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Full Length Research Paper

Comparative analysis of reactive oxygen species in cigarette smoke under two machine smoking regimes (ISO and Canadian intense conditions) from selected Chinese cigarette brands

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Cigarette smoke can cause cellular oxidative stress and contributes to various adverse health effects associated with smoke exposure, partially due to reactive oxygen species (ROS) present in cigarette smoke. The purpose of present work is to evaluate toxic potential of selected Chinese blended and flue-cured cigarettes in terms of ROS quantification and effect of different smoking behaviors on the deliveries of ROS in whole cigarette mainstream smoke (MSS). In this work, fluorescence assay using Dihydrorhodamine 6G molecular was applied to the detection of ROS in MSS. Factors affecting trapping efficiency of ROS and the applicability of the method, such as trapping solution composition and pH were also investigated. Results indicated that, under ISO conditions, the deliveries of ROS in MSS from selected Chinese blended cigarettes were higher than those from flue-cured cigarettes (mean 32.65 versus 15.20 nmol cig^{-1}). Moreover, larger amount of ROS was produced under Canadian intense conditions than under ISO conditions for all the cigarettes tested (mean 31.54 versus 23.93 nmol cig^{-1}), especially for blended cigarettes in which more than 40% increase was found (mean 45.90 versus 32.65 nmol cig^{-1}). These findings showed that smokers might be exposed to elevated levels of ROS as well as other toxic chemicals when switching from regular to low tar or nicotine cigarettes.

Key words: Reactive oxygen species, cigarette smoke, blended cigarette, machine smoking regime, fluorospectrophotometer.

INTRODUCTION

Cigarette smoke can cause cellular oxidative stress and contributes to various adverse health effects associated with smoke exposure, partly due to the presence of reactive oxygen species (ROS) in cigarette smoke. ROS is a collective term used to include oxygen-centered radicals such as superoxide ($O_2^{-}\cdot$), hydroxyl (HO·), alkoxyl (RO·) and organic peroxy radicals (ROO·); and certain non-radicals, such as peroxynitrite ($ONOO^{-}$), hydrogen peroxide (H_2O_2) and hydroperoxide (ROOH), that are oxidizing agents and/or are easily converted into oxygen-centered radicals (Miller et al., 1990). Precursors of ROS such as carbon-centred radicals (R·) can also be considered as ROS owing to their high reactivity. Under normal physiological conditions, ROS mediate diverse functions in a variety of cellular processes and maintain at proper levels by a balance between its generation and elimination (Forman et al., 2010). However, chronic exposure to environment stress (air pollution or smoking) will result in an imbalance between excessive generation of oxidants (e.g. ROS) and/or insufficient antioxidant defense systems, which lead to cellular oxidative stress that can causes cell, tissue or organ injury and is contributable to the development of smoking-related diseases including atherosclerosis, cancer and chronic obstructive pulmonary disease (COPD) (Ambrose and

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Barua, 2004; Balansky et al., 2011; Fearon et al., 2011). Thus, accurate quantification of ROS is of importance to assess potential toxicity of cigarette smoke. However, little was known about the concentrations range of ROS in cigarette mainstream smoke (MSS) from selected Chinese brand cigarettes.

It was well known that electron spin resonance (ESR) and ESR-spin trapping have been widely applied for the detection of ROS-related free radicals, such as R· and RO· in MSS (Lyons et al., 1958; Pryor et al., 1983). However it suffers from some disadvantages. Firstly, due to the complex physical properties and chemical composition of cigarette smoke, most of ESR analyses of cigarette smoke radicals have involved the separation of the phases using a Cambridge filter pad that potentially introduces artifacts of measurement for smoke constituents (Dube and Green, 1982; Shorter et al., 2006). It has also been reported that such filtration significantly reduces the amount of carbon-centered radicals measured in filtered gas-phase smoke compared to whole MSS (Bartalis et al., 2007). Secondly, although the nitrone spin-adducts are more stable than smoke radicals, the radical adducts themselves slowly decay, auto oxidate or react with trapping solvents, which makes it difficult to obtain consistent and accurate results (Church et al., 1994; Baum et al., 2003). Thirdly, although spin trapping reagents, such as alpha-phenyl N-tertiary-butyl nitrone (PBN) and 5,5-dimethyl-pyrroline N-oxide (DMPO), have been used for trapping both carbon and oxygen-centered radicals in cigarette smoke, they have different selectivity to the types of free radicals captured (e.g. PBN mainly to R· and DMPO to RO·) (Baum et al., 2003; Ghosh et al., 2008) and are not sufficiently sensitive to peroxyl radicals (ROO·) that are considered to be the dominant radicals according to kinetic analysis in MSS (Flicker et al., 2001). Thus, a combination of spin traps is usually recommended to characterize free radical chemistry of cigarette smoke. However, it also brings into question the suitability of this assay for quantitative measurements of free radical in MSS.

