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Variation in Free Radical Yields from U.S. Marketed Cigarettes

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Abstract

Free radicals in tobacco smoke are thought to be an important cause of smoking-induced diseases, yet the variation in free radical exposure to smokers from different brands of commercially available cigarettes is unknown. We measured the levels of highly reactive gas-phase and stable particulate-phase radicals in mainstream cigarette smoke by electron paramagnetic resonance (EPR) spectroscopy with and without the spin-trapping agent phenyl-N-tert-butyl nitron (PBN), respectively, in 27 popular US cigarettes and the 3R4F research cigarette, machine-smoked according to the FTC protocol. We find a 12-fold variation in the levels of gas-phase radicals (1.2 to 14 nmol per cigarette) and a 2-fold variation in the amounts of particulate-phase radicals (44 to 96 pmol per cigarette) across the range of cigarette brands. Gas and particulate-phase radicals were highly correlated across brands ($\rho=0.62$, $p<0.001$). Both radicals were correlated with TPM (gas-phase: $\rho=0.38$, $p=0.04$; particulate-phase: $\rho=0.44$, $p=0.02$) and ventilation (gas- and tar-phase: $\rho=-0.58$, $p=0.001$), with ventilation explaining nearly 30% of the variation in radical levels across brands. Overall, our findings of significant brand variation in free radical delivery under standardized machine-smoked conditions suggest that the use of certain brands of cigarettes may be associated with greater levels of oxidative stress in smokers.

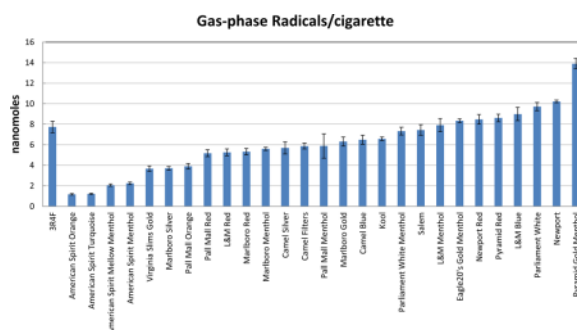
Graphical abstract

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Author Contributions

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INTRODUCTION

Cigarette smoke is a harmful mixture of more than 7000 chemicals generated by a combination of combustion, pyrolysis and distillation with temperature reaching up to 950°C under both oxygen-rich as well as oxygen-depleted conditions.^{1, 2} It has been known for almost seven decades that free radicals are generated in high concentrations as a result of complex reactions in mainstream smoke.^{3–7} Due to the presence of an unpaired electron, radicals are unstable, highly reactive and redox active. The inhaled free radicals, in turn, can cause significant oxidative damage to major biological macromolecules (DNA, lipids, protein and carbohydrates) and trigger the dysregulation of redox signaling pathways impacting numerous critical cellular functions.^{8–10} The resulting oxidative damage and stress has been implicated in the initiation and propagation of many chronic and degenerative diseases in smokers including cancer,^{11–13} cardiovascular diseases,^{12, 14–16} neurodegeneration,¹⁷ and lung diseases.^{12, 18, 19}

Elegant work by Pryor and colleagues in the 1980s characterized cigarette smoke radicals into two types with distinct redox and chemical properties that partition into gas and particulate phases.^{5, 6, 20, 21} The gas-phase of the cigarette smoke contains oxygen- and carbon-centered radicals that are very short-lived and highly reactive. These radicals can penetrate the upper respiratory tract and propagate their damaging effects despite their relatively short half-lives and limited diffusion times.⁸ In contrast to the gas-phase radicals, the particulate-phase contains radical species such as semiquinone, which are relatively longer-lived and can persist in some cases up to several months. Exposure to these “Environmentally Persistent Free Radicals” (EPFRs) can occur directly in the smoker or indirectly in the passively exposed individual and can penetrate deep within the respiratory tract.

Research on the exposure and biological impact of free radicals, especially gas-phase radicals, has been hampered by their extremely short half-lives (milliseconds) and high reactivity making measurements difficult. Consequently, little is known regarding the levels and variation of free radical exposure in smokers and the impact that differences in exposure may have on health status. Our main goal was to systematically quantify free radicals produced from commercial cigarettes available in the US market as a means of estimating the range of tobacco-derived free radical exposure in smokers.

Numerous techniques have been used to measure free radicals in tobacco smoke yielding widely different results, and few attempts have been made to standardize these methodologies.^{22–29} Thus, as part of this research, we developed a standardized protocol for the reproducible and quantitative determination of free radicals in mainstream smoke using electron paramagnetic resonance (EPR) spectroscopy. EPR is a sensitive and selective technique for directly detecting and quantitating free radicals in complex mixtures such as cigarette smoke.³⁰ In the current investigation, mainstream smoke from cigarettes was generated under the Federal Trade Commission (FTC) regimen, and both highly reactive gas-phase radicals and stable particulate-phase radicals were measured by EPR.

