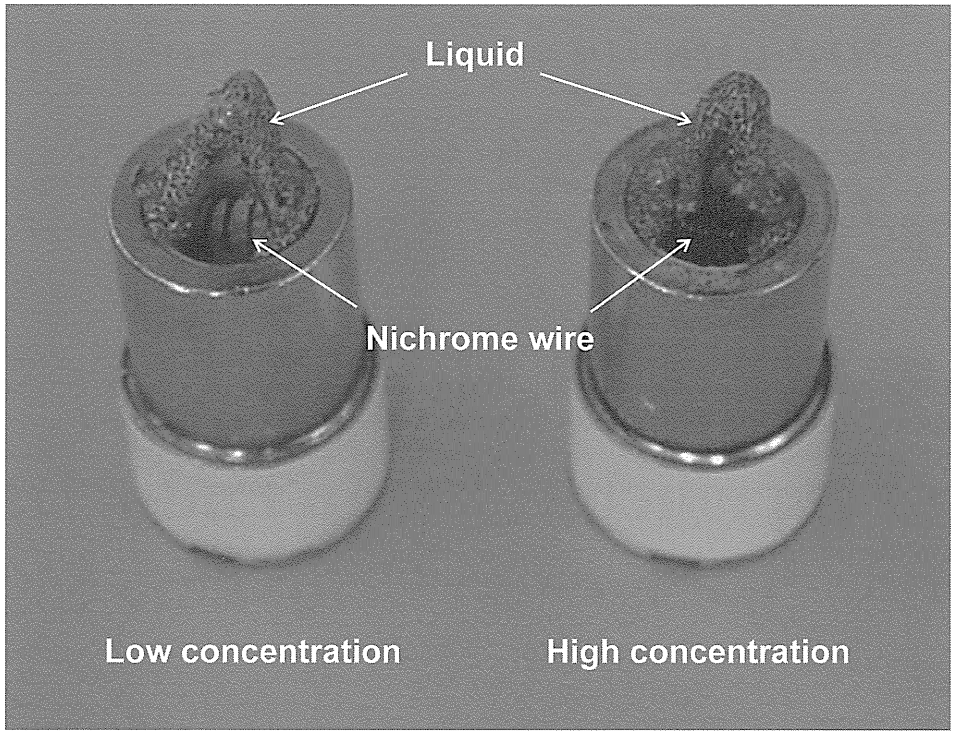

Fig. 3 E-cigarette atomizers that generated low and high concentrations of carbonyl compounds.

enhancing formaldehyde-induced cytotoxicity.[11]

After smoking an E-cigarette, the atomizer that generated the high concentrations of carbonyl compounds was burned black. Figure 3 shows atomizers after smoking 10 puffs. The left atomizer generated a low concentration. The right atomizer generated a high concentration of carbonyl compounds, and the color around Nichrome wire changed from white to black. These results suggest that the compounds in the E-cigarette liquid, such as glycerol and glycols, incidentally touch the heated Nichrome wire and are oxidized to formaldehyde, acetaldehyde, acrolein, glyoxal, and methylglyoxal.

Conclusions

E-cigarettes incidentally generate carbonyl compounds in the E-cigarette smoke mist. A possible cause for carbonyl generation is the oxidation of liquids in the E-cigarette, such as glycerol and glycols, when they incidentally touch the heated Nichrome wire in the atomizer, and are oxidized to formaldehyde, acetaldehyde, acrolein, glyoxal, and methylglyoxal. In some cases, these hazardous compounds are generated with extremely high concentrations. Suppliers and users of E-cigarettes should pay attention to this phenomenon.

Acknowledgements

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Smoking topography and biomarkers of exposure among Japanese smokers: associations with cigarette emissions obtained using machine smoking protocols

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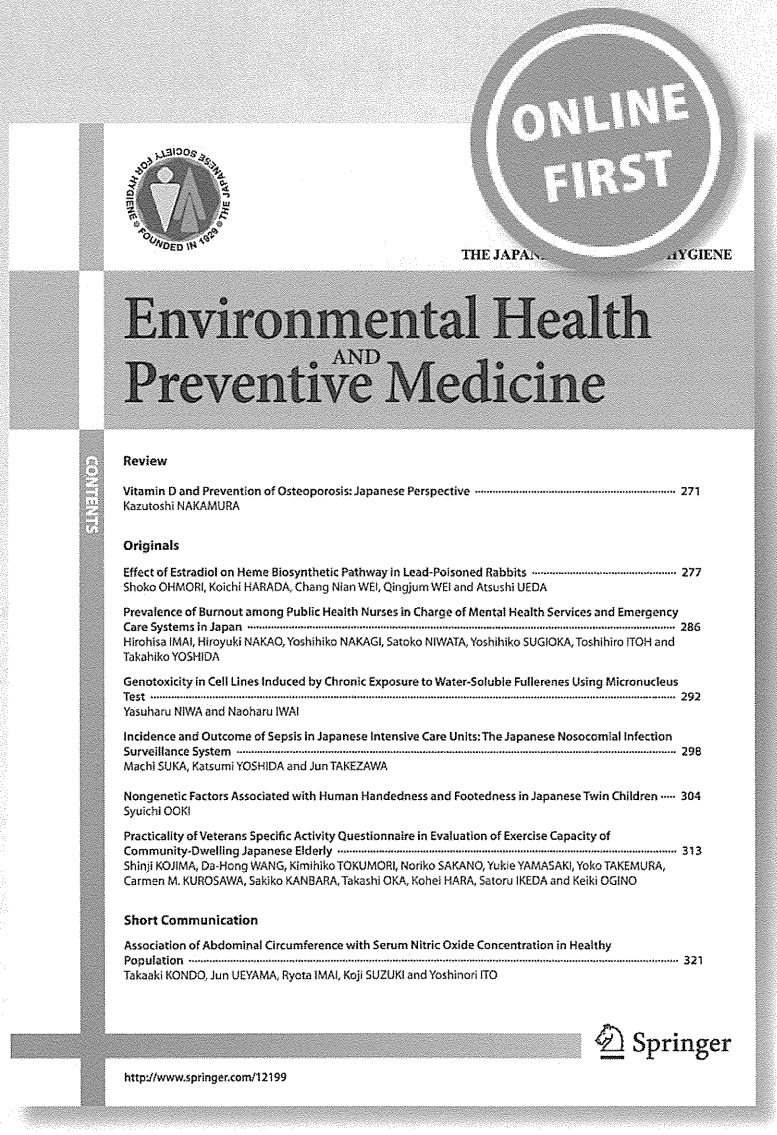

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REGULAR ARTICLE

Smoking topography and biomarkers of exposure among Japanese smokers: associations with cigarette emissions obtained using machine smoking protocols

Mariko Matsumoto · Yohei Inaba · Ichiro Yamaguchi · Osamu Endo · David Hammond · Shigehisa Uchiyama · Gen Suzuki

Received: 21 July 2011 / Accepted: 21 June 2012

Abstract
Objectives Although the relative risk of lung cancer due to smoking is reported to be lower in Japan than in other countries, few studies have examined the characteristics of Japanese cigarettes or potential differences in smoking patterns among Japanese smokers.
Methods To examine tar, nicotine and carbon monoxide (TNCO) emissions from ten leading cigarettes in Japan, machine smoking tests were conducted using the International Organization for Standardization (ISO) protocol and the Health Canada Intense (HCI) protocol. Smoking topography and tobacco-related biomarkers were collected from 101 Japanese smokers to examine measures of exposure.
Results The findings indicate considerable variability in the smoking behavior of Japanese smokers. On average, puffing behaviors observed among smokers were more similar to the parameters of the HCI protocol, and brands with greater ventilation that yielded lower machine values using the ISO protocol were smoked more intensely than brands with lower levels of ventilation. The smokers of "ultra-low/low" nicotine-yield cigarettes smoked 2.7-fold more intensively than those of "medium/high" nicotine-yield cigarette smokers to achieve the same level of salivary cotinine ($p = 0.024$). CO levels in expiratory breath samples were associated with puff volume and self-reported smoking intensity, but not with nominal values of nicotine-yield reported on cigarette packages.
Conclusions Japanese smokers engaged in "compensatory smoking" to achieve their desired nicotine intake, and levels of exposure were greater than those suggested by the nominal value of nicotine and tar yields reported on cigarette packages.

Keywords CReSSmicro device · Cotinine · Carbon monoxide · HCI protocol · Japanese smokers

Introduction

Tobacco use is responsible for one in ten global deaths and remains the leading cause of preventable death worldwide[1]. The health burden from tobacco reflects the wide range of smoking-related diseases, including cardiovascular disease, respiratory disease, and ten different forms of cancer [2].

In an effort to reduce the health effects of smoking, many smokers have reported switching to cigarette brands that yield lower tar levels under machine smoking test conditions [3]. Indeed, the average tar rating of brands has steadily decreased in many markets throughout the world, including the USA, where machine-measured tar levels decreased by more than 44 % from 1968 to 1998 [4]. However, it is well known that the use of low-tar-yield

M. Matsumoto · Y. Inaba (✉) · I. Yamaguchi · S. Uchiyama
Department of Environmental Health,
National Institute of Public Health, 2-3-6 Minami,
Wako, Saitama 351-0197, Japan
e-mail: yohei_inaba@niph.go.jp

O. Endo
School of Life and Environmental Science,
Azabu University, Sagamihara, Kanagawa, Japan

D. Hammond
Department of Health Studies and Gerontology,
University of Waterloo, Waterloo, ON, Canada

G. Suzuki
International University of Health and Welfare,
Otawara, Tochigi, Japan

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cigarettes has not reduced lung cancer deaths [5, 6]. In machine testing, the yields of nicotine and tar are measured in main stream smoke (MSS) generated by a smoking machine operated under the International Organization for Standardization (ISO) protocol: 35-mL puff volume, 60-s puff interval, 2-s puff duration, and no blocking of ventilation holes. However, it has been demonstrated that smokers smoke their cigarettes more intensely than is simulated by smoking machines operated under the ISO protocol [7–9]. Moreover, smokers tend to adapt smoking behaviors upon changing from high- to low-nicotine-yield cigarettes in an attempt to compensate for the lower emission of nicotine in the MSS [7]. In order to reflect a broader range of smoking behaviors, some governmental agencies have adopted more intensive machine smoking protocols on which to base their regulations, such as the Health Canada Intense (HCI) protocol: 55-mL puff volume, 30-s puff interval, 2-s puff duration, and 100 % blocking of ventilation holes [10]. The World Health Organization (WHO) recommends that national health authorities release their respective data on tar, nicotine, and carbon monoxide (TNCO) yields per cigarette according to both the ISO and HCI protocols [11].