Fluorescence-based assays are simple, rapid, and highly sensitive and have been widely used in the detection of ROS in biology systems (Boulton et al., 2011; Cheng et al., 2010; Rastogi et al., 2010; Peshavariya et al., 2007). Recently, several fluorescence probes have been applied in the detection of ROS of cigarette smoke. For example, 2, 7- Dichlorofluorescein (DCFH) and Dihydrorhodamine 6G (DHR 6G) was utilized to determine the concentrations of reactive oxygen species (ROS) in cigarette smoke (Huang et al., 2005; Zhao and Hopke, 2012; Ou and Huang, 2006). It should be noted that DCFH probe is not commercial availably and always freshly prepared by alkaline hydrolysis of its diacetate derivate (DCFH-DA). In addition, considerable variability (that is, ROS concentrations) might exist when different fluorescent probes were used for the analysis of ROS in cigarette smoke (Ou and Huang, 2006; Zhao and Hopke, 2012).

Total yield and chemical composition of the cigarette smoke are affected by tobacco type and blend mixture, various cigarette design parameters (Adam et al., 2010; Hoffmann et al., 1995). In order to lower human exposure to toxic constituents of cigarette smoke, physical design characteristics of cigarettes, such as physical features, ventilation and pressure drop (resistance to draw) have changed dramatically over the past several decades (Hoffmann et al., 1995), many so-called low emission brands with higher levels of filter ventilation (that is, low tar varieties) have appeared in the market. However, these machine-generated smoke measurements may be somewhat arbitrary and do not provide information about human toxicant exposure. In fact, when switching from regular cigarettes to low tar varieties, smokers tend to take larger or more frequent puffs or block vents with lips or fingers to maintain their daily nicotine dosage (Kozlowski and O'connor, 2002; Strasser et al., 2009; Hammond et al., 2005). To the best of our knowledge, little information is available about the effect of different machine smoking regimes on the deliveries of ROS in MSS.

In this study, fluorescent determination of ROS in MSS based on the oxidation of DHR 6G was optimized, including operation parameters, such as trapping solution composition and pH .This proposed method was applied to the analysis of ROS in MSS from selected Chinese blended cigarettes (Brand A, B, C, D and E) and Chinese flue-cured cigarettes (Brand F, G, H, I and J) purchased in the local market. In addition, the deliveries of ROS in MSS generated under Canadian intense smoking conditions were also evaluated for these selected brand cigarettes. This will provide valuable information to assess potential toxicity of cigarette smoke associated with cigarette types and different smoking behaviors in terms of quantification analysis of ROS in MSS.

MATERIALS AND METHODS

Reagents

Dihydrorhodamine 6G (DHR-6G) was obtained from Anaspec (San Jose, CA, USA). Rhodamine 6G was provided by Acros Organics (New Jersey, USA). Dimethyl Sulfoxide (DMSO) and N, N-Dimethylacetamide (DMA) was analytical grade and obtained from CNW Technologies GmbH (Dusseldorf, Germany).Double distilled water (18.2 $M\Omega.cm^{-1}$) purified with a Milli-Q system was used throughout in this work. Unless otherwise indicated, all the other reagent are analytic grade and used without further purification. Ten brands of Chinese cigarette were purchased from local super market, their characteristic parameters was presented in Table 1.