This is the first study to assess and rank free radical yields across commercially available cigarettes. Using these data, direct exposure of smokers to harmful free radicals can be assessed. These data can have important public health and regulatory implications given the importance of free radical exposure in the development of cigarette-induced diseases.

MATERIALS AND METHODS

Materials

The 3R4F research cigarette was obtained from the University of Kentucky (Lexington, Kentucky, USA). All the commercial cigarettes were purchased locally (Hershey, PA): 26 King size (85 mm) and Virginia Slims Gold (100 mm). The cigarette brand varieties were selected based on their market share or unique characteristics. Marlboro, Newport, Pall Mall and Camel were selected for their popularity, with variants of these brands comprising an estimated total US market share of 66%.³¹ Parliament and American Spirit exemplify the types of unique characteristics such as recessed filters and “additive free” formulation, respectively. The cigarettes were stored in their original packaging long term at -20°C in airtight plastic bags. Analytical grade chemicals: nitron spin trap phenyl-N-tert-butyl nitron (PBN), tert-butylbenzene, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), heptadecane from Sigma-Aldrich (St. Louis, MO, USA), Suprasil® EPR tubes (4mm o.d.; Wilmad-Labglass, Vineland, NJ, USA), Schlenk line (Chemglass Life Sciences, Vineland, NJ, USA) and Cambridge filters pads (Performance Systematix Inc., Grand Rapids, MI, USA) were used as supplied.

Mainstream Smoke Generation

The cigarettes were conditioned for testing by removing them from cold storage and placing them in a constant humidity chamber (60% relative humidity, 22°C), for at least 48 hours before smoking. Mainstream smoke was generated by using a 30-port smoking machine (Jaeger-Baumgartner, CSM JB2080). Cigarettes were smoked on the machine under the FTC smoking parameters: 35 mL puff volume, 2 second puff duration, and 60 second puff interval. For each replicate, five cigarettes were simultaneously smoked to provide for a strong EPR signal required for quantitative analysis. Mainstream smoke was separated into particulate-phase and gas-phase by passing through a Cambridge filters pad (CFP).

Analysis of Particulate-phase Radicals

A CFP, located down-stream of the smoke machine pump, was used to trap the particulate-phase radicals and was subsequently stored at -80°C in an airtight plastic bag until further analysis. EPR spectra were obtained by direct insertion of the CFP into the cavity of a Bruker eScan R spectrometer (Bruker-Biospin, Billerica, MA, USA) operating in X-band. The EPR parameters were as follows: microwave frequency, 9.7 GHz; modulation frequency, 86.0 kHz; microwave power, 6.00 mW; scan range, 50 G; modulation amplitude, 1.10 G; sweep time, 5.243 s; time constant, 10.240 ms; and conversion time, 10.240 ms. All measurements were carried out at room temperature ($22 \pm 1^{\circ}\text{C}$). Spin concentrations were determined by integration of the area under the curve of the EPR signal using WinEPR software (version 0.98, National Institute of Environmental Health Sciences, National Institutes of Health, USA). Standardized concentrations of TEMPOL in methanol or a blank methanol solution pipetted onto CFP were used to quantify the spin concentrations of cigarette particulate-phase radicals.

Analysis of Gas-phase Radicals

The gas-phase of mainstream smoke was passed through an impinger located down-stream of the pump and the CFP. Trapping of gas-phase radicals was accomplished using 4 mL ice-cold tert-butylbenzene and 0.05 M PBN. After smoking, aliquots of the PBN solution were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Upon analysis, PBN solutions were thawed at room temperature, and 400 μL aliquots were placed into EPR tubes and deoxygenated using three freeze-pump-thaw cycles with a Schlenk line, as previously described.²⁵ Briefly, samples were frozen using liquid nitrogen, a vacuum was subsequently applied allowing trapped air to escape and gaseous argon was then introduced to provide an inert atmosphere before the sample was re-frozen. Samples were subjected to a total of three freeze-vacuum pump-thaw-argon cycles (2 minute each). The EPR spectra derived from PBN-radical adducts were measured using the following EPR parameters: microwave frequency, 9.7 GHz; modulation frequency, 86.0 kHz; microwave power, 6.00 mW; scan range, 60 G; modulation amplitude, 1.10 G; sweep time, 41.94 s; time constant, 81.92 ms; and conversion time, 81.92 ms. All measurements were carried out at room temperature ($21.5 \pm 0.5^{\circ}\text{C}$). Spin concentrations were determined by peak to peak height of the EPR signal using WinEPR software and comparison with standardized solutions of TEMPO in tert-butylbenzene. The standard TEMPO solution was not subjected to freeze-pump-thaw cycles, a common practice in the field.

