Levels of Tobacco-Specific Nitrosamines and Polycyclic Aromatic Hydrocarbons in Mainstream Smoke from Different Tobacco Varieties

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Abstract

It has been estimated that one in every five cancer deaths worldwide are related to tobacco use. According to the IARC, 10 polycyclic aromatic hydrocarbons (PAH) and 8 tobacco-specific nitrosamines (TSNA), as well as at least 45 other compounds or substances found in tobacco smoke, are potential human carcinogens. The levels of these carcinogens in contents of tobacco and smoke emissions vary between different tobacco products. We evaluated mainstream smoke emissions from cigarettes made with different types of tobacco to examine the relation between their deliveries of TSNA and PAHs and any possible influence from tobacco nitrate content. To investigate the contribution of tobacco content to mainstream cigarette smoke deliveries without confounders such as filter design, filter ventilation, and paper porosity, we used custom-made, research-grade, unfiltered cigarettes that contained bright, burley, oriental, reconstituted, or mixtures of these tobaccos. Our findings confirm results from other researchers that tobacco type can influence the mainstream smoke delivery of nicotine, TSNAs, and PAHs. However, we found that the effect varies among individual compounds. In addition, we observed a statistically significant relationship between nitrate content and mainstream smoke 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK); nitrate level also influenced the mainstream smoke deliveries of the summed total of the 10 PAHs identified by IARC as potential human carcinogens. The influence of nitrate on mainstream smoke NNK and PAH levels were of different magnitude and direction. Our results tend to indicate an inverse relation exists between NNK and PAH deliveries when considering different tobacco blends.

Introduction

Tobacco use, alcohol consumption, obesity, and physical inactivity are major risk factors for cancer. All of these factors are behavioral in nature and thus partially modifiable. These factors contribute to about two-thirds of all cancers in western countries and at least one-third throughout the developing world. Tobacco use is the main cause of cancers of the lung, larynx, oral cavity, and esophagus and a major cause of bladder and pancreatic cancers. Approximately 20% of all cancer deaths worldwide can be attributed to tobacco use. One estimate places the 20th century’s tobacco-related death toll at 100 million people. According to projections, if current smoking patterns continue, tobacco could contribute to about 10 million annual deaths by 2020; 70% of these deaths will occur in developing nations. Unless effective measures are implemented to prevent young people from smoking and to help users quit, global tobacco deaths could approach 1 billion in the 21st century.
of TSNAs (6). High nitrate content, typically found in burley tobacco, is associated with higher TSNA levels. Previously, Hoffmann reported that ~46% and 26% to 37% of N-nitrosornicotine (NNN) and 4-(N-nitroso-methylamino)-1-(3-pyridyl)-1-butanone (NNK) in smoke are derived from NNN and NNK initially present in the tobacco content, with the remainder being pyrosynthesized from nicotine (7, 8). However, adding nitrate and nicotine to tobacco content did not significantly alter NNN and NNK levels in the mainstream smoke (9), suggesting that NNN and NNK in mainstream smoke transfer directly from tobacco with litter or no pyrosynthesis (10). Regardless of whether smoke TSNAs are directly transferred from preformed tobacco content of TSNAs or pyrosynthesized during smoking, nitrate in tobacco affects smoke TSNA levels.

Nitrate content in tobacco also strongly affects the pyrosynthesis of other carcinogenic agents in the smoke. The nitrogen oxides formed during tobacco combustion serve as scavengers of C and H radicals that are major precursors for the pyrosynthesis of carcinogenic PAHs (11). Different types of tobacco, and the same types grown under different agricultural conditions, can have widely different nitrate contents (6). Flue-cured (bright) and sun-cured (oriental) tobaccos tend to have lower nitrate content than air-cured (burley) tobacco. The pyrosynthesis of benzo[a]pyrene (BaP) in cigarettes made exclusively with flue-cured or sun-cured tobaccos produces higher BaP levels in the smoke than in cigarettes made with burley tobacco (11, 12). Tar, nicotine, BaP, and catechol in mainstream smoke are reduced by spiking cigarettes with nitrate; nitrogen oxides, nitroalkanes, volatile N-nitrosamines, and TSNAs are significantly increased by nitrate spiking (13, 14).