Instrumentation

The cigarette smoke was generated by linear 20-port SM 450 smoking machine (Cerulean, Milton Keynes, UK). All pH measurements were made with a pHs-3C digital pH meter (Shanghai Leici Device Works, China). Fluorescence excitation and

Table 1. Characteristic parameter of selected cigarettes used in this work.

Brand	Type	Average weight (g cig^{-1})	Length (mm)	Filter ventilation (%)	Nicotine content (mg cig^{-1})	
					ISO	Canadian intense
A	Blended	0.8410	84	59.7	0.27	1.33
B	Blended	0.8756	84	36.6	0.42	1.87
C	Blended	0.8875	84	22.1	0.71	1.71
D	Blended	0.9223	84	19.5	0.66	2.44
E	Blended	0.9578	84	71.0	1.02	2.46
F	Flue-cured	0.9333	84	0.3	1.12	2.19
G	Flue-cured	0.9210	84	16.3	1.01	2.48
H	Flue-cured	0.8948	84	17.8	1.16	2.43
I	Flue-cured	0.8771	84	18.1	0.84	1.79
J	Flue-cured	0.9215	84	0.20	1.21	2.75

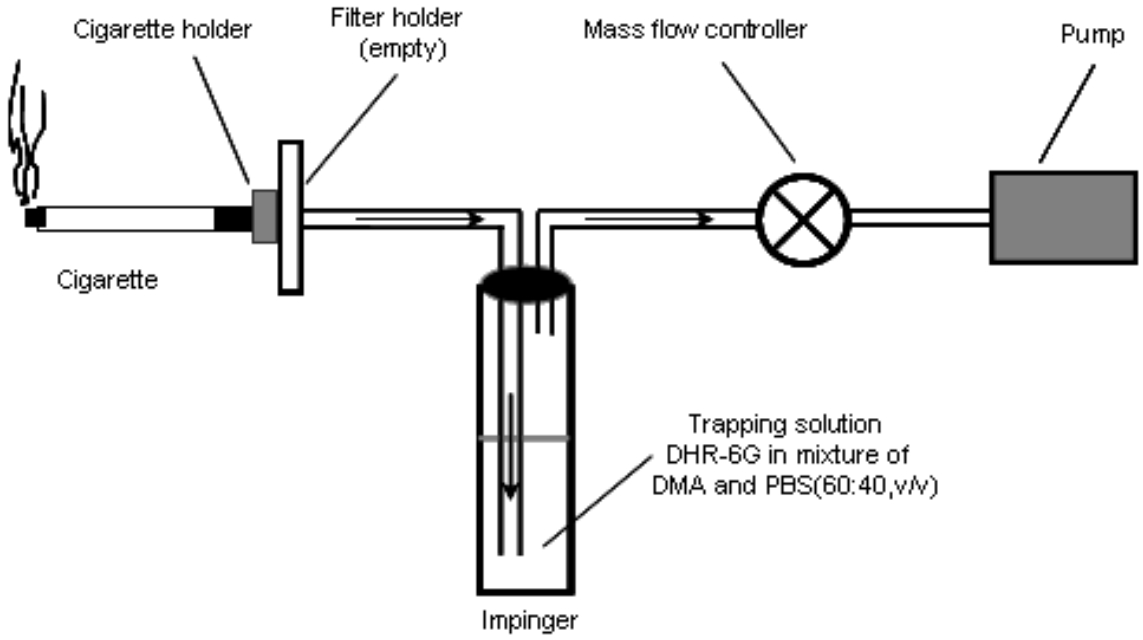

Figure 1. Schematic diagram of experimental set-up for generation and collection of mainstream cigarette smoke.

emission spectra of Rhodamine 6G were obtained with an FP-6200 spectrofluorometer (JASCO, Japan). Fluorescence intensity measurements were carried out on a Model 930A spectro-fluorimeter (Shanghai, China).