The cigarette market in Japan is notable for the very low levels of machine-measured tar in cigarettes, as well as the popularity of charcoal filter cigarettes. Japan is also exceptional because of its lower relative risks for lung cancer compared to many other countries: the relative risk for lung cancer due to smoking among Japanese was recently estimated to be 4.5, and the attributable risk of smoking was about 70 % [12]. Potential explanations for the lower rates of lung cancer include genetic differences, life-style factors other than smoking, and differences in either the patterns of smoking or the types of tobacco products used in Japan. In the study reported here, we sought to characterize patterns of smoking among Japanese smokers using their "usual" brand, as well as the association between biomarkers of exposure and machine-measured TNCO yields.

Materials and methods

Subjects

In 2007, we recruited 101 individuals from in and around the Wako city region, Saitama prefecture, Japan, to our study. The inclusion criteria were daily smokers, age of between 20 and 65 years, no history of heart or lung disease, and reported smoking of one of ten leading Japanese cigarette brands for at least the past 3 months. Table 1 shows a list of the top ten best selling Japanese brands tested in our study. Table 2 shows the demographic characteristics of the participants.

Participants were asked to visit the study laboratory, provide informed consent and to complete a questionnaire on their smoking habits. Based on the information supplied on the completed questionnaires, the maximum smoking history was 528 months, in a 64-year-old individual. Since most participants stated that they had started smoking at 20 years of age, we concluded that the age reflects the smoking history. Furthermore, salivary cotinine level and CO level in the expiratory breath, which we focused on in this study, are biomarkers that decay immediately after smoking; therefore, smoking history was not counted as a statistical variable.

Questionnaire

Participants were asked to indicate their smoking intensity on a scale between 0 and 100 depending on the depth of inhalation and on the number of puffs they took. Participants were also asked to report the number of cigarettes they smoked the day before and the time to their first cigarette of the day, which we used as a proxy measure of nicotine dependence [13].

Salivary cotinine

At the first visit, participants were asked to wear latex gloves in order to collect salivary samples using sterile cotton swabs (Salivette; Sarstedt, Nümbrecht, Germany). Participants kept the Salivettes in their mouth for 2 min. Samples were frozen at −30 °C until analysis. The levels of cotinine in the saliva were not affected by the use of cotton swab collection methods [14].

Cotinine assay

After thawing, the saliva samples were recovered from the cotton swabs by centrifugation (1,000 *g*, 4 °C, 2 min) and arbitrarily diluted tenfold with phosphate buffered saline (Wako Pure Chemical, Osaka, Japan). Duplicate samples (10 μL) were assayed by a Saliva Cotinine Microplate EIA kit (Cozart, Milton Park, UK). Absorbance at 450 nm was measured on an Ultrospec Visible Plate Reader 96 (GE Healthcare Bio-Sciences, Little Chalfont, UK). The determination range of a Microplate EIA kit was from 5 to 50 ng/mL cotinine, and the mean and 95 % confidence interval (CI) of the absorbance value at concentrations of 5, 10, and 50 ng/mL were 0.99 ± 0.04, 0.72 ± 0.05, and 0.29 ± 0.02, respectively ($n = 16$). Standard solutions were assayed in 16 replicates. Samples were assayed in duplicate, and the average of two results was taken as the final salivary cotinine concentration. In the case that the absorbance of the sample was out of range of the standard curve, other dilutions of saliva were re-analyzed. Salivary cotinine data were available for 94 subjects.

Table 1 Top ten best selling cigarette brands in Japan in 2006

Brand name	Categorization according to nicotine yield	Tar (mg/cigarette)	Nicotine (mg/cigarette)	Filter type[a]	Ventilation hole[b]	Market share (%)
Pianissimo One	Ultra-low	1	0.1	P	40	1.5
Mild Seven One	Ultra-low	1	0.1	DC	200	4.3[c]
Mild Seven Extra Lights	Low	3	0.3	DC	100	3
Caster Mild	Low	5	0.4	NC	90	2.7
Mild Seven Super Lights	Low	6	0.5	DC	100	6.8
Cabin Mild	Medium	8	0.6	NC	50	1.9
Mild Seven Lights	Medium	8	0.7	DC	50	6.2
Mild Seven Original	Medium	10	0.8	DC	50	4.9
Hope	High	14	1.1	P	0	1.3
Seven Stars	High	14	1.2	DC	0	6.8[c]

[a] *P* Plain, *DC* dual charcoal, *NC* neo charcoal

[b] Number of ventilation holes

[c] Shares include box-type packaging

Table 2 Sample characteristics and smoking topography of Japanese smokers

Characteristic	Total ($n = 101$)[a]	Ultra-low ($n = 14$)	Low ($n = 38$)[a]	Medium ($n = 27$)	High ($n = 22$)
Male/female	88/13	10/4	33/5	24/3	21/1
Age (years)	40.0 ± 11.0	39.2 ± 10.2	40.1 ± 12.6	41.9 ± 11.2	38.0 ± 8.4
Cigarette consumption (cigarette/day)	18.4 ± 7.5	18.7 ± 8.3	17.8 ± 7.2	18.2 ± 6.6	19.3 ± 8.8
Body mass index	23.2 ± 3.8	22.2 ± 2.6	22.8 ± 3.0	23.9 ± 4.6	23.7 ± 4.5
Self-reported smoking intensity	57.8 ± 17.0	56.1 ± 17.3	57.0 ± 15.5	53.6 ± 15.6	65.5 ± 19.5
Time to first smoking cigarette (%)					
0–5 min	23.8 %	21.4 %	23.7 %	29.6 %	18.2 %
6–30 min	44.6 %	50.0 %	36.8 %	40.7 %	59.1 %
31–60 min	18.8 %	0.0 %	26.3 %	22.2 %	13.6 %
≥61 min	12.9 %	28.6 %	13.2 %	7.4 %	9.1 %
Puff volume (mL)	54.3 ± 14.1	64.6 ± 12.3	56.0 ± 12.2	46.9 ± 12.3	53.8 ± 15.8
Puff volume/cigarette (mL)	767.2 ± 259.5	1160.1 ± 302.7	810.0 ± 179.6	609.4 ± 158.7	638.7 ± 136.9
Puff number/cigarette	14.5 ± 3.6	17.9 ± 3.2	15.0 ± 3.1	13.4 ± 3.4	12.6 ± 3.1
Puff volume/day (mL)	14,456.2 ± 8,769.0	22,579.9 ± 15,557.4	14,423.2 ± 7,249.8	11,435.1 ± 5,232.0	13,049.7 ± 5,556.8

Data are presented as the mean ± standard deviation (SD), unless indicated otherwise

[a] Topographic data were not available for one smoker who smoked low nicotine-yield cigarettes, and there were therefore 100 and 37 smokers in the "Total" and "Low" experimental smoking groups, respectively

CO in expiratory breath

The level of CO in the expiratory breath was measured by the Micro Smokerlyzer (Bendfont Scientific, Rochester, UK) according to the manufacturer's instruction. Repeat measurements of CO were made immediately after the participants had smoked one cigarette. The average CO value of each participant was used for the data analysis, and data were available for all 101 subjects.

Smoking topography

The smoking topography of each participant was measured for a 24-h period using a CReSSmicro device (Plowshare Technologies, Baltimore, MD). CReSSmicro is a battery-operated portable device (2.5 × 2.2 × 1.2 inch, 3.1 oz) that measures a full complement of smoking topography variables, including puff volume, puff count, puff duration, average flow, peak flow, inter-puff interval, time, and date

[7]. Participants were instructed to use the device for all cigarettes smoked during a 24-h period and to keep a diary of their smoking behavior. Puff volume/day was calculated by multiplying the mean puff volume/cigarette by the number of cigarettes smoked in a 24-h period. Smoking topography data were available for 100 subjects.

TNCO yields by machine smoking

Tar and nicotine in the MSS were collected by a smoking machine (Borgwaldt single-channel linear smoking machine model LM1; Borgwaldt KC, Hamburg, Germany) operated under the conditions specified by either the ISO 4387 protocol or the HCI T-115 protocol. Cigarettes were conditioned prior to machine smoking according to the ISO 3402 protocol. MSS was collected on a 44-mm Cambridge filter pad (Borgwaldt KC) and was immediately extracted with 20 mL of 2-propanol (Wako Pure Chemical) by gentle shaking for 20 min using an electric shaker (Personal-11; Taitec, Saitama, Japan). For the nicotine analysis, 50 μL of extracted solution was dissolved in 5 mL of 2-propanol containing 1 μg/mL isoquinoline (Tokyo Chemical Industry, Tokyo, Japan) as an internal standard, and 1 μL (model 7683B autosampler; Agilent Technologies, Santa Clara, CA) was separated by gas chromatographically on a 30-m DB-17 column (J&W Scientific, Cordova, CA; internal diameter 0.25 mm; film thickness 0.25 μm) in a gas chromatography system (HP 6890; Hewlett-Packard, Palo Alto, CA) under the following conditions: (1) temperature program: 2 min at 50 °C, then to 200 °C at the rate of 15 °C/min, then to 280 °C at a rate of 5 °C/min, and hold at 280 °C for 5 min; (2) flow rate: 1 mL/min. Nicotine was quantified using *m/z* 84 amu after electron impact ionization at 70 eV (internal standard: *m/z* 129 amu; mass selective detector model 5975, Agilent Technologies). Determination of water in the 2-propanol extraction solution was carried out in accordance with the ISO 10362-1 protocol using a gas chromatograph equipped with a thermal conductivity detector. Tar was calculated by deducting the nicotine and water content from the total particulate matter of crude smoke condensates according to the ISO 4387 protocol. CO was determined used a modified ISO 8454 protocol.