Determination of Total Particulate Matter and filter ventilation of the Cigarettes

Total particulate matter (TPM) was determined by weighing the CFPs.³² Five replicates of total filter ventilation (%) of the cigarette were measured at the Centers For Disease Control and Prevention (CDC), Atlanta, USA as described in a previous study.³³

Statistical Analysis

We sub-grouped the cigarette brands (Table 1) on the basis of two common cigarette design features; 1) flavor (regular vs. menthol) and 2) filter ventilation. In some analyses, cigarette brands were categorized according to filter ventilation tertiles (low, mid, and high). All

analyses were performed using R statistical package 3.3.³⁴ Summary statistics and boxplots are presented for major variables. The association between different variables is estimated and tested using robust Spearman correlations. Linear regression was used to determine the significance of the ventilation and flavor group effects. Tukey contrasts were used to evaluate all pairwise comparisons of means within the ventilation categories.

RESULTS

Standardized EPR Assessment of Tobacco Smoke Free Radicals

Standardized protocols for measurements of free radicals were developed using 3R4F research cigarettes. EPR analysis of smoke radicals requires the separation of whole smoke into its constituent gas and particulate-phases by use of Cambridge filters. Due to the lack of stability and short half-life of the gas-phase radicals in mainstream smoke, spin trapping was required prior to EPR analysis of the resulting radical adducts. For this purpose, PBN was used as the spin trap based on its ability to form adducts with a broad range of different radicals.³⁰ Since the time between sample collection and EPR measurement was a critical factor, all analyses were performed within 15 minutes. The organic solvent for radical collection can influence trapping efficiency and stability. While benzene is commonly used, we selected to use tert-butylbenzene because it provided identical results as benzene but is far less toxic than benzene and is less subject to vaporization than other organic solvents. Since oxygen can interfere with the EPR signal, optimal results were obtained when deoxygenation was performed prior to EPR measurement.

For quantitative EPR, the use of a reference standard is required. Previous papers have used numerous reference radical standards interchangeably including diphenyl- β -picryl hydrazil, (DPPH), TEMPO, and TEMPOL for tobacco smoke radical quantitation, very often providing few details about conditions such as the solvent used.^{5, 22, 35} In our studies, TEMPO in tert-butylbenzene was selected for use with gas-phase trapped radicals based on its higher degree of sensitivity upon EPR analysis. In contrast, particulate-phase radicals are not trapped, but rather collected and analyzed directly on the CFP. Since CFP emit a small background EPR signal (Figure 1B), measurement blanks were constructed by addition of solvent directly on to filter pads followed by evaporation. For these latter analyses, TEMPOL was selected as the reference standard based on its greater stability and higher sensitivity on CFP.

Utilizing optimized conditions for the collection and analysis of mainstream smoke radicals, representative EPR spectra for both gas-phase PBN radical adducts and particulate-phase stable radicals for the 3R4F reference cigarette are provided in Figure 1. The concentration of both gas-phase radicals and particulate-phase radicals was linearly dependent on the number of cigarettes smoked ($R^2= 0.998$ and 0.996 for gas-phase and particulate-phase radicals, respectively). Results demonstrate that mainstream smoke generated from 3R4F cigarettes contained on average 8 ± 2 nmol (5×10^{15} spins, $n=6$) of gas-phase radicals and 64 ± 13 pmol (4×10^{13} spins, $n=3$) of particulate-phase radicals per cigarette, both in the ranges reported previously.^{5, 22} Overall, these methods provided highly reproducible results for both gas-phase radicals (3.6% precision) and particulate-phase radicals (1.7% precision).

Gas and Particulate phase Radicals in Mainstream Smoke from Popular US Cigarette Brands

Our standardized method for quantitation of tobacco smoke radicals was then used in assessing and ranking free radical yields from 27 popular commercial cigarette brands with diverse characteristics currently in the US market. As shown in Table 1, the gas-phase radical yields under the FTC smoking regimen varied widely (12-fold) among the cigarettes, ranging from 1.2 to 14 nmol per cigarette. Meanwhile, the particulate-phase radical yields varied 2-fold among the cigarettes brands under the FTC smoking regimen ranging from 44 to 96 pmol per cigarette (Table 1).

We examined whether free radical levels in the gas-phase were associated with those in the particulate-phase (Table 2). While levels of variation between products were substantially lower in the particulate-phase, overall, these levels were highly correlated to gas-phase radicals (Spearman coefficient (ρ)=0.62, p =0.0006). Correlational analyses were also performed to examine potential associations between free radicals and both TPM and filter ventilation (Table 2). Significant negative correlations were observed between filter ventilation and both gas-phase (ρ =-0.58, p =0.001) and particulate-phase radicals (ρ =-0.58, p =0.001). Significant associations were also observed between TPM and both gas-phase (ρ =0.38, p =0.04) and particulate-phase radicals (ρ =0.44, p =0.02).

