Nicotine, Tar, and Mutagenicity of Mainstream Smoke Generated by Machine Smoking with International Organization for Standardization and Health Canada Intense Regimens of Major Japanese Cigarette Brands

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Based upon the Framework Convention on Tobacco Control (FCTC), the World Health Organization (WHO) has recommended that health authorities disclose toxicological properties of cigarette mainstream smoke (MSS) obtained not only according to US Federal Trade Commission (FTC)/International Organization for Standardization (ISO) conditions but also by more intense conditions such as the Health Canada Intense (HCI) condition. This is because smokers are believed to smoke more intensely than machine smoking under the ISO regimen. Because there are no previous reports on the toxicological properties of MSS of Japanese cigarettes under the HCI condition, we determined nicotine and water contents by gas chromatography (GC-MS and GC/Thermal Conductivity Detector (TCD)) for three product lots each of the ten bestselling brands of Japanese cigarettes following the WHO protocol. One of the three lots of each MSS condensate was also resolved in dimethylsulfoxide and investigated by Ames preincubation assay using *Salmonella typhimurium* TA100, TA98, and YG1024 strains with and without metabolic activation (rat liver S9 mix). Nicotine and tar yields with the HCI regimen were higher than those with ISO, the latter being very close to the values described on the packages of each cigarette brand. Mutagenicity was mainly observed in TA98 and YG1024 with metabolic activation. Mutagenic activity of MSS with the HCI regimen was 1.4–9 times higher than that with the ISO regimen. Based on YG1024 with activation, high activities were observed in several "low yield" brands. The activity of "low-yield" brands with the HCI regimen was not always lower than that of regular-yield brands with the ISO regimen. These results suggest that "low yield" cigarettes do not result in reduced exposure or reduced risk to humans.

Key words —— cigarette smoke, smoking machine, *Salmonella* mutagenicity, nicotine, tar

INTRODUCTION

Cigarette smoke contains > 4000 chemical toxicants.[1)] Although many reports describe the toxicological properties of cigarette mainstream smoke (MSS), most assessments have been made under the Federal Trade Commission (FTC)/International Organization for Standardization (ISO) condition: 35 ml puff volume, 60 sec puff interval, 2 sec puff duration, and no blocking of ventilation holes. In Japan, only nicotine and nicotine-free dry particulate matter (NFDPM; also known as "tar") yields have been regulated—by the Ministry of Finance—and the measurement criteria are similar to the FTC/ISO methods.[2)] However, it has been demonstrated that smokers smoke their cigarettes more intensely than is simulated with smoking machines operated under the FTC/ISO conditions.[3)] Furthermore, some governmental agencies have adopted regulations that specify more intensive protocols, such as that of Health Canada Intense (HCI): 50 ml puff volume, 30 sec puff interval, 2 sec puff duration, and 100% blocking of ventilation holes.[4)]

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Table 1. Cigarette Samples Tested

Brand name		Tar (mg/cig)	Nicotine (mg/cig)	Filter type[a)]	Vent. Hole	Market Share (%)
A	PIANISSIMO One	1	0.1	P	2	1.6
B	MILD SEVEN ONE	1	0.1	DC	4	3.8[b)]
C	MILD SEVEN EXTRA LIGHTS	3	0.3	DC	2	3.5
D	Caster MILD	5	0.4	NC	2	2.9
E	MILD SEVEN SUPER LIGHTS	6	0.5	DC	2	7.7
F	CABIN MILD	8	0.6	NC	1	1.9
G	MILD SEVEN LIGHTS	8	0.7	DC	1	7.3
H	MILD SEVEN ORIGINAL	10	0.8	DC	1	6.0
I	HOPE	14	1.1	P	0	1.4
J	Seven Stars	14	1.2	DC	0	6.8[b)]

a) P: plain, DC: dual charcoal, NC: neo charcoal. *b*) Shares include box type package. Source: JT Annual Report 2006.

The World Health Organization (WHO) Framework Convention on Tabacco Control (FCTC) criteria were adopted by the World Health Assembly in May 2003 and the Japanese government ratified the FCTC on June 8, 2004. Based on the WHO FCTC, the WHO has organized a laboratory network, TobLabNet, bringing together laboratories belonging to the health sectors in each participating country. TobLabNet's goals include establishing a global tobacco testing and research capacity, testing tobacco products for regulatory compliance, and providing information to risk assessment activities related to the use of tobacco products.[5)] As a part of the TobLabNet research activities, we investigated nicotine and tar yields in MSS of major Japanese cigarette brands and demonstrated *Salmonella* mutagenicity of their tar.