Statistical analysis

We used one-way analysis of variance (ANOVA) to test differences in the CO concentrations of the expiratory breath, salivary cotinine concentrations, and puff volume/day, across categories of nicotine yield. The Bonferroni method was carried out to reduce the chance of false positives in multiple comparisons. The association between cotinine in saliva and nominal brand tar yield, nominal brand nicotine yield, number of cigarettes' filter ventilation holes, cigarette consumption, number of cigarettes smoked the day before, number of cigarettes smoked after getting up in the morning, self-reported smoking intensity, puff volume/day, time to first smoking cigarette, height, and weight was evaluated by a simple linear regression analysis. Variables at either significant ($p < 0.05$) or suggestive levels ($p < 0.1$) were re-analyzed for their association with salivary cotinine by a multivariable regression analysis. The same statistical analyses were conducted using CO in the expiratory breath as the outcome variable. All analyses were conducted using SPSS software ver. 16.0 (SPSS, Chicago, IL).

Results

Analysis of MSS by smoking machine

Figure 1 shows the tar, nicotine, and CO yields in MSS generated by the smoking machine operated under the ISO and HCI protocols. The yields were categorized into four groups according to the nominal brand nicotine yield expressed on the cigarette packages: "ultra-low"- (0–0.1 mg nicotine/cigarette), "low"- (>0.1, <0.6 mg nicotine/cigarette), "medium"- (≥0.6, <1.0 mg nicotine/cigarette), and "high"-yield brands (≥1.0 mg nicotine/cigarette) [15, 16]. Under the ISO protocol, the experimentally determined yields were the same as those appearing on the packages. Under the HCI protocol, the nicotine yields determined in the "ultra-low"-, "low"-, "medium"-, and "high"-yield groups were 5.0-, 3.3-, 2.3-, and 1.9-fold higher, respectively, than those obtained under the ISO protocol (Fig. 1a). Similarly, tar yields under the ISO protocol were the same as the values appearing on the cigarette packages, while under HCI protocol, the yields increased 11.9-, 3.9-, 2.7-, and 2.1-fold for the "ultra-low"-, "low"-, "medium"- and "high"-yield brands, respectively (Fig. 1b). CO concentrations under the ISO protocol were 4.6, 10.6, 13.8, and 17.9 mg/cigarette for the "ultra-low"-, "low"- "medium"-, and "high"-yield brands, respectively (Fig. 1c). In contrast to tar and nicotine yields, CO concentrations under the HCI protocol were very similar across groups, with a mean of 33.6 ± 1.3 mg/cigarette (Fig. 1c).

Smoking topography data of Japanese smokers

Table 2 shows the smoking topography data of 100 Japanese smokers. Overall, the mean puff volume and mean puff volume per cigarette were closer to the puffing parameters of the HCI protocol (55 mL per puff) than the ISO protocol (35 mL per puff). In addition, "ultra-low/

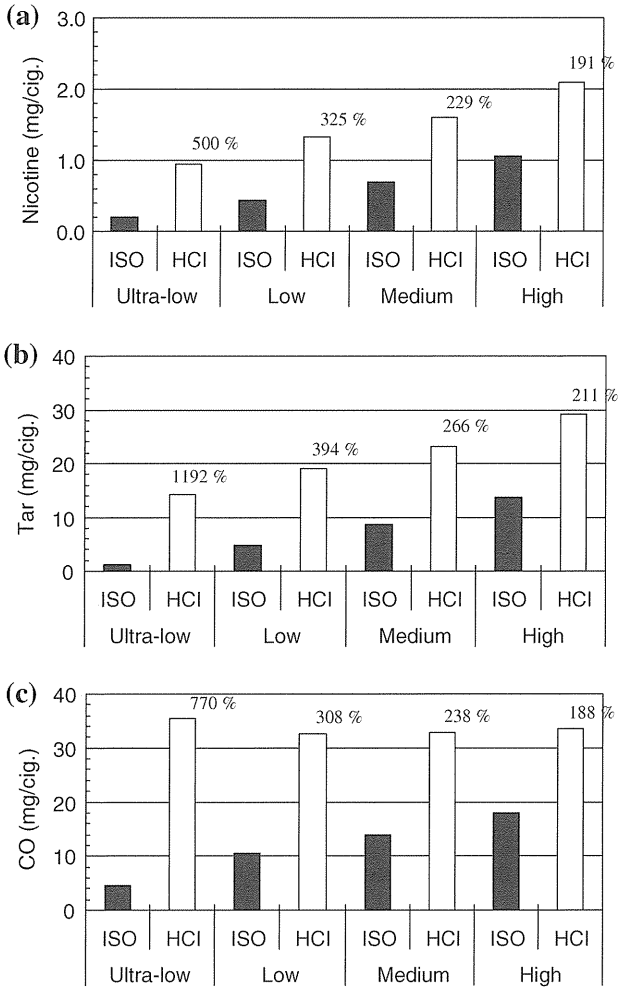

Fig. 1 Tar, nicotine, and carbon monoxide (TNCO) yields by machine smoking tests. Ten cigarette brands were categorized into four groups according to the nicotine yields expressed on the cigarette packages: "ultra-low"- (0–0.1 mg nicotine/cigarette), "low"- (>0.1, <0.6 mg nicotine/cigarette), "medium"- (≥0.6, <1.0 mg nicotine/cigarette), and "high"-yield brands (≥1.0 mg nicotine/cigarette). *Numbers over the columns* of Health Canada Intense (*HCI*) measurements indicate the increase (in percentage) compared with the corresponding International Organization for Standardization (*ISO*) measurements

low" nicotine yield brands were smoked significantly more intensely than "medium"- and "high"-yield brands: for example, the puff volume per day was 37 % greater for "ultra-low/low" brands.

Biomarker levels in smokers

Levels of salivary cotinine were correlated with the nominal nicotine levels of brands smoked by each participant according to ANOVA analysis ($p = 0.002$) and multiple comparison (Fig. 2a). Puff volume/day was negatively correlated with the nominal nicotine levels of brands smoked by the respective smoker by ANOVA analysis ($p = 0.001$) and multiple comparison (Fig. 2b) and by simple linear regression analysis ($r = -0.295$, $p = 0.003$, $n = 100$). In order to examine whether the association between salivary cotinine levels and puff volume/day was constant across different nominal yields, we tested "utra-low/low" yield brands separately from "medium/high" nominal nicotine brands (Fig. 3). From the linear slope of each figure, we determined that smokers of the former smoked 2.7-fold more intensely than those of the latter in terms of puff volume/day to achieve the same levels of salivary cotinine ($p = 0.024$). Thus, the topographical data in Table 2 and biomarker results in Fig. 3 demonstrated that Japanese smokers also practiced so-called compensatory smoking. In contrast, the levels of CO in the expiratory breath were not associated with the nominal nicotine levels of the brands they smoked. The pattern of CO levels in the expiratory breath more closely mimicked the pattern of CO emissions by HCI than that by ISO in Fig. 1c.

Multivariable regression analysis

From the results of simple linear regression analysis, salivary cotinine was associated with nominal brand tar yield ($r = 0.362$, $p = 0.001$), nominal brand nicotine yield ($r = 0.371$, $p < 0.001$), number of cigarettes, filter ventilation holes ($r = -0.341$, $p = 0.001$), cigarette consumption ($r = 0.336$, $p = 0.001$), number of cigarettes smoked the day before ($r = 0.314$, $p = 0.003$), number of cigarettes smoked upon waking up ($r = 0.194$, $p = 0.072$), self-reported smoking intensity ($r = 0.228$, $p = 0.034$), and time to first smoking cigarette ($r = -0.210$, $p = 0.050$). CO in the expiratory breath was associated with number of filter ventilation holes in a cigarette ($r = -0.168$, $p = 0.092$), cigarette consumption ($r = 0.299$, $p = 0.002$), number of cigarettes smoked the day before ($r = 0.306$, $p = 0.002$), number of cigarettes smoked after waking up ($r = 0.230$, $p = 0.020$), self-reported smoking intensity ($r = 0.367$, $p < 0.001$), puff volume/day ($r = 0.312$, $p = 0.002$) and time to first smoking cigarette ($r = -0.183$, $p = 0.067$).