To examine some potential factors that can affect radical production, the impact of common cigarette design features (flavor and filter ventilation) on gas and particulate phase radicals was examined. Analysis of gas-phase radicals based on filter ventilation tertiles indicated that highly ventilated cigarettes tended to produce significantly fewer gas-phase radicals compared to the low and medium ventilated cigarettes (Figure 2A, p <0.05). Meanwhile, particulate-phase radical production was significantly lower for highly ventilated cigarettes compared to those with low ventilation (Figure 2C, p <0.05). Overall, approximately 30% of the variation in both gas-phase (r^2 =0.34, p =0.001) and particulate-phase (r^2 =0.31, p =0.001) radicals was explained by differences in ventilation. Both gas-phase (Figure 2B, p =0.24) and particulate-phase radicals (Figure 2D, p =0.09) were not significantly different between regular and menthol brands.

DISCUSSION

While mainstream tobacco smoke contains over 7,000 chemicals, much of the damage is thought to result from the 93 carcinogens and respiratory, cardiovascular and other toxicants on the FDA's list of hazardous and potentially hazardous chemicals (HPHC's). However risk assessment models based on these smoke constituents underestimate the actual incidences of many smoking induced diseases.³⁶⁻³⁹ Some of the limitations of the models are due to the complexity and incomplete characterization of smoke constituents and lack of human exposure data. Free radicals are not included in the FDA's HPHC list or in most risk models but may account, in part, for the gap between assessment models and disease incidences. Damage resulting from free radical exposure has long been known to have major negative health consequences, and, as clearly delineated in the 2010 Surgeon General's Report, oxidative stress/damage resulting from tobacco free radical exposure was identified as one of the major causes of tobacco induced diseases.⁴⁰ While direct experimental data linking

exposure to free radicals with specific health outcomes are limited due to the difficulties in their measurement, direct evidence for free radical induced harm has been observed for some relevant outcomes. Diminished elastase activity due to the oxidation of alpha-1-proteinase inhibitor was observed after exposure to both gas and particulate-phase radicals, an effect likely linked to the onset of emphysema.⁴¹ Free radicals have also been shown to cause single-strand breaks in DNA.^{13, 42, 43} Cigarette tar fractions that had a strong EPR signal also reduced oxygen to hydrogen peroxide and nicked DNA.⁴³ Inhaled free radicals from cigarette smoke can set off a cycle of secondary radical production resulting from reaction with other chemicals in smoke, including polycyclic aromatic hydrocarbons (PAHs), transition metals, heavy metals and aldehydes,⁴⁴⁻⁴⁶ and by evoking inflammatory responses, which include the generation of endogenous reactive oxygen/nitrogen species.¹⁸ Overall, the chronic exposure of a smoker to such high levels of oxidative stress/damage is commonly accepted as an important factor in the etiology of numerous diseases in the smokers⁴⁷, such as cancer, atherosclerosis, COPD and emphysema.

Here we report for the first time that the levels of free radicals in mainstream smoke can vary substantially by cigarette brand. As observed previously,^{5, 22} in our study both highly reactive gas-phase radicals and more stable particulate-phase radicals were abundant in smoke from all brands tested with single cigarettes producing as much as 16 nmol (8×10^{15} spins) of highly reactive radicals and 114 pmol (5×10^{13} spins) of more stable radicals. However, when different brands were tested, a wide variation in radical production was observed: 12-fold for gas-phase radicals and 2-fold for particulate-phase radicals. The brands tested represent a sampling of 27 of the most commonly used or unique products out of the 260 different brand families identified on the market between 2002 and 2011.⁴⁸ We believe that the differences in free radical production by brand may be a critical factor resulting in differences in exposure and potentially risk for tobacco-related diseases, particularly when one considers the impact of these differences when expressed over the lifetime of a smoker (e.g., 20 cigarettes per day for 40 years). The importance of examining both types of radicals is due to differences in their likely mechanisms of action since gas-phase radicals are highly reactive and can cause extensive oxidative damage and acute responses in the lung and upper digestive tract and whereas particulate-phase radicals are more stable and can penetrate deeper in the body and lead to more systemic consequences. However, to date, there are no studies that distinguish and rank the damage induced by the two types of smoke radicals. Further studies that examine the toxicological implications of both types of radicals are desperately needed prior to development of regulations.