Our previous studies on popular commercial cigarettes revealed that both PAH and TSNAs yields in tobacco smoke vary significantly in cigarettes from different parts of the world (15). Formation of mainstream smoke PAHs and TSNAs are influenced by the tobacco blend. In cigarette brands containing different types of tobacco that delivered low TSNA levels in smoke, PAH levels increased as TSNA levels decreased. However, the relation between PAHs and TSNA was not statistically significant over a wider range of TSNA deliveries; confounding cigarette design factors that made direct product comparisons tenuous likely caused this result (15). King et al. further illustrated the inverse relation between BaP and NNK emissions (nicotine-adjusted) in Australian and Canadian cigarettes (16). Such findings are important because smokers concerned about their health risk may switch to a brand that delivers lower levels of a particularly worrisome chemical but may not actually reduce risk because of their overall exposure to other harmful chemicals.

The current study was designed to further investigate whether a statistically significant relation exists between TSNAs and PAHs in cigarette mainstream smoke and determine the influence that tobacco type and nitrate content play. We investigated whether differing tobacco type contributed to mainstream TSNA and PAH smoke deliveries without introducing confounding variables such as filter design, filter ventilation, and paper porosity. In this study, we used custom-made unfiltered cigarettes that contained bright, burley, oriental, reconstituted, or mixtures of these tobaccos, made with the same paper and no filter ventilation.

Materials and Methods

Reagents, Standards, and Materials. TSNAs, PAHs, and their isotopically labeled analogues were purchased from Cambridge Isotope Laboratories. Nicotine, acetonitrile, acetone, and cyclohexane were obtained from Sigma and were high-performance liquid chromatography (HPLC) grade. Isotopically labeled nicotine was purchased from Toronto Research Chemicals. Cambridge filter pads (44 mm glass fiber filter pad) were obtained from Whatman. Unfiltered custom-blended cigarettes were purchased from Murty Pharmaceuticals. Six different types of nonfiltered cigarettes were selected. They each contained either a single type of tobacco (burley, bright, oriental, or reconstituted) or a mix of tobacco types (blend I: 40% burley, 30% bright, 15% oriental, and 15% reconstituted; blend II: 25% each of burley, bright, oriental, and reconstituted). Blend I was selected to mimic popular American-blended cigarette brands.

Smoke Particulate Matter Collection. Before being smoked, the cigarettes and Cambridge filter pads were conditioned for at least 24 h at 22°C and 60% relative humidity. Mainstream smoke total particulate matter was generated by following the ISO smoking regimen (60-s puff interval, 2-s puff duration, and 35-mL puff volume) and collected on individual Cambridge filter pads using a Cerulean ASM500 16-port smoking machine (Milton Keynes). The cigarettes were smoked to a butt length of 23 mm or the length of the filter overwrap plus 3 mm, whichever was longer. One cigarette was smoked per pad for each individual sample. A total of 30 cigarettes for each type were smoked to obtain average analyte levels in the smoke particulate. During each smoking run, Kentucky Research 2R4F (University of Kentucky) cigarettes were smoked as quality-control samples. Cigarettes and their corresponding butts were weighed before and after smoking to estimate tobacco weights consumed during smoking.

