead of whole cigarette smoke. We have established a standard method for the preparation of CSE. One cigarette per trial is continuously combusted under a reduced pressure generated by an aspiration pump with a velocity of 1.050 L/min; the main stream of the smoke is passed through a Cambridge filter to remove tar, and subsequently, bubbled through a glass ball filter (pore size, 20–30 µm) into 15 mL of phosphate-buffered saline (PBS). To express the concentration of CSE, a virtual tar concentration is introduced, which is calculated assuming that tar trapped on the Cambridge filter is dissolved in the PBS. CSEs prepared from smaller numbers of cigarettes (original virtual tar concentration ≤ 15 mg/mL) show similar concentration–response curves for cytotoxicity versus virtual tar concentrations. CSEs prepared from various brands of cigarettes and by different smoking regimes (continuous and puff smoking) show similar cytotoxic potency if the virtual tar concentrations are the same. In conclusion, using the standardized method for CSE preparation in combination with the virtual tar concentration, it becomes possible to simply and rapidly prepare standard CSEs with defined concentrations from any brand of cigarettes, which are toxicologically equivalent to CSE prepared by puff smoking.

Key words cigarette smoke extract; virtual tar concentration; smoking regime; cytotoxicity

1. INTRODUCTION

The mainstream of cigarette smoke consists of a tar (particle) phase containing nicotine and a remaining gas phase.1,2 In view of human health, the gas phase is more important, because it can pass through the lung alveolar epithelium and induce injury in tissues remote from the lung.3,4 The gas phase is comprised of 400–500 chemical compounds.5,6 The stable cytotoxic compounds in the gas phase of cigarette smoke induce various cytotoxic effects in various types of cells.6–8 Recently, we have reported that the gas phase extract of cigarette smoke (nicotine- and tar-free cigarette smoke extract; CSE) induces cell death and cell membrane injury through reactive oxygen species generation mediated by protein kinase C (PKC) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX).9–11 The gas phase extract of cigarette smoke (nicotine- and tar-free cigarette smoke extract; CSE) induces cell death and cell membrane injury through reactive oxygen species generation mediated by protein kinase C (PKC) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX).9–11 The gas phase extract can also oxidize low density lipoprotein in vitro and promote atherosclerosis in vivo.12,13 Importantly, we have identified acrolein (ACR), methyl vinyl ketone (MVK), and 2-cyclopentene-1-one (CPO) as the major cytotoxic components in the CSE using LC/MS and GC/MS in combination with functional assay.14 Furthermore, we have demonstrated that ACR and MVK-induced cell damage is PKC- and NOX-dependent like that by CSE, although CPO-induced cell damage is independent of PKC and NOX.14

In general, a Cambridge filter is used for preparation of the gas phase of cigarette smoke.15–17 The Cambridge filter is a glass fiber filter and can retain 99.9% of the particulate matters larger than 0.1 µm.15 The fraction trapped on the Cambridge filter is defined as the tar phase, while the fraction which passes through the filter is the gas phase. The CSE is prepared by bubbling the gas phase in an aqueous solution such as phosphate buffered saline (PBS) and culture medium. However, because no standard methods for the preparation of CSE have been established in spite of the importance for human health, researchers prepared CSE according to their own methods. One of the main differences in preparation methods consists in the way of combustion of cigarettes (called smoking regime): puff smoking vs. continuous smoking. Another difference is cigarette brand. Finally, the most critical difference is the concentration of CSE preparation, owing to the absence of a clear definition for the concentration of CSE. Therefore, comparison of experimental results published from various laboratories has been impossible.

In this review, we will briefly review smoking regimes (puff smoking vs. continuous smoking) and the expression of concentrations of CSE. After that, we will introduce a newly established standardized method for the preparation of a gas phase extract of cigarette smoke.

2. THE METHODS FOR CIGARETTE COMBUSTION: PUFF SMOKING VS. CONTINUOUS SMOKING

For research and development purposes, cigarettes are smoked mechanically using specialized machines. There are two methods of combustion of cigarettes: puff smoking vs. continuous smoking. In “puff smoking” which superficially simulates the act of smoking by humans, cigarettes are ac-
Table 1. Representative Machine Smoking Regimes

| Puff   | Ventilation |
|--------|-------------|
| Volume (mL) | Duration (s) | Interval (s) | Block (%) |
| ISO    | 35          | 2            | 60         | 0         |
| FTC    | 35          | 2            | 60         | 0         |
| MDPH   | 45          | 2            | 30         | 50        |
| HCl    | 55          | 2            | 30         | 100       |

ISO: International Organization for Standardization; FTC: US Federal Trade Commission; MDPH: Massachusetts Department of Public Health; HCl: Canadian Health Ministry. Cigarette filter ventilation consists of several holes of filter paper to dilute mainstream of cigarette smoke. The smoke yield of mainstream is increased by the block of ventilation. Modified from ref. 20.