In the present study, we focused primarily on the measurement of total radicals without specifically identifying individual radical species and developed the methodology accordingly. To this end, of the many available spin traps, PBN was selected for use based upon its reactivity with a wide range of radical types, high level of stability of resulting radical adducts, and high trapping efficiency. Indeed, PBN trapping is a standard technique for measurement of tobacco smoke radicals that has been accepted and used for decades.^{5, 22} It is possible that there are radicals in tobacco smoke that we were unable to detect based upon limited reactivity with PBN or extreme short life span, but we expect these to be minimal. In the current studies, the specific identities of the individual radicals could not be determined due to the resolution of the hyperfine coupling constants obtained with or

without PBN. Speciation of radicals is also complicated by their short half-life and high reactivity leading to a high degree of variation based upon reactions occurring in the smoking machine tubing and on the CFP.^{49, 50} It is possible that in addition to differences in total radicals produced, the specific radical species may differ by cigarette brand as may the toxicologic impact of each species. Previous investigations have suggested that cigarette smoke likely contains more than 35 different species⁴⁹ with 18 carbon-centered radical species having been identified by HPLC-MS/MS in mainstream whole smoke.^{28, 29, 50} The unique chemistry of individual radicals requires further evaluation to help clarify the impact they may have on a smoker.

Filter ventilation is a significant variable differentiating the different cigarettes and allows mixing of the air with smoke, thus reducing the temperature of combustion and also diluting the smoke.^{51, 52} The degree of ventilation in the cigarette filter design has been responsible for confusion regarding the potential risk of different brands and sub-brands among the consumer due to the common practice of blocking the ventilation holes in the cigarette filters. When we tested these products on the FTC smoking regimen (without blocking filter ventilation), we found that highly ventilated cigarettes produce lower numbers of free radicals likely due to the dilution of the tobacco smoke. However, based on the common practice of blocking the ventilation holes, smokers of these highly ventilated products will not necessarily be exposed to reduced levels of radicals.

Menthol is the only marketed characterizing flavor in cigarettes to enhance taste and has been shown to alter smoking behavior.⁵³ Menthol cigarettes account for approximately 25 percent of all cigarette sales in the U.S.⁵⁴ Particular interest in menthol as a potential additional risk factor for tobacco related illnesses has been debated over the past decade.^{55–59} In order to determine if free radical formation may be related to menthol concentrations, we compared levels of radical production in menthol versus regular cigarettes. Overall, no significant difference in amounts of either gas or particulate-phase radicals was observed, suggesting that menthol does not impact radical generation during the combustion process.

Interestingly, we find that variation in filter ventilation across the brands explains about 30% of the brand to brand variation in free radical levels. Overall, the nature of the observed variation in radical generation after adjusting for ventilation is not known but may depend on other cigarette characteristics, such as rod length and circumference, filter length and type, tobacco weight and blend, and addition of tobacco additives. Combustion of different tobacco blends, such as burley, oriental and bright, show variations in the free radical production with yields of gas-phase radicals highest in burley > oriental > bright and particulate-phase radicals highest in oriental > burley > bright.^{7, 29} The pyrolysis temperature also has been shown to affect radical yields.⁶⁰ The particulate-phase radical yield is temperature dependent, increasing from 0.8×10^{15} spins/gram tobacco at 240°C to $3.5\text{--}4.0 \times 10^{15}$ spins/gram tobacco at 450–510°C, and is inversely dependent on the oxygen concentration.⁷ Meanwhile, oxidation of the tobacco is required for formation of gas-phase radicals. In our study, the fact that gas-phase radical yields are highly correlated to particulate-phase yields suggest that they are produced from similar tobacco constituents and thermal decomposition processes. Further, we find that both phases of radicals were

correlated with total particulate matter (TPM). Since the majority of TPM constituents result from the incomplete combustion of the tobacco leaf, this further supports a shared mechanism for formation among free radical species.

In the current study, we machine smoked cigarettes using the FTC smoking regimen for comparison purposes. However, differences in human smoking behaviors, such as intensity of smoking, number of puffs per cigarette, and depth of inhalation, are well-known to impact delivery of tobacco smoke and its constituents, such as tar, nicotine and PAHs.^{61, 62} Thus, it is possible that these factors could similarly impact free radicals leading to greater levels of exposure to free radicals when more intense puffing protocols are used. As such, our findings likely underestimate the overall exposure to smokers and studies are underway to assess these possible differences.

Free radicals have not been included in FDA's toxicant list perhaps due to the analytical challenges in identifying and quantifying them. Previously, several laboratories have made efforts to measure free radicals; however, most of the studies lack quantitation and have not used standardized smoking protocols, experimental conditions, cigarettes brands, and analytical techniques, probes, or spin traps. Studies that used fluorescent probes to assess free radicals in cigarette smoke lack specificity and can introduce artifacts.^{63–65} Several groups have used more promising nitroxide-based probes to quantitate and identify tobacco radicals; however, nitroxide-based probes only trap carbon-centered radicals.^{23, 28, 29, 66} Although it has its own technical limitations, EPR is still the only method that allows for the direct measurement of free radicals. Baum et al. reported on some of the variables that may impact radical quantitation including solvent, spin trap, collection and analysis volume, and EPR tube positioning and thickness.²² We have expanded on their work and developed a robust technique for the measurement of gas-phase as well as particulate-phase radicals in mainstream cigarette smoke with high reproducibility. We found that careful deoxygenation is a critical step to obtain consistent and accurate measurement of gas-phase radicals since any residual oxygen significantly impacts the yields. Results were expressed in nmol (relative to the reference standards used) rather than the number of spins to allow for a more direct comparison with other smoke toxicants as other authors have also suggested.^{50, 67, 68}