MATERIALS AND METHODS

Reagents —— *Nicotine*: (−)-Nicotine (Wako Pure Chemical Ltd., Osaka, Japan) was used.

Solvents: Super special grade 2-propanol (Wako Pure Chemical Ltd.) and fluorometric analysis grade dimethylsulfoxide (DMSO; Dojin Chemical Laboratory Ltd., Kumamoto, Japan) were used.

Metabolic Activation System —— S9/Cofactor A set for the Ames test (S9 mix; Oriental Yeast Co. Ltd., Osaka, Japan) was used. S9 mix contained 50 μl of S9 (23.1 mg protein/ml) prepared from livers of male Sprague-Dawley rats intraperitoneally administered phenobarbital and 5,6-benzoflavone at a total volume of 500 μl.

Cigarettes Tested —— We tested the ten best-selling Japanese cigarette brands. According to the TobLabNet protocol, each brand was purchased in ≧ 3 shops in the Tokyo area (*i.e.*, three product lots were obtained). Table 1 shows characteristics of the cigarettes tested.

Machine Smoking —— Smoke was collected by ISO harmonized Borgwaldt LM1 smoking machine (Borgwaldt KC GmbH, Hamburg, Germany). Cigarettes were conditioned according to ISO 3402 (ratified in 1999).[6)] Total particulate matter (TPM; also known as "crude tar") was collected by 44-mm filter pad (Cambridge). ISO smoking was carried out in accordance with ISO 4387 (ratified in 2000).[7)] The HCI protocol was performed according to Health Canada method T-115.[4)]

Extraction —— After determination of TPM, pads were extracted with 2-propanol by gently shaking using an electric shaker at room temperature for 20 min in accordance with ISO 4387.

Nicotine and Water Contents —— Nicotine in the 2-propanol solution was determined in accordance with ISO 10315 (2000),[8)] with slight modification using GC-MS (GC: Hewlett Packard, CA, USA, HP6890, MS: Agilent, CA, USA, 5975 inert/N) and a capillary column, DB-17 (30 m × 0.25 mm I.D. × 0.25 μm, J&W Scientific, CA, USA).[9)] The oven temperature program was as follows: initial temperature was at 50°C for 2 min, increased to 200°C at the rate of 15°C/min, then up to 280°C at the rate of 5°C/min, and held at 280°C for 5 min. Carrier gas was helium; flow rate was 1.0 ml/min. The injection was splitless mode at 250°C. Ionization mode was the electron impact ionization (EI) method, and temperature of the ionization chamber was 230°C. Selective ion monitoring (SIM) mode was used for measurement. The mass numbers of target ion and monitor ion (m/z) were 84 and 161, respectively for nicotine and 129 for isoquinoline as an internal standard. Determination of water in 2-propanol solution was carried out in accordance with ISO 10362-1 (1999) using GC/TCD

Table 2. Nicotine, Water, and Particulate Matter Yields of Ten Major Japanese Cigarette Brands

Cigarette	Regimen	Chemical yields (mg/cigarette)							
		TPM		Nicotine		Water		NFDPM	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
A	ISO	1.62	0.11	0.20	0.01	0.43	0.06	1.00	0.08
	HCI	21.02	0.95	0.89	0.01	5.17	0.64	14.96	0.85
B	ISO	1.96	0.44	0.19	0.03	0.36	0.21	1.41	0.39
	HCI	20.26	1.91	0.97	0.07	5.70	1.30	13.60	0.61
C	ISO	4.25	0.80	0.32	0.02	0.67	0.06	3.26	0.77
	HCI	25.18	1.48	1.28	0.19	8.13	0.65	15.78	1.75
D	ISO	6.56	0.93	0.47	0.06	1.13	0.31	4.98	0.56
	HCI	30.93	0.36	1.29	0.07	10.08	0.33	19.56	0.56
E	ISO	7.75	0.61	0.53	0.05	1.13	0.15	6.07	0.47
	HCI	33.38	0.90	1.40	0.08	10.52	0.57	21.44	1.37
F	ISO	10.32	0.16	0.63	0.03	1.93	0.15	7.77	0.12
	HCI	34.19	1.29	1.46	0.08	10.77	0.48	21.94	0.99
G	ISO	10.55	0.16	0.66	0.05	1.63	0.06	8.26	0.12
	HCI	35.46	1.05	1.52	0.09	11.84	0.70	22.10	1.25
H	ISO	12.61	0.77	0.78	0.12	1.57	0.12	10.26	0.55
	HCI	39.51	2.12	1.81	0.17	12.60	1.66	25.07	0.90
I	ISO	18.29	1.91	0.95	0.06	4.11	0.64	13.23	1.23
	HCI	47.51	4.58	2.04	0.04	15.97	0.95	29.50	4.04
J	ISO	18.70	1.59	1.11	0.13	3.14	0.69	14.55	0.84
	HCI	45.31	1.23	2.21	0.20	14.40	0.78	28.70	0.83

(Shimadzu, Kyoto, Japan GC2014) and a column, Porapack Q (2.0 m × 3.0 mm I.D., 80–100 mesh deactivated stainless) at a temperature of 170°C.[10] Carrier gas was helium; flow rate was 30 ml/min. Both injector and detector temperature was 250°C.