Nicotine and TSNAs. Before smoking, each Cambridge filter pad was pretreated with ascorbic acid solution and air-dried in a chemical fume hood. Nicotine and four TSNAs (NNN, NNK, N-nitrosoanabasine, and N-nitrosoanatabine) were measured for each mainstream smoke particulate sample. After smoking, each Cambridge filter pad was spiked with isotopically labeled TSNA and nicotine internal standard solutions followed by solvent extraction using ammonium acetate solution on a Lab-line shaker operated at 250 rpm for 1 h. A 1-mL aliquot was placed in a 2-mL amber vial that was loaded on the autosampler where 20 μL were injected into an Agilent Technologies 1100 HPLC coupled with an API 4000 triple-quadrupole mass spectrometer [MS; Applied Biosystems; HPLC-tandem MS (MS/MS)] to analyze TSNAs. The HPLC column selection and MS variables were described previously (17). For nicotine analysis, the extract was diluted 1:1,000 with ammonium acetate solution and an aliquot of 20 μL was injected into the same HPLC-MS/MS system. The masses for the
quantification and confirmation ion pairs for nicotine were 163.2, 130, 163.2, and 117 amu, respectively. The masses for the quantification and confirmation ion pair for nicotine internal standard (nicotine-D₃) were 166.2 and 130 amu.

**PAHs.** A total of 21 PAHs was measured for each sample. The sample preparation scheme was based on two previously published methods (18, 19) with the following modifications. After smoking, cyclohexane extraction and solid-phase extraction were conducted on the total particulate matter. Eluents collected from solid-phase extraction cartridges were then dried using a Zymark Turbovap LV evaporator to 3 mL. A 200 µL aliquot was transferred to an autosampler vial and 1 µL was injected into a gas chromatography-MS to analyze eight PAHs (naphthalene, acenaphthylene, acenaphthen, fluorene, phenanthrene, anthracene, fluoranthene, and pyrene). The gas chromatography column selection and variables were as described (18). The following temperature program was used: 45°C/min ramp from 65°C to 152°C, 15°C/min ramp to 160°C, 45°C/min ramp to 175°C, 15°C/min ramp to 210°C, 5°C/min ramp to 220°C, and 15°C/min ramp to 260°C. Total run time was 9.80 min. An Agilent model 5973 MS was used for detection and data acquisition in selected ion monitoring mode.

The remaining eluent was evaporated and reconstituted in 200 µL acetonitrile. For quantitative analysis, 10 µL were analyzed using HPLC-MS/MS. The reconstituted smoke-extract solutions were injected into the HPLC-MS/MS system twice, on different columns, in a standard, dual-column configuration. A Thermo HyperSil Green PAH column (Thermo Electron) separated 8 of the PAHs (benz[a]anthracene, chrysene, 5-methylchrysen, benzo[j]fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, BaP, and benzo[py]pyrene). A Waters Xterra C₁₈ MS column (Waters) separated the remaining 11 PAHs (dibenzo[a,h]pyrene, 5-methylchrysen, benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[c]fluoranthene, BaP, dibenzo[a,h]anthracene, indeno[1,2,3-cd]pyrene, dibenzo[a,c]-pyrene, and dibenzo[a,i]pyrene) to obtain IARC 10 PAHs. The other 11 PAH levels were summed to obtain the remaining 11 PAHs.

**Nitrate Content.** The tobacco in the cigarette was weighed and nitrate levels were measured by a nitrate probe using a previously published method (15). The results were normalized by tobacco weight.

**Data Analysis.** The gas chromatography-MS reconstructed ion chromatograms used for quantification were processed using Xcalibur software (Thermo Electron). All HPLC-MS/MS data were processed using the Analyst software version 1.4.1 (Applied Biosystems). Each quantitation ion peak area was automatically selected and integrated. Integrations were manually inspected for errors and reintegrated if needed. Mainstream smoke nicotine, TSNA, and PAH results were normalized by tobacco weight consumed during smoking.

**Statistical Analysis.** Linear regression analysis was used to analyze relationships between TSNA or PAHs and nitrate and between TSNAs and PAHs. An ANOVA statistical procedure from the Statistical Analysis System was used to calculate the relational coefficients and significance. All calculated P values <0.05 were considered statistically significant.