Smoke collection

All cigarettes were conditioned at 22 ± 1°C with 60 ± 2% relative humidity for at least 48 h before smoking. Cigarette smoking was carried out according to two machine smoking conditions: (1) ISO 3308 (35 ml puff, drawn for 2 s at 60-s intervals) and (2) the Canadian intense *regime* (55 ml puff, drawn for 2 s at 30-s intervals, and 100% filter ventilation blocked) (ISO 3308, 2000; Health Canada, 2000). A schematic diagram of experimental set-up for generation and collection mainstream cigarette smoke was shown in Figure 1. For each sample, smoke from two cigarettes was drawn through one home-made glass impinger (50 ml). The impinger contained 30 ml of trapping solvent, which was consisted of DHR-6G (25 mg in 200 ml of 60% DMA in 0.01 mol L^{-1} phosphate buffer saline (PBS) solution (pH = 7.4).

Determination of nicotine in cigarette smoke

Mainstream smoke nicotine deliveries were determined as follows. First, cigarettes were smoked using two different smoking regimes (ISO and Canadian intense conditions) and cigarette smoke was passed through a 44 mm Cambridge filter pad (CFP). Secondly, the CFP were extracted with 2-propanol by gently shaking using an

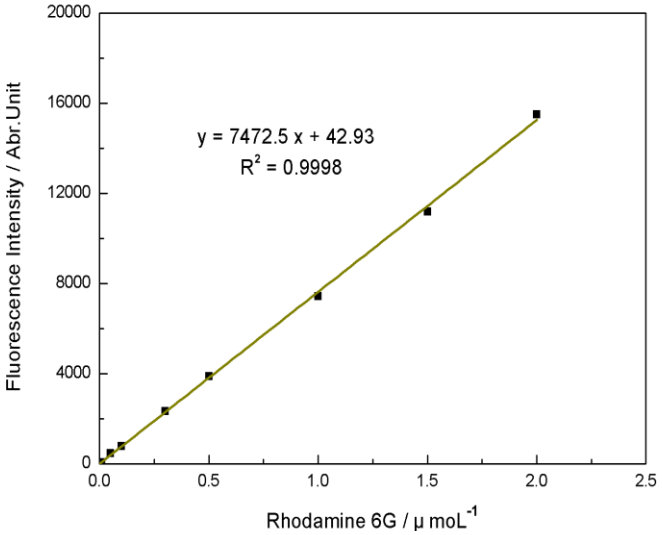

Figure 2. Calibration curve for the determination of Rhodamine 6G in DMA and 0.01 mol L^{-1} PBS solution (60:40, V/V). The initial solution pH was 7.4.

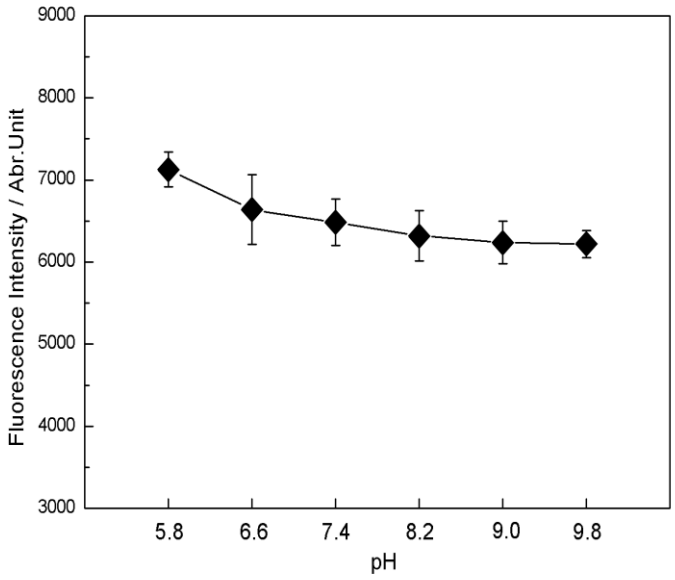

Figure 3. Effect of pH on the fluorescence intensity of the trapping solution of cigarette smoke generated from two cigarettes (Brand A) under ISO machine smoking conditions.