A multivariable regression analysis was performed to examine predictors of salivary cotinine and CO in the expiratory breath. Variables that were correlated at $p < 0.1$ in the simple regression analysis and which had no multicollinearity were selected and included in the model as independent variables (Table 3). As shown in Table 3, salivary cotinine levels were positively associated with puff volume/day ($\beta = 0.27$, $p < 0.01$) and nominal brand nicotine yield ($\beta = 0.44$, $p < 0.01$), and negatively associated with time to first smoking of a cigarette ($\beta = -0.18$, $p = 0.05$). Thus, even after the nominal nicotine levels were adjusted, smokers smoked more intensely in terms of puff volume/day to increase plasma nicotine levels as

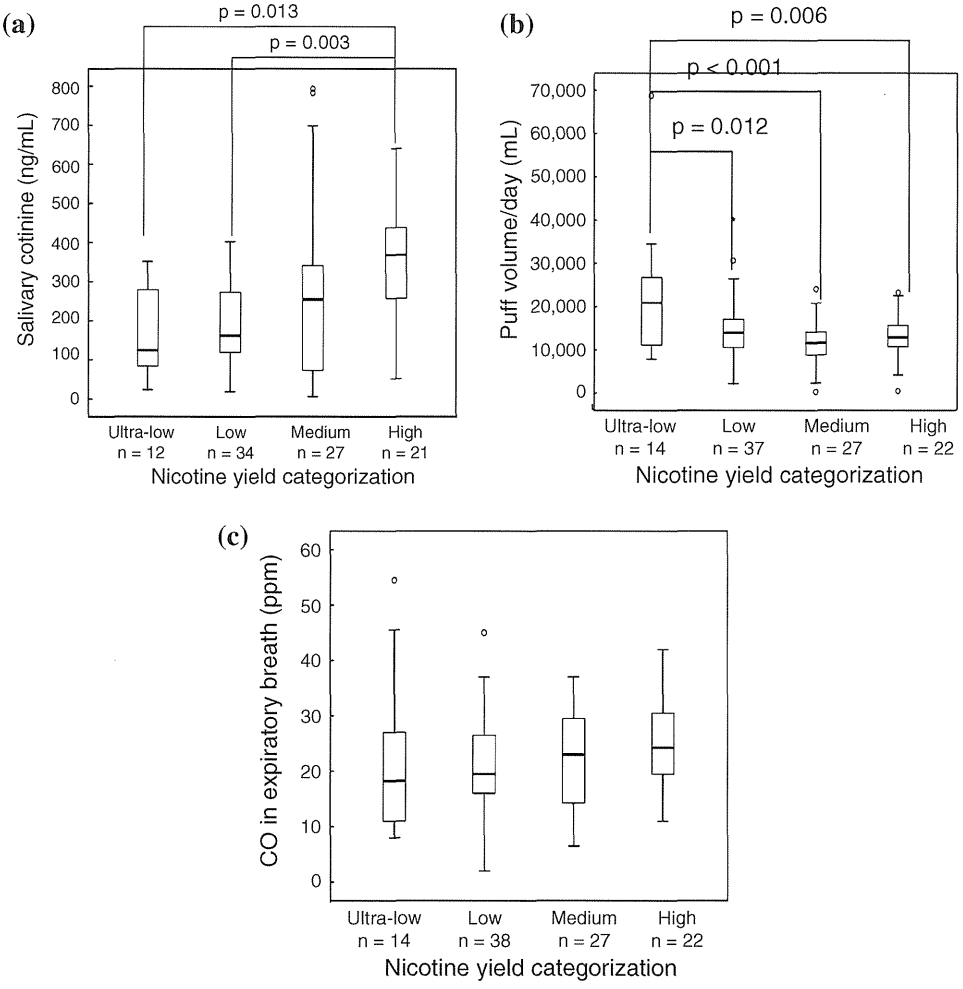

Fig. 2 **a** Salivary cotinine concentrations, **b** puff volume/day, **c** CO concentrations in expiratory breath by cigarette nicotine yield categorization. The number of participants was 94 for the saliva samples, 100 for the puff volume/day measurements, and 101 for the CO in expiratory breath samples. The correlation between puff volume/day and nominal nicotine yield was $r = -0.295$, $p = 0.003$ ($n = 100$). p values in the figures are results of multiple comparison applying the Bonferroni method. *Horizontal lines* Median salivary cotinine concentrations, puff volume/day, and CO concentrations. *Open symbol* Outliers

judged by salivary cotinine. We also performed multivariable regression analysis between the levels of CO in the expiratory breath and other independent variables. CO levels in expiratory breath was positively associated with puff volume/day ($\beta = 0.27$, $p = 0.01$) and self-reported smoking intensity ($\beta = 0.30$, $p < 0.01$), and negatively associated with time to first smoking of a cigarette ($\beta = -0.19$, $p = 0.03$), but not with nominal nicotine levels of the different brands.

Discussion

This study is among the first to characterize the TNCO emissions of the best-selling cigarettes in Japan, a critically important tobacco market. We observed significant increases in emissions under the more intensive smoking protocol of the Health Canada. The association between the ISO and HCI values varied depending upon the emission parameter measured: whereas tar and nicotine yields under the ISO and HCI yields appeared to be highly correlated, CO yields under the HCI protocol showed very little difference across cigarette brands, in contrast to the ISO values.

This study is also the first to report measures of puffing behavior among Japanese smokers and to examine the behavioral measures of smoking compensation. Topographical data on the smoking behavior of each participant were collected by a CReSSmicro device for one whole day. Puff volume and puff volume per cigarette, as measured by machine smoking under the ISO or HCI protocols, were 35 or 55 mL, and 203–245 or 385–534 mL, respectively (our unpublished data). These findings demonstrate that Japanese smokers engage in compensatory smoking: brands with lower nominal tar and nicotine yields and higher levels of ventilation are smoked systematically more intensely than higher yield brands. The average puff volume per cigarette recorded among our Japanese smokers (767.2 mL) is somewhat higher than previous estimates from the UK [17] and the USA [8], but similar to estimates from Canadian smokers who switched to cigarettes with 4 mg of ISO tar [7]. The differences between our values and those of the UK and USA studies may reflect the higher levels of filter ventilation and lower tar and nicotine

machine yields included in our study. The average puff volume in our study for "ultra-low/low" brands (58 mL) was similar to that reported in previous studies conducted with similar brand categories in the USA (57 mL) [8] and in Canada (58 mL) [7]. Together, these data suggest that behavioral compensation in response to more heavily ventilated, "lower tar" cigarettes may be a universal phenomenon among smokers. The findings also underscore the fact that the Health Canada "Intense" protocol does not represent an "upper limit" in terms of its smoking parameters. Indeed, the puff volume used in the HCI protocol was lower than the average puff volume observed among the smokers of "ultra-low" and "low" brands in our study. Although the HCI protocol also blocks filter ventilation holes, the Our findings nevertheless suggest that a considerable number of smokers may be exposed to greater levels of chemical emissions than the "intensive" method would suggest.

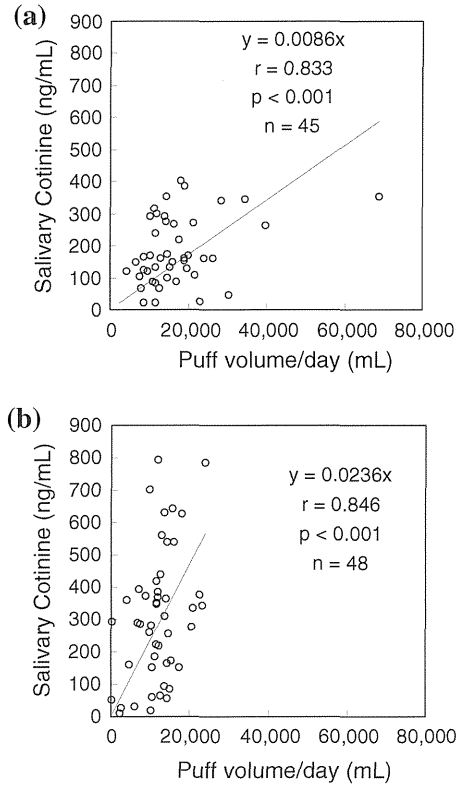

Fig. 3 Correlation between puff volume/day and salivary cotinine for "ultra-low/low" nicotine yield smokers (**a**) and for "medium/high nicotine" yield smokers (**b**). Salivary cotinine concentrations were normally distributed whether or not they were log-transformed. When a subject $[(x, y) = (68764.8, 351.4)]$ was excluded in **a**, regression parameters changed only slightly: $y = 0.0097x$, $r = 0.845$, $p < 0.001$, $n = 44$

There was positive association with the levels of salivary cotinine levels and the nominal nicotine levels of brands being smoked. The same trend has also been reported between nominal nicotine levels and urinary cotinine levels [15], serum cotinine levels [16], and salivary cotinine levels [18]. It is noteworthy, however, that the median value of salivary cotinine concentration, 124 ng/mL, in smokers who smoked "ultra-low" nicotine yield brands and the concentration, 368 ng/mL, in smokers who smoked "high" nicotine yield brand are much closer than differences in the nominal nicotine value would suggest. Although nominal nicotine levels on packages differed more than tenfold under the ISO protocol, the difference in salivary cotinine levels was only threefold. Nakazawa et al. also reported that there was only a twofold difference in urinary cotinine concentration between Japanese smokers who smoked "ultra-low" nominal nicotine brands and those who smokes "high" nominal nicotine brands [19]. Although the association between filter ventilation levels, puffing behaviors, and nominal yields under the ISO protocol were not explicitly tested in the current study due to missing data for some "ultra-low/low" brands,

Table 3 Predictors of biomarkers of exposure

Predictors	Partial regression coefficient B	Standardized partial regression coefficient β	Significance probability p	95 % confidence interval for B	
				Lower limit	Upper limit
Salivary cotinine (ng/mL)					
Puff volume/day (mL)	0.01	0.27	<0.01	<0.01	0.01
Nominal brand nicotine yield (mg/cigarette)	214.32	0.44	<0.01	118.25	310.38
Self-reported smoking intensity	1.25	0.11	0.22	−0.74	3.23
Time to first smoking cigarette	−0.24	−0.18	0.05	−0.47	<0.01
CO in expiratory breath (ppm)					
Puff volume/day (mL)	<0.01	0.27	0.01	<0.01	<0.01
Nominal brand nicotine yield (mg/cigarette)	2.35	0.09	0.40	−3.05	7.76
Self-reported smoking intensity	0.17	0.30	<0.01	0.06	0.29
Time to first smoking cigarette	−0.02	−0.19	0.03	−0.03	<0.01

Multiple correlation coefficient is $r^2 = 0.275$ (upper model) and $r^2 = 0.239$ (lower model), respectively

the data depict a positive association of salivary cotinine with puffing behavior and a strong negative association of puffing behavior with nominal nicotine yields under the ISO protocol. This pattern is consistent with previous research indicating that the primary design strategy for lower tar and nicotine yields under the ISO protocol is to increase filter ventilation as opposed to "genuine" reductions in the nicotine content [20]. CO in the expiratory breath showed very modest differences across brands regardless of the nominal nicotine yields listed on the cigarette package or nominal CO yield under ISO protocol, similar to previous findings [15, 16]. This finding is also consistent with the notion that CO is primarily a measure of smoking intensity and that it is less dependent on differences in tobacco blend or product design.