It is of interest to compare levels of free radicals in tobacco smoke with other major toxic tobacco smoke constituents currently on the FDA HPHC list including numerous carcinogens. Per cigarette, the observed levels of gas-phase radicals (6.2 nmol) were similar to those reported for other constituents including nitromethane (9.8 nmol), nickel (8.5 nmol), cobalt (1.7 nmol), 2-toluidine (1.3 nmol), hydrazine (1.8 nmol), heterocyclic A- α -C aromatic amine (1.4 nmol), N'-nitrosornicotine (NNN, 1.5 nmol), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 1.1 nmol), N-nitrosodiethanolamine (2 nmol), and N-nitrosopiperidine (2.2 nmol).⁶⁸ Likewise, observed particulate phase radicals per cigarette (65 pmol) were similar to several tar based constituents including benz[a]anthracene (117 pmol), benzo[j]fluoranthene (13 pmol), benzo[a]pyrene (60 pmol), chrysene (108 pmol), dibenz[a,j]acridine (3.6 pmol), and dibenzo[c,g]carbazole (2.6 pmol).

The Family Smoking Prevention and Tobacco Control Act gives FDA the authority to regulate cigarettes to reduce the impact on public health. FDA's Center for Tobacco

Products can consider regulatory proposals to disclose and set limits on total free radical yields from cigarettes. Much research is warranted to identify all the cigarette radical species and understand the biological implications of individual radicals. We believe our work is a step forward to bringing cigarette free radical emissions under regulatory framework.

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Abbreviations List

CFP	Cambridge filter pad
EPR	electron paramagnetic resonance spectroscopy
EPFRs	Environmentally Persistent Free Radicals
FTC	Federal Trade Commission smoking regimen
FDA	Food and Drug Administration
HPHC	hazardous and potentially hazardous chemical
PBN	spin-trapping agent phenyl-N-tert-butyl nitrene
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl

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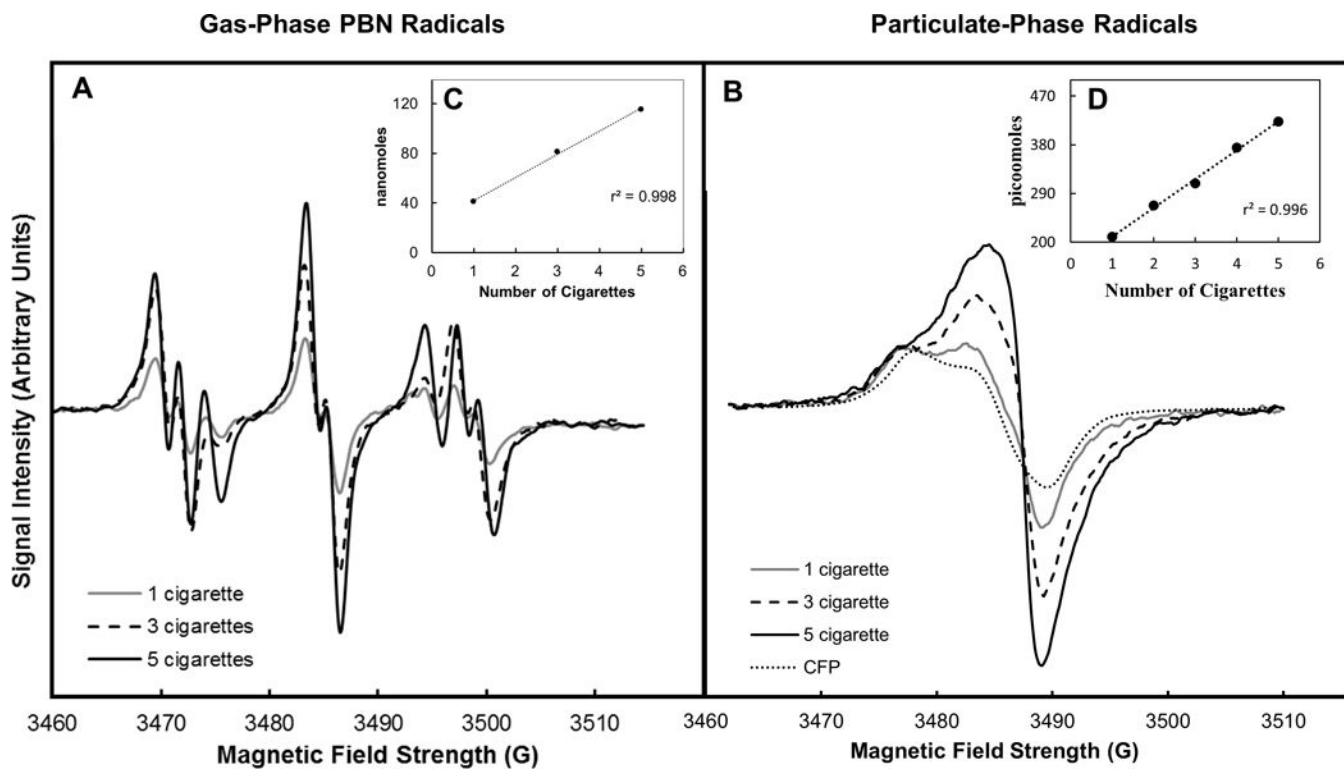