electric shaker at room temperature for 20 min. Finally, the resulting extraction was utilized for the determination of nicotine yields by a Hewlett Packard Model 6890 gas chromatograph equipped with a thermal conductivity detector and with an HP-Ultra 2 cross-linked 5% phenyl-methyl silicone capillary column (25 m × 0.25 mm ID, 0.52 µm film thickness) (Hewlett Packard, San Jose).Helium was used as carrier gas at constant flow rate of 1.7 ml/min. Automatic injections (2 µl) were made in the split mode with a split ratio of 40 to 1. The initial temperature of the column program was set at 190°C for 3 min and then programmed to 230°C at 40°C/min, with a final hold at 230°C for 6 min.

Quantitative analysis of ROS in cigarette smoke

The fluorescence intensity of Rhodamine 6G was measured with an excitation maximum at 538 nm and an emission maximum at 559 nm. Quantification of ROS in cigarette smoke was adapted from the reported method with some modification (Ou and Huang, 2006). Briefly, five replicates (two cigarettes per replicate) of each brand were smoked through a SM-450 smoking machine without using the Cambridge filter under ISO and Canadian intense smoking conditions. The whole MSS was collected as described above and the fluorescence intensity of trapping solution was then measured by spectrofluorimeter.

Statistical analyses

Each experiment was performed five duplicates. The data are reported as the mean ± standard deviation and were analyzed using SPSS data editor 16.0 and Microsoft Office Excel 2003. Statistical analyses were performed using a one-way analysis of variance. A probability value of $P < 0.05$ was considered significant.

RESULTS

Effect of trapping solution composition

To study the influence of solvent composition on the fluorescence intensity of smoke trapping solution, single solvent (DMA and DMSO) and their mixtures with PBS (0.01 mol L^{-1}, pH = 7.4) were used as trapping solution for analysis of ROS in cigarette smoke, respectively. As shown in Figure 2, at the same conditions, the fluorescence intensity of pure DMA and DMSO trapping solution were lower than that of phosphate buffer. Moreover, the fluorescence intensity of solvent mixture containing 60:40 DMA and PBS (v/v) was highest among all the trapping solutions used in this study. Therefore, this mixture solution was selected as optimal trapping solution.

Effect of pH value

The effect of pH on the fluorescence intensity of trapping solution was investigated (Figure 3). The results showed that the fluorescence intensity decreased with increasing pH and kept almost constant after pH 8.2. In order to evaluate actual amount of ROS exposure at the normal physiological conditions, all the experiments should be performed with the trapping solution at pH 7.4, which would be favorable to evaluate toxic potential of cigarette smoke in terms of ROS quantification analysis.

Calibration curve

Rhodamine 6G calibration standards of 0.01, 0.05, 0.1, 0.3, 0.5, 1.0, 1.5 and 2.0 µmol L^{-1} were prepared in DMA-0.01 mol L^{-1} PBS at pH = 7.4 (60:40, v/v). Under the selected experimental conditions, calibration curve was