More generally, the findings highlight the elastic nature of cigarette design and the fact that all conventional cigarette brands are capable of delivering a wide range of emission levels. For example, salivary cotinine levels varied by more than tenfold for the same brand. In other words, the consumer controls his/her level of chemical exposure from each brand by changing his/her smoking behavior to a much greater extent than does the design of different cigarette brands. Our data on biomarkers of Japanese smokers resembled the smoking pattern of the HCI protocol.

Overall, our results suggest that smokers who smoke "ultra-low/low" nominal nicotine brands tend to draw smoke more deeply into the lung and take more puffs. The findings underscore the fact that tar and nicotine levels from machine smoking protocols should not be used as indicators of risk. They also highlight the importance of using measures of human exposure to understand cigarette delivery and potential differences between brands. Finally, our findings provide additional evidence on the misleading nature of tar and nicotine values from machine smoking protocols as a source of consumer information on packs. Cigarette properties, smoking patterns, lifestyles (including dietary habits), and genetic difference are considered to be reasons why lung cancer risk is lower in Japan than in other countries. In our study, we have found that smoking pattern was not the main cause of this difference. Exposure to tobacco-specific nitorosamines and aldehydes may also differ according to the charcoal filter. Future research should therefore focus on cigarette properties.

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Conflict of Interest All authors declare that we have no financial relationship with a biotechnology manufacturer, a pharmaceutical company, or other commercial entity that has an interest in the subject matter or materials discussed in the manuscript.

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資　料

国産たばこ主流煙中たばこ特異的ニトロソアミン類の異なる捕集法を用いた測定

杉山　晃一 *1,2, 稲葉　洋平 *2, 大久保忠利 *2, 内山　茂久 *2,
髙木　敬彦 *1, 欅田　尚樹 *2

*1 麻布大学大学院獣医学研究科
*2 国立保健医療科学院生活環境研究部

Determination of Tobacco-Specific *N'*-Nitrosamines in Mainstream Smoke from Japanese Cigarettes

Koichi SUGIYAMA*1,2, Yohei INABA*2, Tadamichi OHKUBO*2, Shigehisa UCHIYAMA*2, Yukihiko TAKAGI*1 and Naoki KUNUGITA*2

*1*Department of Veterinary Medicine, Azabu University*
*2*Department of Environmental Health, National Institute of Public Health*

Abstract　Objectives: Mainstream smoke from cigarettes contains tobacco-specific *N'*-nitrosamines (TSNAs) listed as Group 1 and 3 carcinogens by the International Agency for Research on Cancer (IARC). Herein, we report on a method of measuring the concentrations of TSNAs in mainstream smoke from the ten top-selling Japanese cigarette brands using an ISO regime by International Organization for Standardization (ISO) and HCI regime of Health Canada.

Methods: Tar in mainstream smoke was collected on a Cambridge filter pad using a smoking machine. The filter pad was immersed in 40 mL of ammonium acetate (pH 6.8) and shaken for 30 min. The extract was then loaded into a C18 column. After washing with 5 mL of 10% methanol and eluting with 5 mL of 70% methanol, the eluate was concentrated to 1 mL for LC-MS/MS analysis.

Results: The concentrations of TSNAs in all cigarette brands were higher when determined using the HCI regime than when determined using the ISO regime. Furthermore, the concentrations of TSNAs measured using both the ISO and HCI regimes showed negligible correlation to the tar and nicotine concentrations indicated on package labels. The cigarette samples used in the study were categorized into four classes: ultralow-, low-, medium-, and high-yield brands, which corresponded to 1, 3–6, 8–10, and 14 mg tar/cigarette, respectively. The concentration of TSNAs in ultralow-yield cigarettes was 210 ng/cigarette, as measured using the HCI regime, which was nearly equal to that in high-yield cigarettes (180 ng/cigarette).

Conclusions: Exposure to TSNAs from mainstream smoke from ultralow-yield cigarettes is comparable to that from high-yield cigarettes. To properly evaluate the risk of smoking, not only the concentrations of tar and nicotine but also those of other chemicals, including TSNAs, should be printed on package labels.

Key words: tobacco-specific *N'*-nitrosamines (たばこ特異的ニトロソアミン類), Japanese cigarettes (国産たばこ), mainstream smoke (主流煙), smoking protocol (喫煙法), solid-phase extraction (固相抽出), high-performance liquid chromatography/tandem mass spectrometry (高速液体クロマトグラフィー／タンデム質量分析計)

緒　言

喫煙の習慣化は発がんリスクを高める可能性があると考えられており (1), その理由としてたばこ煙中には, 発がん性が認められている化学物質が約 250 種, 発がん性が疑われる物質は約 50 種が含まれていることが挙げら

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Reprint requests to: Yohei INABA
Department of Environmental Health, National Institute of Public Health, Minami 2-3-6, Wako, Saitama 351-0197, Japan
FAX: +81(48)458-6270
E-mail: yohei_inaba@niph.go.jp

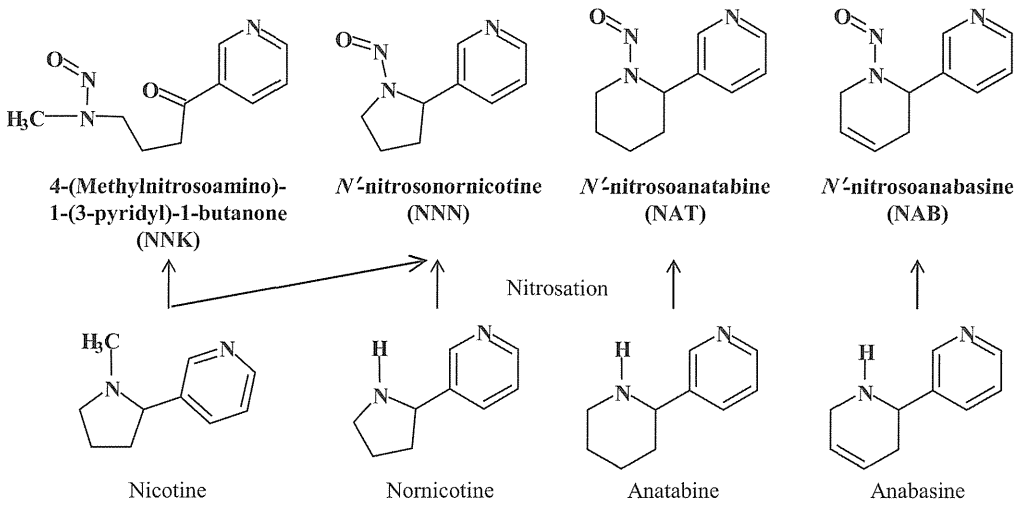

Fig. 1　Formation and generation of TSNAs. 4 tobacco-specific *N'*-nitrosamines (above), 4 alkaloids in tobacco (below).

れる (2)。このたばこ煙中発がん関連物質には，たばこ特異的ニトロソアミン類 (tobacco-specific *N'*-nitrosamines; TSNA) も含まれている。TSNA は，ヒトの肺腺癌に関係があり，さらに実験動物による先行研究でも肺腺癌の発生が報告されている (3)。特に，*N'*-nitrosonornicotine (NNN) と 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) は肺での悪性腫瘍を誘発すると報告されている (3)。TSNA はたばこ葉中のアルカロイド (nicotine, nornicotine, anatabine および anabasine) と亜硝酸や硝酸が反応することで，上記アルカロイドがニトロソ化し，4 種類生成される (4) (Fig. 1)。このニトロソ化反応はたばこ葉の発酵，たばこの製造過程や燃焼時に熱合成により起こるといわれている (5)。さらに，TSNA は，たばこ葉の発育時に環境中の温湿度が高いほど生成量は増加することが分かっている (6)。このように，TSNA は，たばこ葉の発育，たばこ製造工程そして喫煙時等の多くの過程で生成し，最終的に主流煙に含まれ喫煙者の体内に吸収されることから，たばこ主流煙中 TSNA 濃度を測定することは喫煙者の健康影響を評価する上で必要な資料になると考えられる。