## Results

### Comparison of Mainstream Smoke Constituents Yields by Tobacco Variety and Blends

In addition to four single types of cigarettes, we also tested cigarettes made with a mix of tobacco types. Tobacco contents in blend I mixture were similar to a typical American-blend cigarette (Marlboro). As expected, the mainstream smoke nicotine deliveries for certain constituents were dependent on the tobacco type. Cigarettes containing 100% burley tobacco delivered the highest mainstream nicotine levels followed by those containing 100% bright, blend I, blend II, 100% oriental, and 100% reconstituted tobacco (Table 1). The differences in mainstream smoke nicotine levels among these different types of cigarette were statistically significant. In general, deliveries of the four mainstream smoke TSNA levels from cigarettes made solely from burley tobacco were much higher than those from bright or oriental tobacco. This was especially obvious for NNN and N-nitrosoanatabine levels in mainstream smoke from 100% burley cigarettes, which were nearly three times higher than those in cigarettes made from 100% oriental tobacco.

### Table 1. Mainstream smoke nicotine, TSNA, PAH levels, and nitrate content in research cigarettes made from different tobacco varieties (with 95% confidence intervals)

<table>
<thead>
<tr>
<th>Smoke nicotine (mg/g tobacco)</th>
<th>Smoke TSNA (ng/g tobacco)</th>
<th>Smoke PAH (ng/g tobacco)</th>
<th>Nitrate content (ppm/g tobacco)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NNN</td>
<td>NNK</td>
<td>IARC 10 PAHs</td>
</tr>
<tr>
<td>Burley</td>
<td>5.40 ± 0.09</td>
<td>1,970.27 ± 81.87</td>
<td>174.12 ± 3.84</td>
</tr>
<tr>
<td>Bright</td>
<td>3.97 ± 0.10</td>
<td>35.30 ± 3.16</td>
<td>35.94 ± 4.00</td>
</tr>
<tr>
<td>Oriental</td>
<td>1.53 ± 0.03</td>
<td>84.23 ± 5.42</td>
<td>25.12 ± 2.32</td>
</tr>
<tr>
<td>Reconstituted</td>
<td>0.48 ± 0.02</td>
<td>241.70 ± 7.13</td>
<td>326.92 ± 7.38</td>
</tr>
<tr>
<td>Blend I</td>
<td>3.14 ± 0.07</td>
<td>486.68 ± 22.39</td>
<td>143.69 ± 7.51</td>
</tr>
<tr>
<td>Blend II</td>
<td>2.42 ± 0.06</td>
<td>534.92 ± 23.58</td>
<td>179.70 ± 5.82</td>
</tr>
</tbody>
</table>

**NOTE:** Ten PAHs listed in IARC monograph 83. All cigarettes were smoked under ISO smoking regimen. Mainstream smoke emission constituents were normalized by tobacco weight consumed during smoking. Nitrate contents were normalized by tobacco weight.
an order of magnitude higher than from cigarettes containing 100% bright or 100% oriental. The mainstream smoke deliveries for both NNK and NNK were also high in cigarettes made solely from reconstituted tobacco (Table 1) but still much lower than the level of NNN in smoke from cigarettes made with burley tobacco.