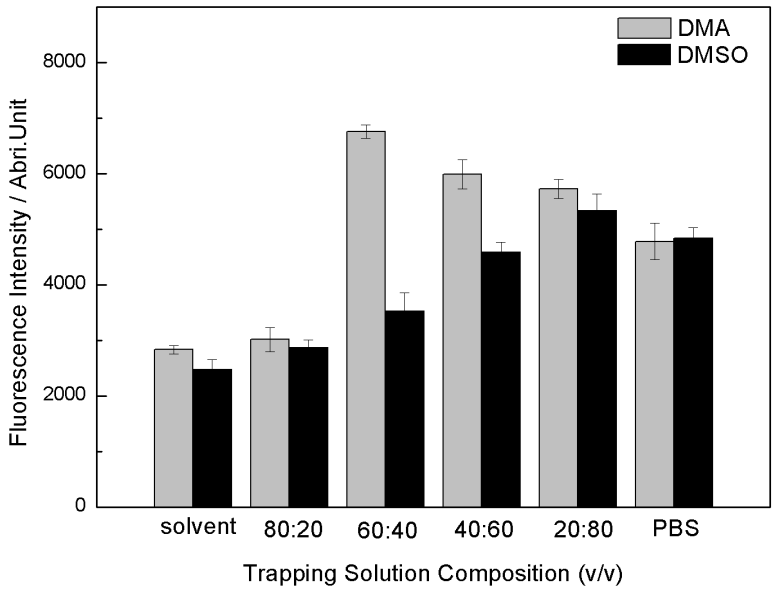

Figure 4. Effect of solvent composition on the fluorescence intensity of the trapping solution of cigarette smoke generated from two cigarettes (Brand A) under ISO machine smoking conditions.

achieved by plotting the fluorescence intensity of Rhodamine 6G in each standard solution against the corresponding concentration (Figure 4). The regression equation was y = 7472.5 x + 42.92 (R2 = 0.9998), where x and y was Rhodamine 6G content and fluorescence intensity of solution, respectively).The detection limit and qualification limit, defined as three times and ten times of the standard deviation of measured lowest standard solution (10 n mol L^{-1}) was 1.94 and 6.64 n mol L^{-1}, respectively.

In order to evaluate the precision of the method, five replicates analysis of ROS deliveries in cigarette smoke from brand A generated under ISO and Canadian intense conditions were carried out and the relative standard deviations(RSD) of 3.9 and 2.2% was obtained, respectively. Recovery was determined by spiking known amounts of Rhodamine 6G (after smoking) into trapping solution of MSS from the same brand cigarettes generated under two conditions aforementioned, and the spiked solution was detected with the same method as samples. Un-spiked solution was also analyzed as blank. Recovery was between 90.3 and 104.7% for these Chinese cigarettes.

ROS levels in MSS from selected Chinese cigarettes

All cigarettes contained a cellulose acetate filter. Cigarettes of brand A, B, C, D and E were blended cigarette containing bright and burley tobaccos. The remaining cigarettes (Brand F to J) were categorized as being Virginia-style cigarettes that contained only flue-cured tobacco. It was obvious that, on a per cigarette basis, smoke from Virginia tobacco had a higher level of nicotine than did smoke from blended tobacco except for brand E (Table 1).

In this work, no Cambridge filter was used during all the experiments. So the smoke was regarded as whole MSS. The concentrations of ROS detected in MSS for Chinese blended and flue-cured cigarette were presented in Table 2. At least five replicate measurements were made for each brand reported. Under ISO machine smoking regime, the deliveries of ROS detected in the MSS of blended and flue-cured cigarettes were in a range of 15.50 - 54.34 and 12.63 - 19.41 nmol cig^{-1}, respectively.

To test whether smoking behavior changes will affect ROS deliveries in MSS, all brands cigarettes were analyzed for their ROS deliveries under Canadian intense conditions. Results showed that the deliveries of ROS detected in MSS of blended and flue-cured cigarettes varied from 32.70 to 70.48 and from 14.79 to 21.11 nmol cig^{-1}, respectively. As anticipated, all cigarettes tested have higher levels of ROS in MSS under Canadian intense conditions than ISO conditions (mean 31.54 versus 23.93 nmol cig^{-1}, $p < 0.05$), especially for low tar or nicotine blended cigarettes in which more than 40% increase was found (mean 45.90 versus 32.65 nmol cig^{-1}, $p < 0.05$).

DISCUSSION

Fluorescent probe DHR-6G is frequently used as a marker of oxidative stress in biological systems. Recently, this redox-active compound was selected as

Table 2. ROS Levels in selected cigarettes under different smoking regimes a(n=5).