現在，たばこ煙中化学物質の測定は，国際標準化機構 (International Organization for Standardization; ISO) が定める機械喫煙法 (ISO 法) によりたばこ主流煙を捕集し，各種化学分析手法を用いて行われている。これらを用いたたばこ主流煙中 TSNA の測定報告は海外で多数されている (6–14) が，国産たばこ主流煙中 TSNA 測定に関する報告 (15, 16) はわずかである。また，近年，国内のたばこ販売は低タール・低ニコチンたばこが多くを占め，特に外箱表示量がタール 1 mg・ニコチン 0.1 mg のたばこ銘柄の販売本数は，2009 年度に全体の 24.5% であった (17)。この低タール・低ニコチンたばこを ISO 法により捕集した場合，主流煙中 TSNA の濃度は海外の報告よりも低いと推測した。したがって，国産たばこ主流煙中 TSNA 測定法には濃縮操作を含めた前処理法を確立することが必要になる。一方，これまでの研究から，喫煙者が日常的に用いているたばこを低タール・低ニコチンたばこに替えたとき，必要摂取量のニコチンを体内へ取り込もうとするため，喫煙行動パターンが変化することが分かっている (18)。具体的には，吸煙回数が増加したり，一服をより深く吸い込むといった変化が生じることが報告されている。加えて鈴木らは，ニコチン量が 0.6 mg 未満の日本人喫煙者は，ニコチン欲求を満たすため，1 回の吸煙量が多くなる (代償性補償喫煙) 傾向があると報告している (19)。また，その平均吸煙量は 58 mL であり，このような吸煙量の増加が TSNA の曝露量の増加に繋がると推測される。さらに近年，カナダ保健省はヒトに近い喫煙法として別の機械喫煙法 (HCI 法) を提唱している。この HCI 法での吸煙量は 55 mL であることから，HCI 法を用いて主流煙中 TSNA を測定することは，曝露実態の推定に繋がると考えられる。

これまでの先行研究で報告された主流煙中 TSNA 測定法は，主にガスクロマトグラフィー / 熱エネルギーアナライザ (GC/TEA) を用いている (6–9, 15, 16)。一方，近年では検出感度が良く，選択性の高い高速液体クロマトグラフィー / タンデム質量分析計 (LC-MS/MS) を用いた手法も報告されている (10–14)。この LC-MS/MS を用いた測定は，前処理方法にフィルター濾過を行う方法 (11, 12) の他に，合成吸着剤による固相抽出法 (13)，液–液抽出法と固相抽出法を組み合わせた前処理方法 (14) なども報告されている。

そこで本研究では，海外たばこより外箱表示タール量が低い国産たばこに適した固相抽出法を用いて主流煙抽出液から測定妨害物質を除去および TSNA を濃縮する前処理法を確立し，LC-MS/MS を組み合わせた TSNA の測定法を検討した。また，確立した手法を用いて，ISO 法および HCI 法により捕集した国産たばこ 10 銘柄の主流煙中 TSNA を測定した。

材料と方法

1）各種試薬

N'-nitrosonornicotine（NNN），4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone（NNK），*N'*-nitrosoanatabine（NAT）と*N'*-nitrosoanabasine（NAB）およびTSNAの重水素体（NNN-d_4, NNK-d_3, NAT-d_4, NAB-d_4）はTronto Research Chemicals社製を使用した。メタノールおよび酢酸は和光純薬社製のLC/MS用を使用した。アセトニトリルはSigma-Aldrich社製，酢酸アンモニウムは和光純薬社製のHPLC用を使用した。なお，HPLCと試薬調製用の純水にはMillipore社製のMilli-Qシステムを使用した。

2）各種試薬の調製

各TSNA（NNN, NNK, NAT, NAB）およびTSNAの重水素体（NNN-d_4, NNK-d_3, NAT-d_4, NAB-d_4）はアセトニトリルに溶解し，TSNAとTSNA-*d*は各々1 μg/mLになるよう混合調製した。酢酸アンモニウムはMilli-Q水に溶解して100 mMに調製し，TSNAの抽出に用いた。酢酸は，各々0.1%になるようにメタノールおよびMilli-Q水に添加し，HPLCの移動相に用いた。

3）たばこ試料および主流煙の捕集

たばこ試料は2006年度の国内販売量上位10銘柄を用いた。たばこの恒温・恒湿化は，ISO 3402（1999）（20）に準じ，たばこ主流煙の捕集前に48時間（最短）から10日間（最長），温度22±2°C，湿度60±3%で行った。

たばこ主流煙の捕集には，半自動喫煙装置（Borgwaldt KC社製）を用いた。捕集中はISO 3308（2000）（21）に準じ，温度22±2°C，湿度60±5%の条件下で以下に示すISO法およびHCI法で捕集を行った。

ISO法：ISO 4387（2000）（22）に準じ，以下の条件で捕集した。

- 吸煙量：35 mL
- 吸煙時間：2秒
- 吸煙間隔：60秒
- フィルター部の通気孔：塞がずに捕集

HCI法：T-115(1999)(23)に準じ，以下の条件で捕集した。

- 吸煙量：55 mL
- 吸煙時間：2秒
- 吸煙間隔：30秒
- フィルター部の通気孔：メンディングテープで完全に塞いで捕集

上記条件により，たばこ主流煙はcambridge filter pad（CFP）（44 mm, Borgwaldt KC社製）1枚につきたばこ5本分（ISO法）もしくは3本分（HCI法）の粒子成分を捕集し，1試料とした。各銘柄毎に5試料作り，それぞれ測定に供した。

4）主流煙中TSNAの前処理

たばこ主流煙を捕集したCFPは，100 mL容共栓付三角フラスコに入れ，TSNA-*d*溶液（1 μg/mL）400 μLを添加後，100 mM酢酸アンモニウム溶液を40 mL加えた。三角フラスコはアルミホイルで遮光し，250 rpmで30分間振盪抽出を行った。次に抽出液は50 mL容プラスチック遠沈管に移し，アルミホイルで遮光して4～10°Cで一時保存した。抽出液は，固相抽出直前に3,000 rpmで5分間遠心分離（05P-21, HITACHI社製）し，上清を回収した。上清10 mLは，3 mLのメタノールとMilli-Q水で活性化を行ったDiscovery DSC-18Lt カラム（500 mg/3 mL, Supelco社製）に導入した。次に10%メタノール（5 mL）で洗浄後，70%メタノール（5 mL）で溶出することとした。得られた溶出液は窒素気流下にて1 mL以下にした。この濃縮液は100 mM酢酸アンモニウム溶液で1 mL容メスフラスコに定容し，遠心用チューブに移した。この溶液は8,000 rpmで5分間遠心分離を行い，上清を1 mLシリンジと濾過フィルターで濾過し，分析用試料とした。

5）LC-MS/MSによるTSNAの分析

HPLC測定条件

TSNA分析には，HP 1100シリーズのデガッサー，ポンプおよびカラムオーブン（Hewlett Packard社製）にAgilent 1200シリーズのオートサンプラー（Agilent Technologies社製）を備えた装置を使用した。高速液体クロマトグラフィー用分離カラムは，プレカラムフィルター（0.5 μm, Supelco社製）を繋げたZorbax Eclipse XDB C-18カラム（2.1×150 mm, 3.5 μm, Agilent Technologies社製）を使用した。カラムオーブン温度は40°Cとし，試料注入量は10 μLとした。また，移動相には0.1%酢酸水溶液（A液）と，酢酸0.1%を含むメタノール溶液（B液）を用いた。送液プログラムは流量を200 μL/分とし，0分（A液：80%, B液：20%），0→8分（A液：40%, B液：60%），8→10分（A液：40%, B液：60%），10→12分（A液：80%, B液：20%）と設定し，分析時間は35分とした。

質量分析法

質量分析には三連四重極型質量分析器Micromass Quattro LC（Micromass UK社製）を用いた。イオン化モードはESI+/MRMを用い，キャピラリー電圧は3.5 kVとし，各イオンは500 m秒でモニターした。コリジョンエネルギーとコーン電圧は分析種ごとに適切な条件を設定し(24)，イオンモニタリング法には，TSNAおよびTSNA-*d*を検出する時間帯ごとにMRMチャンネルを区切り，各TSNAおよびTSNA-*d*のみ測定する方法（segment法）を用いた（Table 1）。なお，Fig. 2に標準溶液でのクロマトグラフを例示した。

6）統計解析

各銘柄の主流煙中TSNA濃度についてたばこ銘柄の分類と喫煙方法の二要因に関して二元配置分散分析を実施した。さらに喫煙法について層別化後，一元配置分散分析を行った。コンピュータ統計解析用ソフトウェアはSPSS Ver.16.0を使用し，有意水準はすべて5%とした。

Table 1　Monitoring conditions of TSNAs and its internal standards

Analyte	*m/z*			(min)	(eV)	(V)
	Precursor ion		Product ion	Monitoring time	Collision energy	Cone voltage
NNN	178	>	148	0–6	10	17
NNN-d_4	182	>	152		11	17
NAT	190	>	160	6–9	10	17
NAT-d_4	194	>	164		10	17
NAB	192	>	162		11	12
NAB-d_4	196	>	166		11	12
NNK	208	>	122	9–13	12	17
NNK-d_3	211	>	122		11	17

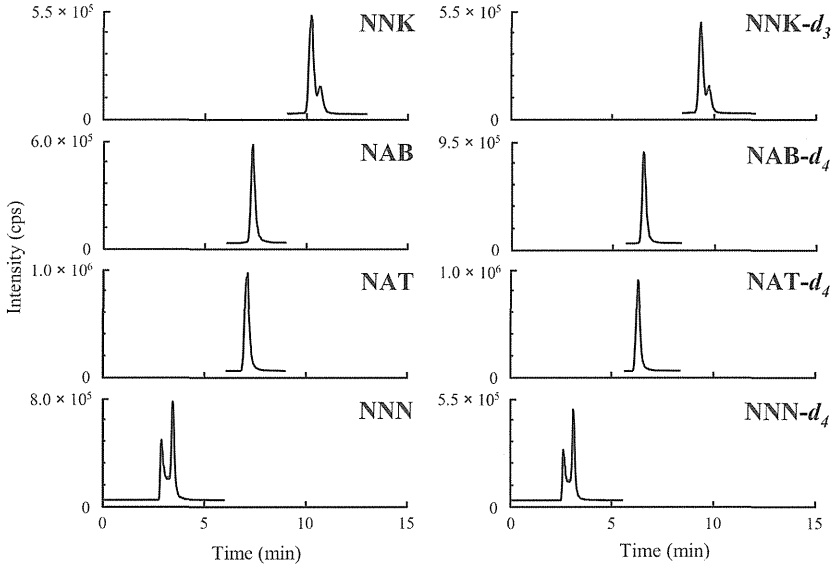

Fig. 2　Chromatogram of TSNAs by LC-MS/MS. The concentrations of TSNAs and TSNAs-*d* are 100 ng/mL.