Figure 1. Representative EPR spectra for (A) gas-phase PBN radical adducts and (B) particulate-phase radicals for the 3R4F reference cigarette and blank CFP are shown. The concentrations of both gas-phase radicals (C) and particulate-phase radicals (D) are linearly dependent on the number of cigarettes smoked.

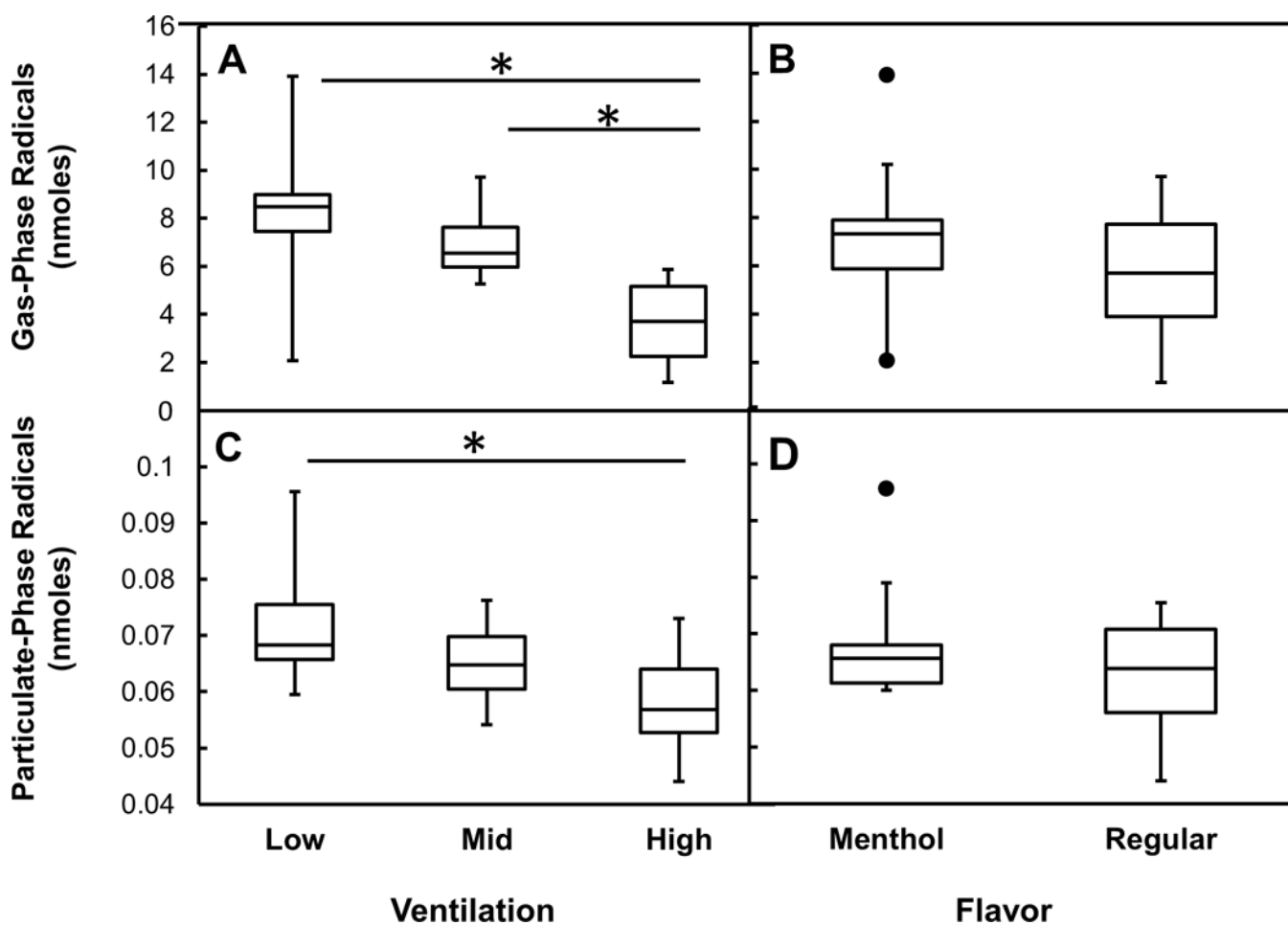


Figure 2. Box-plots of free radicals yields comparing brands by cigarette strength and flavor. Box-plots of gas-phase radicals (A) cigarette filter ventilation and (B) cigarette flavor (regular and menthol). Box-plots of particulate-phase (C) filter ventilation and (D) cigarette flavor (regular and menthol). *p < 0.05 between groups.