Puff smoking, there are several smoking regimes which define puff volume, puff frequency, puff duration, and ventilation blocks (Table 1). Among these, the smoking regime defined by the International Organization for Standardization (ISO) has become the standard regime. Notably, in many countries, the yield of tar and nicotine of cigarettes combusted according to ISO smoking regime is printed on cigarette packages. In the ISO smoking regime, smoking conditions are strictly defined: puff duration, 2.00 ± 0.3 mL; puff volume, 35.0 ± 0.3 mL; puff interval, 60 ± 0.5 s; puff profile, bell-shaped with a maximum between 0.8 s and 1.2 s from the start of the puff. To prepare smoke according to this regime requires specialized smoking machines which are usually too expensive for many researchers.

In contrast, in so-called “continuous smoking,” there is no definite smoking regime, hence researchers arbitrarily combust cigarettes according to their own smoking regimes at various aspiration rates. This method is used by many researchers, because it does not require specialized smoking machines.

Regarding the chemical composition of the two types of puff smoking, some researchers suspect the smoke may be different, since different combustion temperatures resulting from differences in the speed and/or profile of combustion could generate different combustion products. However, there has been no clear evidence suggesting the presence of such a difference, thus, at present, it is unknown whether the chemical composition of the two types of smoke is similar to each other or not.

3. EXPRESSION OF THE CONCENTRATION OF CSE

In the case of whole cigarette smoke extract and the tar phase extract, concentrations have been expressed in terms of the concentrations of endogenous substances such as nicotine, the amount of these substances is relatively easy to determine using gas chromatography or the weight trapped on the Cambridge filter, respectively. However, in the case of nicotine- and tar-free CSE, it is difficult to express its concentration, because it does not contain appropriate endogenous substances like nicotine and tar. In this context, most researchers represent the concentration by the number of cigarettes used for CSE preparation or the number of puffs. Other researchers represent the concentration in terms of the optical density at 302 nm (OD). However, these values do not necessarily represent actual concentrations of CSE, because they do not reflect parameters such as the efficiency of extraction of chemical compounds in the gas phase into the aqueous phase and saturation of aqueous phase with chemical compounds. Therefore, it is an urgent matter to develop a reliable expression system for the concentration of gas phase extract.

4. A METHOD FOR THE PREPARATION OF CSE BY A CONTINUOUS SMOKING REGIME

We have employed continuous smoking for combusting cigarettes to establish a method which is simple and rapid. The schematic diagram of an apparatus for CSE preparation by continuous smoking is shown in Fig. 1. One cigarette per trial is continuously combusted by reduced pressure generated using an aspiration pump, with the flow rate set at 1.050 L/min. The main stream of the cigarette smoke is passed through a Cambridge filter to remove the tar phase and nicotine, and subsequently, bubbled into 15 mL of PBS in a 100-mL graduated cylinder kept at 25°C. For increasing the bubbling efficiency, a glass ball filter with a pore size of 20–30 μm is used. The CSE is aliquoted and stored at −80°C until use. The CSE prepared by continuous smoking regime is designated as cCSE.

The flow rate is based on ISO3308 in which the volume and duration of a puff should be 35 mL and 2 s, i.e. 1.050 L/min. According to ISO4387, cigarettes with and without filters are combusted up to 3 mm from the tipping paper or 23 mm from the end of the cigarette, respectively.

5. VIRTUAL TAR CONCENTRATION

To express the concentration of CSE, we have introduced a virtual tar concentration, which is calculated on the assumption that the tar phase (dry weight) trapped on the Cambridge filter is dissolved in PBS used for CSE preparation. Because the tar phase contains water, the Cambridge filter is air-dried at 25°C for 12 h in order to vaporize the water before measuring the dry weight of tar. Drying at higher temperatures should be avoided because chemical compounds with low vaporizing temperatures may vaporize.