The levels of the 21 PAHs (IARC 10 PAHs + remaining 11 PAHs) in mainstream smoke from the cigarettes studied varied from nearly 1,000 ng/cigarette (naphthalene) to a few nanograms per cigarette (5-methylchry-sene, dibenz[a,h]anthracene, dibenz[a,e]pyrene, and dibenz[a,i]pyrene). The molecular weights for the PAHs ranged from 128 to 302 amu. The compounds with higher molecular weights (>200 amu) were consistently found at lower levels in mainstream smoke than those for the compounds with lower molecular weights. The mainstream smoke PAH distribution profiles from the four single-blend cigarettes differed considerably. The level of naphthalene was higher in the smoke of cigarettes made from burley tobacco where the mean ± 95% confidence interval was 978 ± 50 ng/g tobacco consumed compared with the smoke from cigarettes made from bright tobacco (752 ± 23 ng/g), oriental tobacco (848 ± 31 ng/g), and reconstituted tobacco (588 ± 50 ng/g). When the mainstream smoke levels of the remaining 11 PAHs, which were primarily low molecular weight compounds, were summed, there was significant difference between cigarettes made from burley compared with bright or oriental tobaccos, although their 95% confidence intervals overlapped somewhat (Table 1). In contrast, higher molecular weight PAHs, such as the IARC 10 PAHs, were found at higher levels in the mainstream smoke of cigarettes made from bright and oriental tobaccos compared with burley (Table 1). The reconstituted tobacco used in this particular study had the lowest mainstream smoke delivery of PAHs in all cases (Table 1).

**Nitrate Content in Tobacco Filler and Its Influence on Smoke Constituents.** Nitrate levels were the highest in the reconstituted tobacco followed by burley, blend II, blend I, oriental, and bright (Table 1). The nitrate level differences among the four types were statistically significant. Mainstream smoke levels of NNK increased with increasing nitrate content (P < 0.01; Table 2). Although the relationship between NNN and nitrate also suggested a positive trend, this was not statistically significant (Table 2). Nitrate content was inversely related with IARC 10 PAHs (Table 2). Nitrate content and remaining 11 PAHs suggested the same inverse relation but were not statistically significant (Table 2).

**Intercorrelation in Constituent Yields.** Regression analysis suggested an inverse relationship between all 4 TSNAs and IARC 10 PAHs (Table 2); however, only the relationship between NNK and IARC 10 PAHs was statistically significant in this study (P < 0.01; Table 2). No statistically significant relationships were found between any of the TSNAs and remaining 11 PAHs (Table 2).

**Discussion.**

Most studies quantifying mainstream cigarette smoke constituents have noted a relation between nicotine and other smoke constituents (11, 20). The reported levels, using standardized smoking regimens, are heavily influenced by dilution of mainstream smoke from air entering through the filter ventilation holes especially among those with highly ventilated filter tips, specifically the so-called “light” and “ultralight” cigarette varieties. To reduce complications associated with differing filter ventilation or other physical design variables, we examined unfiltered and unventilated cigarettes made by using different tobacco types. Our results confirm that smoke nicotine, PAH, and TSNAs deliveries can be influenced by tobacco type (9, 11).

The mainstream smoke PAH distribution profiles varied with tobacco type. However, even in the most extreme cases, the delivery of PAHs between the various types of tobacco differed by only 2- to 3-fold (compared with a 50-fold difference in TSNA levels), suggesting that chemical or mechanistic differences are important in the formation of individual PAHs during the tobacco combustion process. Cigarettes made exclusively from oriental tobacco had difficulty burning continuously, often self-extinguishing, and had a much longer static burn rate. This type of slow or incomplete combustion may have contributed to the high PAH levels from the oriental tobacco smoke. Our findings confirm previous research (5, 13) that nitrate affects PAH formation during cigarette combustion. However, the effect of nitrate on different PAH species appears to vary with molecular weight. The influence of nitrate on higher molecular weight PAHs, such as IARC 10 PAHs, appears to be greater than on lower molecular weight PAHs. This further supports the hypothesis that chemical or mechanistic differences influence the formation of individual PAHs.
Because the relative levels of PAHs differ with tobacco type, a single surrogate (BaP) is insufficient to serve as a marker for all PAHs. The relative toxicity or carcinogenicity depends on the specific PAH species (4, 21-23); thus, such factors must be included when evaluating relative risk from cigarette smoke generated by different tobacco types. Therefore, to better understand the relative risks associated with mainstream cigarette smoke PAH exposure from different types of tobaccos, multiple PAH surrogates covering the range of deliveries and carcinogenicity should provide more accurate information.