Mutagenicity Test —— One lot of each cigarette brand was tested for mutagenicity using the Ames assay. After solvent exchange with DMSO, the mutagenicity of TPM was assessed by preincubation method[11] using *Salmonella typhimurium* TA100, TA98, and YG1024[12] strains. Tests were performed both with and without metabolic activation (S9 mix).

RESULTS

Nicotine and Particulate Matter

Table 2 shows means and standard deviations of TPM, nicotine, water, and NFDPM for the three product lots of each cigarette brand under both smoking regimens. Results based on the ISO regimen were close to the values described on the cigarette packages. Results with the HCI regimen, however, were higher for every brand. Relative standard deviations (RSD) across the three lots ranged from 1.2% to 22.4% for TPM, 1.1% to 15.8% for nicotine, 3.3% to 58.3% for water, and 1.5% to 27.7% for NFDPM. RSD for water content was generally higher than for TPM, nicotine, and NFDPM,[13,14] indicating that the results reported here are reasonable.

Mutagenicity

Dose-response curves for the Ames assay of one cigarette sample are shown in Fig. 1. No samples exhibited killing toxicity at least up to the top dose by microscopic examination of the background lawn in this study. The NFDPM contents (mg per cigarette) and mutagenic activities (revertants per cigarette) of each cigarette brand are shown in Table 3. Mutagenic activity was calculated from the slope of the linear portion of the dose-response curve using linear regression. If revertant rate was at least twice the spontaneous revertant rate in the control, it was judged "positive"; if it was between one half and twice the spontaneous rate, it was judged "pseudopositive." Pseudopositive results in Table 3 are shown in parentheses. If revertant rate was less than one half the spontaneous rate, it was judged "negative" ("neg" in Table 3). All brands were mutagenic for frameshift-type strains (TA98 and YG1024) with S9 mix, but most were negative without S9 mix, as exemplified by Fig. 1. With the base-pair substitute-type strain (TA100), among 20 samples, 9 were positive, 7 were pseudopositive,

Table 3. Mutagenic Activity of Ten Major Japanese Cigarette Brands

Cigarette	Regimen	Tar (mg/cig)	Mutagenic activity (revertants/cigarette)					
			TA100		TA98		YG1024	
			−S9	+S9	−S9	+S9	−S9	+S9
A	ISO	0.9	neg	neg	neg	1690	neg	8140
	HCI	15.4	4320	3970	neg	13200	1300	74000
B	ISO	1.5	neg	neg	neg	2040	neg	14100
	HCI	13.6	3030	neg	1590	12200	neg	62700
C	ISO	3.9	(1520)	neg	(680)	4790	neg	24900
	HCI	17.1	(4180)	neg	(2090)	13600	neg	71600
D	ISO	5.5	(4900)	(1760)	neg	4820	neg	29600
	HCI	19.9	15200	11100	neg	18000	neg	85100
E	ISO	6.3	9060	(3590)	neg	9210	neg	38500
	HCI	22.8	26600	9420	neg	23700	neg	122000
F	ISO	8.3	(1620)	3190	neg	5650	(750)	57300
	HCI	20.2	(5560)	7220	neg	14200	(2020)	106000
G	ISO	8.1	11200	5370	neg	13300	neg	78600
	HCI	24.4	24900	(6860)	neg	20300	neg	147000
H	ISO	10.5	(5100)	(3860)	neg	10800	neg	80900
	HCI	25.9	20000	17600	neg	31200	neg	205000
I	ISO	14.2	(3450)	(3250)	neg	7450	(1340)	50500
	HCI	32.8	neg	(9080)	neg	11700	(3270)	108000
J	ISO	14.8	neg	(6110)	neg	7710	neg	43200
	HCI	30.3	29100	(5170)	neg	17000	neg	58500

neg: negative; numbers in parenthesis indicate pseudopositive results.