The relationship between cCSE cytotoxicity and the numbers of cigarettes used for cCSE is examined. The concentration–response curves for the inhibition of 3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reduction activity are comparable among cCSEs prepared from 2–6 Hi-Lite brand cigarettes (equivalent to the original virtual tar concentrations of 5–15 mg/mL; as described later, cigarette brands do not affect the cytotoxic activities of CSEs, as long as the virtual tar concentrations of the CSEs are similar). However, concentration–response curves shift to the right, when more than 8 Hi-Lite brand cigarettes are suspended. The rightward shift of the curves means that the cytotoxic activities of cCSEs prepared from more than 8 Hi-Lite cigarettes.
are lower than expected at a given tar concentration. These results suggest that with the virtual tar concentrations of up to 15 mg/mL, the cytotoxic activity of cigarette smoke gas phase is extracted into PBS at a constant efficiency, but when concentrations exceed 20 mg/mL, some part of the cytotoxic activity leaks without extraction. In fact, significant leakage of cytotoxic activity is detected in the second reservoir incorporated downstream of the first reservoir in the smoking apparatus, at a high virtual tar concentration (35 mg/mL) but not a low concentration (10 mg/mL). Therefore, cCSE should be prepared at the original tar concentrations of \( \leq 15 \text{ mg/mL} \).

6. THE EFFECTS OF CIGARETTE BRANDS

Many researchers use commercially available cigarette brands for their research, although the Center for Tobacco Reference Products, University of Kentucky (U.S.A.) provides reference cigarettes (Kentucky reference cigarettes) for research use. It is possible that cigarette brands affect the cytotoxicity of cCSE, because different brands of cigarettes, which are made from different tobacco leaves and contain different additives, can generate smoke with varying chemical composition. To clarify this point, we have examined the cytotoxic activities of cCSEs prepared from 8 types of representative cigarette brands (5 brands from JT, Japan; 3 brands from other countries) with nominally different tar yields. Notably, the concentration–response curves of the cCSEs prepared from all 8 brands of cigarettes are comparable, when the corresponding cCSEs at the original tar concentration of 10 mg/mL are used (Fig. 3). These results demonstrate that the cytotoxic activities of cCSEs depend on the virtual tar concentration but not on the brand or nominal tar yield of cigarettes: hence, the virtual tar concentration can be used as a universal unit of cCSE concentration.

7. COMPARISON OF THE GAS PHASE EXTRACTS PREPARED BY CONTINUOUS AND PUFF SMOKING REGIMES

Finally, the properties of cCSE are compared with those of CSE prepared by the puff smoking regime (pCSE) in terms of cytotoxic activities and chemical constituents. The concentration–response curve for the inhibition of MTS reduction activity of cCSE is comparable to that of pCSE (Fig. 4), and the cytotoxic activities of both CSEs are PKC- and NOX-dependent. Furthermore, the concentrations of the major cytotoxic compounds such as ACR and MVK are also comparable in both CSEs (Table 2). These results show that cCSE is identical to pCSE from the viewpoint of toxicology and pharmacology.

8. CONCLUDING REMARKS

Understanding the toxicological properties of CSE is important for research on smoking science, since the gas phase of cigarette smoke is mainly responsible for various disorders in organs remote from the lung. As the first step toward the development of smoking science, we have established a standard method for practical CSE preparation and also introduced a new representation method for CSE concentration, i.e. the vir-
Virtual tar concentration. Using these methods, we have shown that the virtual tar concentration can be used as a reference value to normalize the cytotoxic activities of cCSE, irrespective of the number of combusted cigarettes, cigarette brands or smoking protocols (continuous smoking vs. puff smoking), as long as the tar concentrations in the original cCSEs are \( \leq 15 \) mg/mL of PBS. The standardized method for CSE preparation will contribute to the development of smoking science.