Brand	ROS level (nmol/cig)			
	ISO	RSD (%)	Canadian intense	RSD (%)
A	15.50±0.61	3.96	38.13±0.82	2.17
B	22.96±1.87	8.12	32.70±1.38	4.22
C	28.98±0.84	2.89	43.40±1.55	3.57
D	41.49±2.01	6.70	44.81±0.30	2.74
E	54.34±1.78	3.28	70.48±4.45	6.32
F	15.84±1.33	8.43	16.74±1.53	9.16
G	14.99±0.70	4.70	17.41±0.87	5.03
H	19.41±1.03	5.30	21.11±1.87	8.83
I	12.63±0.59	4.69	14.79±1.04	7.01
J	13.14±0.68	5.20	15.84±0.98	6.17

[a] ISO: ISO conditions (35 ml puff volume, 2 s duration, 60 s puff interval); Canadian intense: Canadian intensive conditions (55 ml puff volume, 2 s duration, 30 s puff interval, 100% vent holes block).

the molecular probe to qualification of ROS in cigarette smoke (Ou and Huang, 2006; Liu et al., 2012). DMSO was usually recommended by manufacturer to dissolve DHR-6G due to its excellent solubility for this lipophilic compound. A solvent mixture for trapping ROS in cigarette smoke usually required a high boiling point and should have good solubility for both lipophilic probe and cigarette smoke. Although several researchers reported that DMSO, DMA and PBS solution (pH 7.2) could be used as trapping solution for ROS present in cigarette smoke(Ou and Huang, 2006; Liu et al., 2012; Huang et al., 2005), none of the previous published works had compared all three trapping solutions to determine the differences in trapping efficiency. Our study showed that solvent mixture containing 60:40 DMA and PBS (v/v) was most for trapping ROS in MSS among all the trapping solution used in this study. This result was consistent with those of other studies (Ou and Huang, 2006). It was obvious that the deliveries of ROS in MSS reported in the literature could be lower than actual values (Huang et al., 2005; Zhao and Hopke, 2012).

The pH dependence of production, transformation, and decomposition for some ROS in some simple chemistry systems had been reported (Nakayama et al., 2002; Georgi et al., 2007; Carbajo et al., 2000). The rate constant for superoxide ($O_2^{\cdot-}$) / hydroperoxyl radical (HOO·) with spin trapping nitrones was also affected by pH of reaction medium (Allouch et al., 2007). To our knowledge, there is little or no literature about effect of pH value of trapping solution on the deliveries of ROS in cigarette smoke. Our results indicated that the deliveries of ROS in cigarette decreased with increasing pH value of trapping solution in a range of 5.8 to 9.8.

Measurements of ROS yields collected under standard conditions with vents unblocked (International Organization for Standardization) and intense conditions with vents fully blocked (Health Canada) were present in Table 2. Under ISO machine smoking conditions, the deliveries of ROS in Chinese blended MSS was higher than those of in flue-cured cigarettes smoke (mean 32.65 versus 15.20 nmol cig^{-1}, $p < 0.05$), which was consistent with findings by previous researchers that flue-cured tobacco typically delivered less ROS than blended tobacco types but difference of 10-fold did not existed in our study (Ou and Huang, 2006). This may be because that Chinese blended cigarettes contained more flue-cured tobacco compared to foreign blended cigarettes and the difference of physical design and production maybe also existed. Overall, mainstream ROS deliveries obtained under Canadian intense conditions were higher than those under ISO regime (ISO mean 23.93 v Canadian mean 31.54 nmol cig^{-1}, $p < 0.05$), especially for blended cigarettes in which more than 40% increase was found (Canadian mean 45.90 versus ISO 32.65 nmol cig^{-1}). Similarly, nicotine emissions generated under Canadian intense regime were more than double those collected under ISO conditions (ISO mean 0.84 v Canadian mean 2.15 mg cig-1, $p < 0.05$).