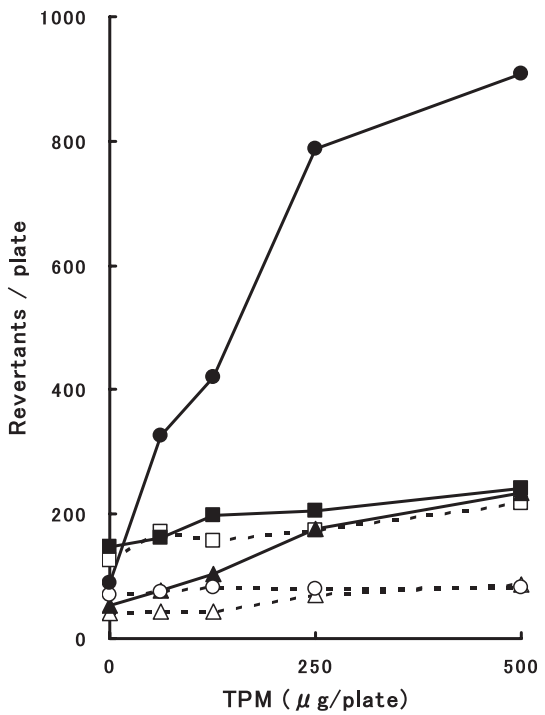

Fig. 1. Ames Assay dose Responses for Cigarette Mainstream Smoke (MSS) Obtained with a Smoking Machine (Sample H/HCI regimen)

The assays were conducted using the preincubation method with *Salmonella* TA100, TA98, and YG1024 strains either with (+) or without (−) rat liver S9 mix. □ TA100−S9; △ TA98−S9; ○ YG1024−S9; ■ TA100+S9; ▲ TA98+S9; ● YG1024+S9.

and 4 were negative without S9 mix; 7 were positive, 8 were pseudopositive, and 5 were negative with S9 mix. The highest activity was observed in Sample H (205000 revertants per cigarette for YG1024+S9).

DISCUSSION

Since the late 1950 s, consumption of cigarette products with filter tips (advertised as having reduced tar and low-yield nicotine) has increased significantly in most countries. Presently, few people smoke unfiltered cigarettes such as those commonly consumed in the 1950 s. Although the first filtered cigarette brand, Hope (sample I in this study), was introduced into the Japanese market as early as 1957, filtered cigarettes caught on rather late. In 1960, the market share was only 3%, but it then increased more rapidly than in other countries, reaching 96% in 1973.[15] Lower emissions of tar and nicotine—as measured by machine—were achieved by product engineering within the tobacco industry, most notably through the use of highly efficient filter tips, filter tip ventilation, reconstituted tobacco, and porous cigarette paper.[16] In spite of advertisements by tobacco companies, it is question-

able whether cigarettes having a low yield of tar are truly "mild" or "health concerned."

All ten brands tested in this study are filtered cigarettes. Yields of nicotine and tar have declined in proportion to improvements such as number of ventilation holes. Our results with the ISO regimen were almost identical to yields presented on package labels by the Tobacco Institute of Japan. On the other hand, results with the HCI regimen were higher than those with ISO for every brand: the ratio (HCI/ISO) ranged from 1.99 (brand J) to 5.11 (B) for nicotine, and from 1.97 (J) to 14.96 (A) for tar. Generally, the lower the yield the higher the HCI/ISO ratio for nicotine and tar.

Neither the FTC nor the ISO method for determining tar and nicotine yield is based on scientific assessment of human smoking behavior.[3] Recognizing the limitations of these methods, alternative protocols have been developed in Canada and by the state of Massachusetts in the U.S.A. The Canadian method aims to provide smokers with more meaningful values to estimate levels of toxicity. The Massachusetts method produced nicotine yields twice as high as those found with the FTC method.[17]

Another problematic issue is compensatory smoking behavior. Smokers have been found unsatisfied by low-nicotine or nicotine-free cigarettes and often modify their smoking pattern so as to regulate their nicotine intake.[3] Many smokers switching from high- to low-yield products will adopt several maneuvers simultaneously to compensate for lower nicotine yield in MSS, and will maintain these behaviors for as long as they continue to smoke lower-yield cigarettes. Our unpublished study on smoking behavior in Japanese smokers supports these results (Matsumoto *et al.*, manuscript in preparation).