Acknowledgments This work was supported by a Grant from Smoking Research Foundation to S.M., by a Grant-in-Aid for Young Scientists (B) (26860166) from the Japan Society for the Promotion of Science to T. Higashi, by a Grant from Akiyama Life Science Foundation to T. Higashi, and by a Grant from NISHINOMIYA Basic Research Fund (Japan) to T. Higashi.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Pryor WA, Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann. N. Y. Acad. Sci.*, 686 (1 Tobacco Smoke), 12–27, discussion, 27–28 (1993).
2) Smith CJ, Fischer TH. Particulate and vapor phase constituents of cigarette mainstream smoke and risk of myocardial infarction. *Atherosclerosis*, 158, 257–267 (2001).
3) Kunitomo M, Yamaguchi Y, Kagota S, Yoshikawa N, Nakamura K, Shinozuka K. Biochemical evidence of atherosclerosis progression mediated by increased oxidative stress in apolipoprotein E-deficient spontaneously hyperlipidemic mice exposed to chronic cigarette smoke. *J. Pharmacol. Sci.*, 110, 354–361 (2009).
4) Yamaguchi Y, Nasu F, Harada A, Kunitomo M. Oxidants in the gas phase of cigarette smoke pass through the lung alveolar wall and raise systemic oxidative stress. *J. Pharmacol. Sci.*, 103, 275–282 (2007).
5) Adam T, Baker RR, Zimmermann R. Characterization of puff-by-puff resolved cigarette mainstream smoke by single photon ionization-time-of-flight mass spectrometry and principal component analysis. *J. Agric. Food Chem.*, 55, 2055–2061 (2007).
6) Lambert C, McCue J, Portas M, Ouyang Y, Li J, Rosano TG, Lazis A, Freed BM. Acrolein in cigarette smoke inhibits T-cell responses. *J. Allergy Clin. Immunol.*, 116, 916–922 (2005).
7) Su Y, Han W, Giraldo C, De Li Y, Block ER. Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. *Am. J. Respir. Cell Mol. Biol.*, 19, 819–825 (1998).
8) Takano S, Matsuo I, Magami W, Watanabe C, Nakanishi H. Possible existence of platelet aggregation inhibitor(s) in a gas-phase extract of cigarette smoke. *Fukushima J Med. Sci.*, 43, 1–11 (1997).

9) Asano H, Horinouchi T, Mai Y, Sawada O, Fujii S, Nishiyama T, Minami M, Katayama T, Iwanga T, Terada K, Miwa S. Nicotine- and tar-free cigarette smoke induces cell damage through reactive oxygen species newly generated by PKC-dependent activation of NADPH oxidase. *J. Pharmacol. Sci.*, 118, 275–287 (2012).

10) Higashi T, Mai Y, Noya Y, Horinouchi T, Terada K, Hoshi A, Nepal P, Harada T, Horinouchi M, Hatake C, Kuge Y, Miwa S. A simple and rapid method for standard preparation of gas phase extract of cigarette smoke. *PLoS ONE*, 9, e017856 (2014).

11) Mai Y, Higashi T, Terada K, Hatake C, Nepal P, Horinouchi M, Harada T, Miwa S, Horinouchi T. Nicotine- and tar-free cigarette smoke extract induces cell injury via intracellular Ca²⁺-dependent subtype-specific protein kinase C activation. *J. Pharmacol. Sci.*, 120, 310–314 (2012).

12) Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipidprotein properties in human blood plasma. Protective effects of ascorbic acid. *Biochem. J.*, 277, 133–138 (1991).

13) Yamaguchi Y, Kagota S, Hagiwara J, Kunitomo M. Participation of peroxynitrite in oxidative modification of LDL by aqueous extracts of cigarette smoke. *FEBS Lett.*, 512, 218–222 (2002).

14) Noya Y, Seki K, Asano H, Mai Y, Horinouchi T, Higashi T, Terada K, Hatake C, Hoshi A, Nepal P, Horinouchi M, Kuge Y, Miwa S. Identification of stable cytotoxic factors in the gas phase extract of cigarette smoke and pharmacological characterization of their cytotoxicity. *Toxicology*, 314, 1–10 (2013).

15) Bodnar JA, Morgan WT, Murphy PA, Ogden MW. Mainstream smoke chemistry analysis of samples from the 2009 U.S. cigarette market. *Regul. Toxicol. Pharmacol.*, 64, 35–42 (2012).

16) Pang X, Lewis AC. Carbonyl compounds in gas and particle phases of mainstream cigarette smoke. *Sci. Total Environ.*, 409, 5000–5009 (2011).

17)UCHIYAMA S, HAYASHIDA H, IRU R, INABA Y, NAKAGOME H, KUNUGI N. Determination of nicotine, tar, volatile organic compounds and carbonyls in mainstream cigarette smoke using a glass filter and a sorbent cartridge followed by the two-phase/one-pot elution method with carbon disulfide and methanol. *J. Chromatogr. A*, 1426, 48–55 (2015).

18) Counts ME, Hsu FS, Laffoon SW, Dwyer RW, Cox RH. Mainstream smoke constituent yields and predicting relationships from a worldwide market sample of cigarette brands: ISO smoking conditions for tobacco control policy. *Tox. Control*, 16, 8–14 (2007).

19) Hammond DT, Carchman RA. Limitations of cigarette machine smoking regimes. *Toxicol. Lett.*, 203, 20–27 (2011).

20) Roemer E, Carchman RA, Baier A. Free radicals in gas-phase mainstream cigarette smoke by infrared spectroscopy. *Vib. Spectrosc.*, 27, 29–42 (2001).