結　　果

1) TSNA 検量線と固相抽出法条件検討

本測定での LC-MS/MS による TSNA 標準溶液の検量線結果を Table 2 に示す。各 TSNA の定量範囲はいずれも 0.5 または 1.0 ～ 200 ng/mL であり，相関係数も 0.9998 ～ 0.9999 と良好であった。

次に固相抽出カラムの洗浄および溶出液の条件検討を行った。まず TSNA（10 ng/mL）および TSNA-*d*（10 ng/mL）溶液 10 mL を固相抽出カラムに導入し，5，10，20，30，50，70% メタノール（5 mL）にて溶出試験を行った（n＝5）。その結果，メタノール濃度が 5，10，および 20% では全 TSNA，TSNA-*d* の溶出が認められなかった。30% メタノール溶出では NNK と NNK-d_3 の一部および NNN と NNN-d_4 の溶出が確認され，50% 以上で全ての TSNA，TSNA-*d* の溶出が認められた。また，TSNA および TSNA-*d* の回収率は，メタノール濃度が 50% では 102 ～ 113%，70% では 100 ～ 107% であった。以上の結果から，洗浄液のメタノール濃度は 10% とした。また，溶出液は 70% メタノール溶液とした。

また，固相抽出カラムへの試料導入液量を 5, 10, 15, 20, 30 mL として検討した。この条件検討には，国産たばこへの適用を目的として，たばこ外箱表示タール量が 1, 6, 14 mg のたばこ銘柄 Mild Seven One, Mild Seven Super Lights, Seven Stars を ISO 法と HCI 法の両喫煙法で捕集した計 6 検体を用いた。その結果，すべての試料とも試料導入量が 20 mL までに，TSNA 回収量が試料導入量に依存して増加したが，試料導入量が 30 mL の条件では，捕集タール量が多い HCI 捕集試料は，TSNA の回収量の低下が認められた。以上の結果から，試料導入量が 5 ～ 20 mL の範囲内で行うことが可能であった。そこで，本研究の試料導入量は 10 mL とした。

2) 同時再現性試験

上記固相抽出条件検討で用いた 3 銘柄の 2 喫煙法の主流煙抽出液（6 検体）を用いて，固相抽出と LC-MS/MS を組み合わせた主流煙中 TSNA の測定法の同時再現性試験を行った（検体毎に 7 回実施）。6 検体の各 TSNA の濃度範囲は，NNN が 9.4 ～ 86 ng/cig，NNK が 6.4 ～ 58 ng/cig，NAT が 11 ～ 100 ng/cig，NAB が 3.1 ～ 29 ng/cig であった。こ

Table 2　Regression equation, correlation coefficient values (r), limits of detection and liner range of TSNAs

Analyte	Internal standard	Regression equation	Correlation coefficient (r)	Limits of detection (ng/mL)	Liner range (ng/mL)
NNN	NNN-d_4	y=0.01689x+0.00806	0.9999	0.3	1.0–200
NNK	NNK-d_3	y=0.00970x+0.00640	0.9999	0.2	0.5–200
NAT	NAT-d_4	y=0.01084x+0.00613	0.9998	0.2	0.5–200
NAB	NAB-d_4	y=0.00581x+0.00128	0.9999	0.4	1.0–200

Table 3　Results of simultaneous reproductivity of TSNAs analysis in mainstream smoke from Japanese cigarettes (n=7)

Regime	Cigarette brands	TSNA (ng/cig)											
		NNN			NNK			NAT			NAB		
		Ave	SD	CV (%)	Ave	SD	CV (%)	Ave	SD	CV (%)	Ave	SD	CV (%)
ISO	Mild Seven One	9.4	± 0.2	2.1	6.4	± 0.1	1.9	11	± 0.1	1.2	3.1	± 0.1	2.4
	Mild Seven Super Lights	31	± 1.0	3.3	18	± 0.3	1.7	33	± 0.5	1.5	10	± 0.2	2.1
	Seven Stars	25	± 1.9	7.5	19	± 0.3	1.5	38	± 0.5	1.4	10	± 0.2	1.9
HCI	Mild Seven One	58	± 1.2	2.0	42	± 0.6	1.3	59	± 0.5	0.9	17	± 0.6	3.8
	Mild Seven Super Lights	86	± 3.1	3.6	58	± 0.7	1.1	100	± 2.5	2.4	29	± 0.5	1.6
	Seven Stars	57	± 2.0	3.5	50	± 0.8	1.5	78	± 1.9	2.4	21	± 1.0	4.9

※ Ave, average; SD, standard deviation; CV, coefficient of variation.

Table 4　Results of TSNAs in mainstream smoke from Japanese top 10 seller cigarette brands at 2006 (n=5)

Regime	Cigarette brands	Tar (mg)	Nicotine (mg)	Categori-zation	TSNA (ng/cig)							
					NNN		NNK		NAT		NAB	
					Ave	SD	Ave	SD	Ave	SD	Ave	SD
ISO	Pianissimo One	1	0.1	Ultra low	10	± 1.6	8.4	± 0.1	11	± 0.4	3.1	± 0.1
	Mild Seven One	1	0.1		9.4	± 1.7	6.3	± 0.5	12	± 1.4	3.3	± 0.3
	Mild Seven Extra Lights	3	0.3	Low	20	± 4.6	10	± 1.9	22	± 2.9	6.3	± 0.9
	Caster Mild	5	0.5		18	± 1.2	10	± 0.8	20	± 1.5	5.7	± 0.6
	Mild Seven Super Lights	6	0.5		35	± 1.4	20	± 0.4	39	± 0.6	11	± 0.6
	CABIN Mild	8	0.6	Medium	46	± 5.5	31	± 6.7	45	± 4.2	13	± 1.0
	Mild Seven Lights	8	0.7		43	± 2.3	26	± 2.4	45	± 2.9	12	± 0.6
	Mild Seven Original	10	0.8		46	± 3.4	28	± 4.2	54	± 6.4	15	± 1.9
	HOPE	14	1.1	High	26	± 3.0	20	± 3.1	38	± 3.2	10	± 0.5
	Seven Stars	14	1.2		21	± 3.0	18	± 1.9	36	± 2.5	9.3	± 1.0
HCI	Pianissimo One	1	0.1	Ultra low	59	± 4.8	67	± 12	65	± 2.5	16	± 1.1
	Mild Seven One	1	0.1		67	± 3.9	49	± 3.7	78	± 3.0	12	± 0.6
	Mild Seven Extra Lights	3	0.3	Low	85	± 17	45	± 3.8	89	± 10	24	± 2.7
	Caster Mild	5	0.5		53	± 3.2	36	± 2.9	66	± 3.3	18	± 1.0
	Mild Seven Super Lights	6	0.5		100	± 7.0	63	± 4.9	120	± 7.2	31	± 2.5
	CABIN Mild	8	0.6	Medium	110	± 12	68	± 5.9	110	± 16	32	± 4.9
	Mild Seven Lights	8	0.7		98	± 8.2	67	± 9.0	120	± 4.7	33	± 1.7
	Mild Seven Original	10	0.8		110	± 6.6	70	± 8.9	130	± 2.6	36	± 1.0
	HOPE	14	1.1	High	43	± 3.1	36	± 4.1	65	± 6.4	18	± 2.2
	Seven Stars	14	1.2		55	± 5.2	40	± 3.0	82	± 2.8	22	± 1.4

※ Ave, average; SD, standard deviation.

のときの各TSNA濃度の変動係数（CV）は，NNNが2.0～7.5%，NNKが1.1～1.9%，NATが0.9～2.4%，NABが1.6～4.9%であった（Table 3）。

3）国産たばこ主流煙中TSNAの測定

Table 4には，上記測定手法による国産たばこ10銘柄の主流煙中TSNA測定結果を示した。ISO法では，NNNが9.4～46 ng/cig，NNKが6.3～31 ng/cig，NATが11～

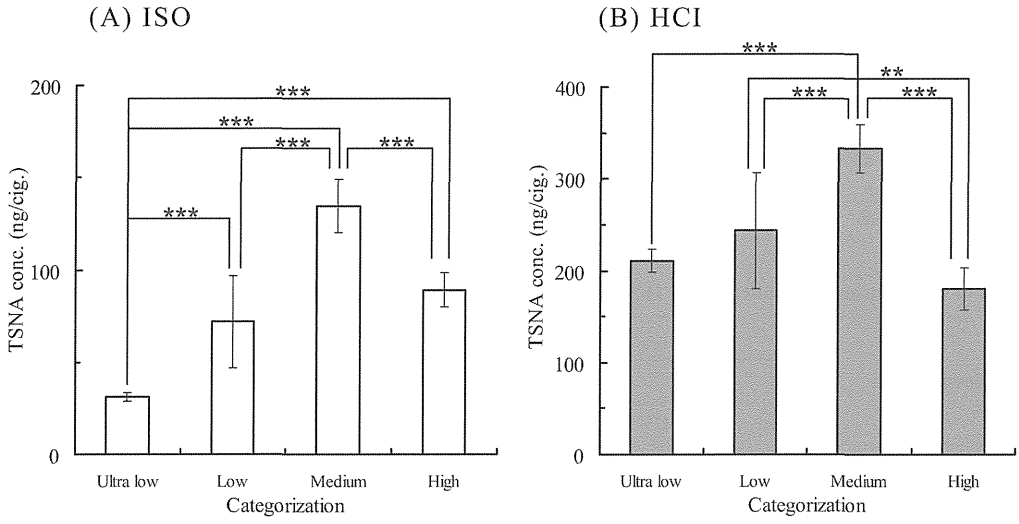

Fig. 3　Amounts of TSNAs in mainstream smoke from Japanese cigarettes. Category of cigarette samples is Ultra low (tar; 1 mg), Low (tar; 3–6 mg), Medium (tar; 8–10 mg) and High (tar; 14 mg). The differences in concentration among groups were as analyzed with one-way ANOVA following Scheffe's *post hoc test* (**: p<0.01, ***: p<0.001).