Although the Canada intensive machine smoking regime is designed to mimic human puffing more closely, it does not represent human behavior in terms of compensatory smoking. In fact, most smokers usually regulate their nicotine intake to maintain a relatively constant across brands. To minimize the differences between the machine emissions and actual uptake by cigarette smokers, ROS: nicotine ratios of selected brands cigarettes were determined in our work (Figure 5). If smokers self-titrate their nicotine intake, brands with lower ROS: nicotine ratios might deliver fewer ROS to smokers. These findings suggested that smokers might be exposed to elevated levels of ROS as well as other toxic chemicals when switching from regular to low tar or nicotine cigarettes. In addition, the differences between brands and types for RSO: nicotine levels were sufficiently large. For example, ROS: nicotine levels showed more than a 5-fold difference across selected

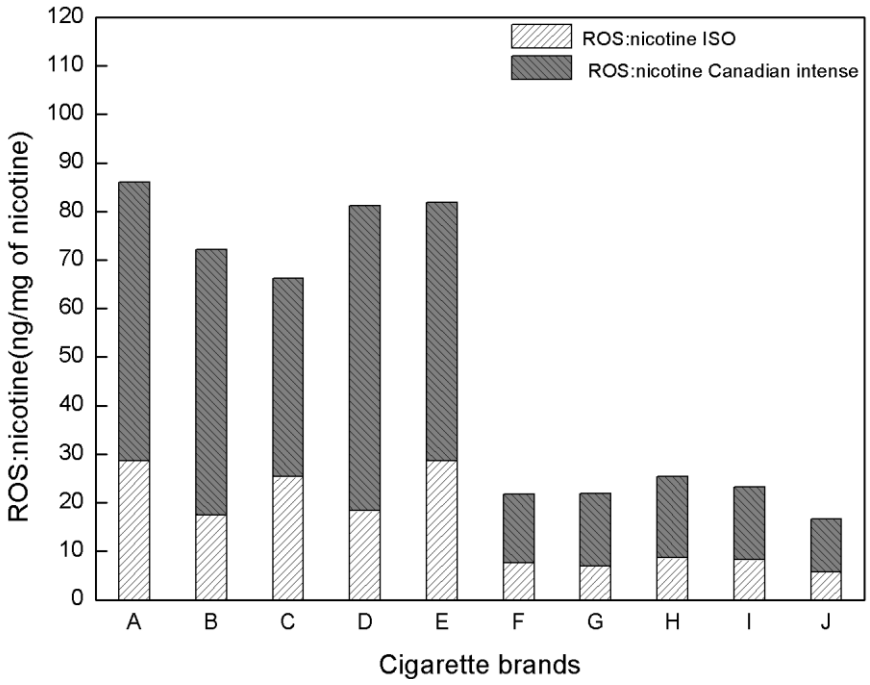

Figure 5. ROS: nicotine ratios for selected Chinese brand cigarette tested under ISO and Canada intense smoking regimes (cigarettes A to E are Chinese blended cigarettes and F to J are Virginia cigarettes).

brands cigarettes in local market. However, no significant difference was found in ROS: nicotine levels between ISO and Canadian intense smoking regimes.

Conclusion

An improved fluorescence determination based on DHR-6G probe was applied to analysis of ROS in MSS from selected Chinese blend and flue-cured cigarettes under ISO and Canadian intense regimes. The deliveries of ROS in MSS from Chinese blended cigarettes were higher than those from flue-cured cigarettes (mean 31.54 versus 23.93 nmol cig^{-1}, $p < 0.05$) under ISO machine smoking conditions. For all the cigarettes tested, more ROS emissions were achieved under Canadian intense regime, which indicated that smokers might be exposed to elevated levels of ROS as well as other toxic chemicals when switching from regular to low tar tobacco. This will give important information about potential toxicity of cigarette smoke and is favorable to assess health risk to smokers in terms of ROS quantification.

ACKNOWLEDGEMENT

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Abbreviations: ROS, Reactive oxygen species; **ROO,** peroxy radicals; **RO,** alkoxyl radicals; **R,** Alkyl radicals; **DCFH,** dichlorofluorescein; **DHR 6G,** dihydrorhodamine 6G; **DCFH-DA,** dichlorofluorescein diacetate; **ISO,** International Organization for Standardization; **DMSO,** dimethyl sulfoxide; **DMA,** N, N-Dimethylacetamide; **MS,** mainstream smoke; **ESR,** electron spin resonance; **PBN,** Alpha-phenyl N-tertiary-butyl nitrone; **DMPO,** 5,5-dimethyl-pyrroline N-oxide; **PBS,** phosphate buffer saline.

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