21) Asano H, Horinouchi T, Mai Y, Sawada O, Fujii S, Nishiyama T, Minami M, Katayama T, Iwanga T, Terada K, Miwa S. Nicotine and tar-free cigarette smoke induces cell damage through reactive oxygen species newly generated by PKC-dependent activation of NADPH oxidase. *J. Pharmacol. Sci.*, 118, 275–287 (2012).

22) Hammond DT, Carchman RA, Baier A. Free radicals in gas-phase mainstream cigarette smoke by infrared spectroscopy. *Vib. Spectrosc.*, 27, 29–42 (2001).

23) Kołoszówski LT, O’Connor RJ. Cigarette filter ventilation is a defective design because of misleading taste, bigger puffs, and blocked vents. *Tox. Control*, 11 (Suppl. 1), i40–i50 (2002).

24) ISO 3308: Routine analytical cigarette-smoking machine-definitions and standard conditions (2000).

25) Lendvay AT, Laszlo TS. Cigarette smoke peak carbon temperature measurements. *Beitr. Tabakf.*, 7, 276–281 (1974).

26) Parrish ME, Lyons-Hart JL, Shafer KH. Puff-by-puff and intrapuff analysis of cigarette smoke using infrared spectroscopy. *Vib. Spectrosc.*, 27, 29–42 (2001).

27) Arimilli S, Damratsko BE, Prasad GL. Combustible and non-combustible tobacco product preparations differently regulate human blood mononuclear cell functions. *Toxicol. In Vitro*, 27, 1992–2004 (2013).

28) Thacher MO, Tippett GS, Nelson MB, Swenssen AC, Winden DR, Hansen ME, Anderson MC, Johnson JE, Porter JP, Reynolds PR, Bkman BL. Ceramides mediate cigarette smoke-induced metabolic disruption in mice. *Am. J. Physiol. Endocrinol. Metab.*, 307, E919–E927 (2014).

29) Wu W, Zhang W, More S, Booth JL, Duggan ES, Liu L, Zhao YD, Metcalfe JP. Cigarette smoke attenuates the RIG-I-initiated innate antiviral response to influenza infection in two murine models. *Am. J. Physiol. Lung Cell. Mol. Physiol.*, 307, L848–L858 (2014).

30) Aufderheide T, Greissmann H. A modified Ames assay reveals the mutagenicity of native cigarette mainstream smoke and its gas- and vapour-phase. *Exp. Toxicol. Pathol.*, 58, 383–392 (2007).

31) Greabu M, Battino M, Totan A, Mohora M, Mitrea N, Totan C, Spina T, Didilescu A. Effect of gas phase and particulate phase of cigarette smoke on salivary antioxidants. What can be the role of vitamin C and pyridoxines? *Pharmacol. Rep.*, 59, 613–618 (2007).

32) Stinn W, Arts JH, Buettner A, Duistermaat E, Janssens K, Kuper CF, Haussmann HJ. Murine lung tumor response after exposure to cigarette mainstream smoke or its particulate and gas/vapour phase fractions. *Toxicology*, 275, 10–20 (2010).

33) Adam T, McAughey J, McGrath C, Mocker C, Zimmermann R. Simultaneous on-line size and chemical analysis of gas phase and particulate phase of cigarette mainstream smoke. *Anal. Bioanal. Chem.*, 394, 1193–1203 (2009).

34) Culcas M, Muller A, Mercier A, Clement JL, Payet O, Rockenbauer A, Marchand V, Pietri S. Early specific free radical-related cytotoxicity of gas phase cigarette smoke and its paradoxical temporary inhibition by tar. An electron paramagnetic resonance study with the spin trap DEPMPO. *Chem. Biol. Interact.*, 164, 215–231 (2006).

35) Lu X, Hua Z, Du G, Ma X, Cao J, Yang Z, Chen J. Scavenging of free radicals in gas-phase mainstream cigarette smoke by immobi- lized catalase at filter level. *Free Radic. Res.*, 42, 254–262 (2008).

36) Sdratia ND, Patmanidi AL, Velentzas AD, Margaritis LH, Baltatzis GE, Hatzinikolaou DG, Stavridou A. The mode of lymphoblastoid cell death in response to gas phase cigarette smoke is dose-depen- dent. *Respir. Res.*, 10, 82 (2009).

37) Purkis SW, Cahours X, Rey M, Teillet B, Troude V, Vernon T. Some consequences of using cigarette machine smoking regimes with different intensities on smoke yields and their variability. *Regul. Toxicol. Pharmacol.*, 59, 293–309 (2011).