54 ng/cig, NAB が 3.1 ～ 15 ng/cig であった。一方, HCI 法では, NNN が 43 ～ 110 ng/cig, NNK が 36 ～ 70 ng/cig, NAT が 65 ～ 130 ng/cig, NAB が 12 ～ 36 ng/cig であった。Table 4 の各 TSNA の測定結果を喫煙法とたばこ外箱表示タール量の分類を要因とする二元配置の分散分析を行ったところ, TSNA 量には二要因の群間により有意に異なった (喫煙法 : $F(1, 92) = 468.186$, $p < 0.001$; たばこ外箱表示タール量の分類 : $F(3, 92) = 53.106$, $p < 0.001$)。また, 交互作用の効果も確認された (交互作用 : $F(3, 92) = 9.522$, $p < 0.001$)。次に喫煙法で層別化後, たばこ外箱表示タール量の分類の影響について一元配置分散分析を行った。その結果, ISO 法における表示タール量の分類による群間において TSNA 量は有意差が認められた ($F(3, 46) = 84.757$, $p < 0.001$)。Scheffe を用いた多重比較によれば, Ultra low は, Low, Medium 及び High との間に有意差があった ($p < 0.001$) (Fig. 3A)。同様に HCI 法における分散分析を行った。その結果は, ISO 法の結果と同様に表示タール量の分類群間において TSNA 量に有意差が認められた ($F(3, 46) = 35.740$, $p < 0.001$)。Scheffe を用いた多重比較によれば, Ultra low は, ISO の結果と異なり Medium との間にのみ有意差があった ($p < 0.001$) (Fig. 3B)。さらに, ISO 法と HCI 法の High の TSNA 量は, Medium より有意に低いことが確認された ($p < 0.001$)。

考　　察

1) 測定法の精度

本測定法で使用した LC-MS/MS の TSNA 定量下限濃度は, Wu らが報告した 0.2 ng/mL より高く 0.5 または 1.0 ng/mL であった (12)。しかしながら, 本測定法は TSNA 抽出液を固相抽出によって 10 倍濃縮するために, 最終的なたばこ 1 本あたりの主流煙中 TSNA 検出下限濃度は, ISO 捕集試料が 0.4 ～ 0.8 ng/cig, HCI 捕集試料が 0.68 ～ 1.36 ng/cig となった。この濃度は, 本研究の国産たばこ 10 銘柄の TSNA 測定に十分適用できる感度であり, Wu らの ISO 捕集による定量下限濃度範囲 0.360 ～ 0.656 ng/cig と同等の感度であった (12)。さらに最近の高感度 LC-MS/MS は, TSNA 定量下限濃度が 0.005 ～ 0.069 ng/mL といった報告もあるが (25), 国産たばこの主流煙中 TSNA を測定する LC-MS/MS には本測定法で使用した装置で十分であった。次に本測定法と TSNA 抽出液を直接試料注入し測定する Xiong らの手法と比較すると本手法の NNN の CV が 2.0 ～ 7.5% に対して 3.3 ～ 7.8%, NNK は 1.1 ～ 1.9% に対して 4.9 ～ 8.5%, NAT は 0.9 ～ 2.4% に対して 3.9 ～ 5.3% そして NAB は 1.6 ～ 4.9% に対して 4.6 ～ 7.3% であった (25)。本手法が若干ではあるが精度が良いことが分かった。この一因は, 固相抽出を行なうことによって TSNA の濃縮が行なわれ, その際に測定妨害物質も除去されているために若干の精度向上が認められたと推測している。

2) 国産たばこ銘柄の TSNA の傾向

Fig. 3 は, たばこ外箱表示タール量の分類 (4 区分) と総 TSNA 濃度との関係を示した。横軸は, 各たばこ外箱表示タール量を基に Ultra low (1 mg), Low (3 ～ 6 mg), Medium (8 ～ 10 mg), High (14 mg) の 4 区分に分類した。一方, 縦軸は, 各銘柄の総 TSNA 濃度を分類ごとに平均した値である。TSNA は, 国際がん研究機関 (International Agency for Research on Cancer; IARC) の発がん性リスク一覧において NNK と NNN が IARC グループ 1 に, NAT と NAB が IARC グループ 3 に分類された 4 種類で構成されており, たばこ 1 本を喫煙する場合, 4 種に同時に曝露される。そこで 1 本あたりの総曝露量を算出し, 先行研究との比較を行うために区分毎にこの平均値を算出し

た。ISO法によるUltra low, Low, MediumおよびHighの平均TSNA測定結果は，各々31，72，130および89 ng/cigであった（Fig. 3A）。また，HCI法の測定結果は各々210，240，330および180 ng/cigであった（Fig. 3B）。主流煙中TSNA濃度が高い区分は，Mediumであった。また，HCI法で捕集したUltra lowとHighのTSNA測定結果は，210と180 ng/cigであり，喫煙法によっては必ずしもHighたばこが有害化学物質の濃度が高くなることはなかった。本研究測定結果と統計解析から，ISO及びHCIそれぞれの喫煙法による主流煙中のTSNA濃度は，表示タール量による分類において有意に異なったが，外箱表示量に示された濃度差は認められなかった。さらにHCI法とISO法で捕集した測定結果の比（HCI/ISO）は，Ultra lowが6.7, Lowが3.3, Mediumが2.5でHighが2.0であることから，タール，ニコチン表示量が低いたばこの主流煙TSNA量は，喫煙法の影響を受けやすいことが分かった。このHCI/ISO比の結果は，Endoらの同10銘柄のタール，ニコチン濃度のHCI/ISO比と同様の傾向であった（26）。

次に，喫煙者のTSNA曝露量を推計する喫煙法について評価を行なった。Jarvisらは，ニコチン表示量が0 mg以上のたばこを最大1 mgまで0.1 mgずつ区分し，それぞれの区分に該当する喫煙者1本あたりのニコチン取り込み量と比較したところ，ニコチン取り込み量は，0.97～1.39 mgの範囲であった（27）。またこのときニコチン取り込み量と表示ニコチン量との比は，低ニコチン喫煙者ほど高い数値が得られた。次に，Kozlowskiらは低ニコチン表示たばこのフィルター通気孔が極めて重要な特徴であると指摘している（28）。これによって喫煙者が通気孔を唇や指で塞ぎ，深く吸い込むといった代償性補償喫煙の大きな原因の一つになると報告している。さらに，鈴木らは，代償性補償喫煙行動が認められた喫煙者（Ultra low及びLowたばこ喫煙者）の一回の吸煙量が58 mLであると報告している（19）。よって，一回の吸煙量が55 mLで，通気孔を塞ぐ喫煙法であるHCI法で捕集し，有害化学物質量を測定することは有効であると考えられる。さらに鈴木らの報告によると上記以外のMediumとHighの喫煙者も吸煙量が50 mLであることから，Medium以上のたばこ喫煙者でもISOの測定結果以上の曝露量になることが推測できる。特に，国内販売本数の多いUltra lowたばこは，喫煙行動によって，主流煙中TSNA濃度の変動が大きいことが推測されることから，ISO法とHCI法で主流煙を捕集し測定することで，喫煙者の曝露されるTSNA量の下限から上限と考えることが良いのではないかと推測できる。

本研究において，Highに分類されたたばこ主流煙のTSNA量がMediumと比較すると低い濃度であった（Fig. 3）。紙巻たばこは，数種類のたばこ葉を混合して製品化している（29）。よって，今回，測定対象としたHighに分類されたたばこ銘柄ではTSNA量が低いたばこ葉を使用しているのではないかと考えている。

現在，主流煙中TSNAのような発がんリスクのある有害化学物質の濃度が外箱に表示されていない。そのため，喫煙による有害性を判断するには，タール，ニコチン以外の化学物質を公表することも重要であると考えられる。

3）国産たばこと海外たばこ主流煙TSNAとの比較

今回測定したTSNAについて厚生労働省が平成11–12年度の測定結果を公開している（16）。国産たばこのISO法による主流煙中TSNA量は今回測定した国産たばこと同銘柄同士を比較すると総TSNA量は27～57%減少していることが分かった。しかしながら，Rickertらがカナダ産たばこ6銘柄の主流煙中TSNA量を同様に報告しており，2003–2005年の間に主流煙中総TSNA量がISO法で最大66%，HCI法で最大71%削減していた（30）。また，主流煙中総TSNA濃度は2005年の時点でも最大でISO法では39 ng/cig, HCI法では73 ng/cigと，日本産たばこのそれぞれ130 ng/cig（ISO），330 ng/cig（HCI）よりも大幅に低減していることが分かった。このことから，国産たばこにおいてもTSNAを含む有害物質の削減を実施していることが示唆されたが，海外と同程度の有害物質の大幅な減量には至っていないことが分かった。

現在，たばこ主流煙中TSNAのリスク評価書は作成されていないため，生体影響評価をすることは難しいが，今回の研究データが喫煙者の生体影響評価に資するデータになるものと期待している。

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