Table 1

Cigarette brand identification information with UPC, listing of flavor, filter ventilation and their yields of gas-phase and particulate-phase radicals.

Brands	UPC	Flavor ^d	Filter Ventilation (%) ^b	Gas-Phase Radicals ^c (nmol/cigarette)	Particulate-Phase Radicals ^d (pmol/cigarette)
3R4F	N/A	R	38	7.72 ± 2.22	63.87 ± 13.66
American Spirit Mellow Menthol	047995855055	M	39.2	2.06 ± 0.53	63.10 ± 9.47
American Spirit Menthol	047995855222	M	13.4	2.23 ± 0.63	64.22 ± 2.67
American Spirit Orange	047995855239	R	56.3	1.17 ± 0.28	44.08 ± 6.22
American Spirit Turquoise	047995855246	R	28.9	1.21 ± 0.15	52.72 ± 2.88
Camel Blue	01230739	R	32.3	6.49 ± 2.20	54.17 ± 11.38
Camel Filters	01230331	R	29.5	5.86 ± 0.84	60.33 ± 1.92
Camel Silver	012300200158	R	50.1	5.69 ± 1.78	64.04 ± 1.89
Eagle20's Gold Menthol	01117307	M	10.6	8.33 ± 0.94	95.59 ± 16.76
Kool	02725228	M	29.6	6.58 ± 0.90	60.72 ± 9.29
L&M Blue	028200310026	R	16.4	8.99 ± 2.59	75.50 ± 9.61
L&M Menthol	028200310323	M	21.8	7.90 ± 0.91	60.38 ± 8.35
L&M Red	028200309822	R	19.4	5.26 ± 1.34	70.79 ± 12.14
Marlboro Gold	02838423	R	26.4	6.31 ± 1.74	65.71 ± 13.99
Marlboro Menthol	02836124	M	18.9	5.60 ± 0.61	67.03 ± 11.49
Marlboro Red	02835727	R	15.4	5.32 ± 1.26	59.53 ± 11.81
Marlboro Silver	02847722	R	45.9	3.70 ± 0.87	52.59 ± 7.92
Newport	02657512	M	2.4	10.2 ± 0.39	65.70 ± 5.07
Newport Red	02665618	R	3.5	8.48 ± 1.88	66.81 ± 5.09
Pall Mall Menthol	02785929	M	43.3	5.87 ± 1.25	60.00 ± 14.63
Pall Mall Orange	02717520	R	57.9	3.90 ± 1.34	56.87 ± 5.88
Pall Mall Red	02785123	R	36.2	5.17 ± 1.33	56.12 ± 21.44
Parliament White	02878421	R	31.8	9.71 ± 1.60	74.17 ± 9.75
Parliament White Menthol	02878722	M	26.1	7.32 ± 2.28	76.28 ± 3.21
Pyramid Gold Menthol	01149403	M	10	13.9 ± 1.97	78.98 ± 9.75
Pyramid Red	01149209	R	12.1	8.61 ± 1.85	74.11 ± 10.66

Brands	UPC	Flavor ^a	Filter Ventilation (%) ^b	Gas-Phase Radicals ^c (nmol/cigarette)	Particulate-Phase Radicals ^d (pmol/cigarette)
Salem	01232038	M	18.5	7.44 ± 2.06	68.36 ± 9.17
Virginia Slims Gold	02873329	R	41	3.67 ± 0.78	73.06 ± 3.52

Note: All cigarettes were from king-sizes hard packs (85mm), except 3R4F (soft pack) and Virginia Slims Gold (100s).

^aR = Regular; M = Menthol

^bValues are mean (n=5)

^cValues are mean ± SD (n=3-7)

^dValues are mean ± SD (n=2-4)

Table 2

Spearman's rank correlation of mainstream smoke free radicals for the 27 brands of cigarettes.

	Gas-phase radicals	Tar-phase radicals
Tar-phase radicals	0.617 (0.0006)	
Filter ventilation	-0.586 (0.001)	-0.582 (0.001)
TPM	0.380 (0.04)	0.438 (0.02)

Note: Values are Spearman's Rank correlation coefficients (ρ) with corresponding p-values in parentheses.