All brands tested herein were mutagenic for frameshift-type strains with metabolic activation, and their mutagenic activities with the HCI regimen were higher than those with ISO (with ratios ranging from 1.5 to 7.8 for TA98 and from 1.4 to 9.1 for YG1024). Tar yields and mutagenic activities with YG1024+S9 are illustrated in Fig. 2. Tar yields increased with the values presented on package labels under both ISO and HCI as shown in Fig. 2(A). On the other hand, as shown in Fig. 2(B), mutagenic activities of regular-yield brand cigarettes (I and J) were less than those of low-yield brands (G and H). In particular, the specific activity of J (tar yield on package label: 14 mg) under the HCI regimen was less than those of G (8 mg) and H (10 mg) under the ISO regimen. Furthermore, activities of B (1 mg), C (3 mg), E (6 mg), G, and H under the HCI regimen were higher than that of J with the ISO regimen, despite the observation that brands B, C, E, G, and H are low-yield series of brand J. In short, the activities of so-called low-tar brands under the HCI regimen are not always less than that of the parent brand under the ISO regimen. This tendency was more clear in the case of revertants per mg of tar as shown in Fig. 2(C). These results suggest that "low tar" cigarettes do not result in reduced exposure or reduced risk to humans.

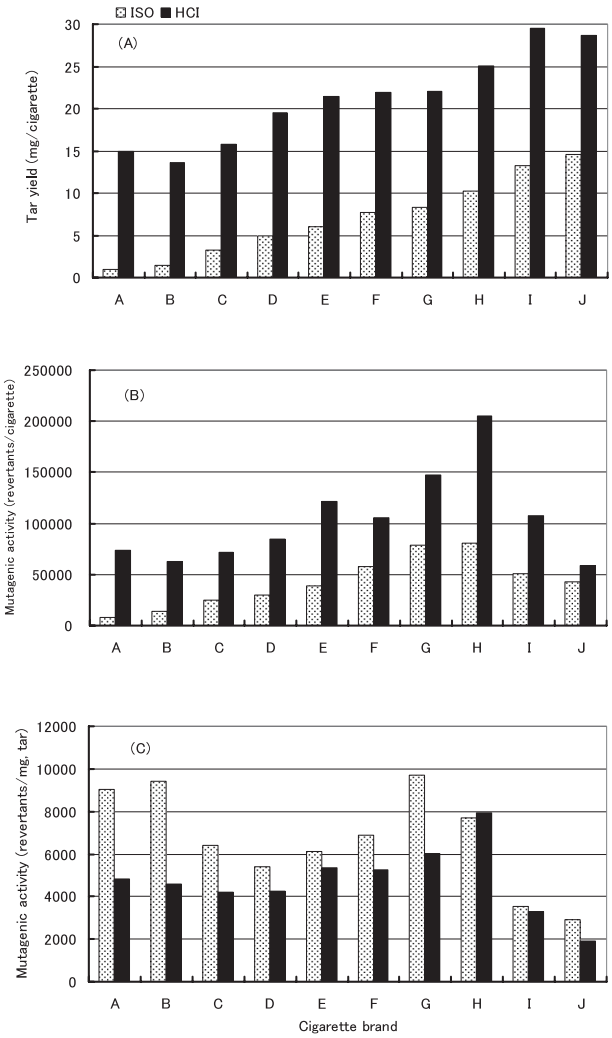

Fig. 2. Tar Yield (A; mg/cigarette) and Mutagenic Activity (B; revertants/cigarette, C; revertants/mg, tar) of Ten Major Japanese Cigarette Brands

Dotted bars represent results with the ISO regimen; closed bars those with the HCI regimen. "Tar" means NFDPM. Mutagenicity was tested using the preincubation procedure with *Salmonella* YG1024 strain and S9 mix.

From our results, the most effective mutagens are assumed frameshift-type with metabolic activation. Furthermore, their mutagenic activity

is enhanced by o-acetyltransferase. Heterocyclic amines are representative of mutagens having these properties. Indeed, there have been numerous reports on heterocyclic amines and cigarette smoking.[1)] Our results support those reports. However, several brands in our study evinced potent mugenicity with the TA100 strain, a base-pair substitution-type mutagenicity, both with and without metabolic activation. This is indicative of the presence of mutagens or carcinogens in cigarette smoke in addition to heterocyclic amines such as tobacco-specific nitrosoamines (TSNA). TSNA include N-nitrosonornicotine (NNN) and 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), which are International Agenoy for Research on Cancer (IARC) class 1 carcinogens. Furthermore, it has been suggested that several additives such as flavor-related compounds may introduce additional smoking-related health risks.[18)] Recently, a cigarette importer displayed on its website a list of ingredients added to tobacco in brands manufactured for sale in Japan.[19)] However, the list contained < 120 ingredients. This is insufficient to assess the risk of tobacco and cigarette smoking, because > 3000 constituents have been isolated from tobacco and approximately 4000 substances from MSS.[20)] In addition, it is necessary to investigate again the chemical toxicants in MSS as well as sidestream smoke since the new international standard (ISO 20773) was issued in 2007.[21)]

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