The mainstream smoke NNN levels from the burley tobacco cigarettes are much higher than from any of the other single tobacco types or the blended tobacco cigarettes. When burley tobacco is excluded from the comparison, the largest effect on the total carcinogenic TSNA levels (NNN + NNK) is from the reduction in NNN levels. Although it is not of the same magnitude, removing burley from the comparison dramatically reduces the NNK smoke levels from cigarettes with higher percentages of bright or oriental tobaccos. The variation in TSNA levels with tobacco type was also evident in previous studies of TSNAs in tobacco and smoke from cigarettes purchased globally (24, 25).

Depending on tobacco type (bright or American blended), total smoke TSNAs vary significantly. Thus, when evaluating the differences in the levels of the carcinogenic TSNAs in tobacco smoke from changing tobacco blends, NNK will be a factor, but the primary effect will be variation in the NNN levels. We analyzed tobacco content of nitrate, mainstream smoke nicotine, and TSNA levels from our custom-blended research cigarettes and observed a statistically significant relationship only between nitrate and NNK delivery. Reconstituted tobacco used in this study had the highest nitrate level per gram of tobacco, but cigarettes made with this material delivered lower NNN levels than cigarettes made with burley tobacco. Clearly, there are other factors, independent of nitrate, which significantly influence NNN deliveries.

Reconstituted tobacco was introduced in cigarette manufacturing to reduce cost by using tobacco stems, fines, and dust to supplement tobacco lamina used in cigarette content (11). Tobacco stems and ribs added to reconstituted tobacco are naturally low in nicotine but high in nitrate (11). We observed the highest NNK levels and higher levels of NNN in mainstream smoke from cigarettes made exclusively of reconstituted tobacco compared with single blended bright and oriental cigarettes. The NNK mainstream smoke levels from burley cigarettes, which had the second highest nitrate content, were significantly higher than all other tobacco types. The high mainstream TSNA levels from cigarettes made with reconstituted tobacco could result partially from an assortment of chemical reactions facilitated by the high ambient nitrate levels, although the nicotine levels are relatively low. It has been recommended that manufacturers extract nitrate from the stems and ribs before producing reconstituted tobacco (11). This could be especially important for the nitrate-rich stems and ribs from burley tobacco (11).

Our data, in accordance with previous findings (11), suggest that nitrate has an opposite effect on the levels of NNK and IARC 10 PAHs in mainstream tobacco smoke. The NNK levels in smoke are positively correlated with nitrate and IARC 10 PAHs are negatively correlated with nitrate (Table 2). Although possibly acting as a precursor for TSNA formation, nitrate affects the formation of smoke PAHs by serving as a free radical scavenger (11). Reducing nitrate content to reduce NNK may result in increasing smoke PAH deliveries. Because mainstream smoke levels of total carcinogenic TSNAs are determined primarily by NNN, we found no statistically significant relationship between total carcinogenic TSNAs and PAHs. Given that smokers consume cigarettes to obtain a desired dose of nicotine, we normalized smoke TSNAs and PAHs by nicotine in smoke as a statistical covariate and examined whether there was a relation between TSNAs and PAHs. With nicotine normalization, no statistically significant relationship was found between any of the TSNAs and PAHs. In agreement with previous studies by Counts (26), King et al. (16) and Ding et al. (15), the results of this study suggest that it is possible to reduce high TSNA levels found in some commercial cigarettes without a substantial increase in BaP. Thus, it is important to consider what range of TSNA levels are being compared when evaluating the relationship between TSNAs and PAHs.

Although our current findings cannot be immediately generalized to all products because only a single source of each tobacco types was tested, we found that our results are comparable with published data on commercial cigarettes using mainstream nicotine levels to adjust for differences in cigarette design. To fully examine the interrelationships among these different tobacco types, additional sources of different tobacco samples grown and processed in different locations should be examined. In addition to NNK and NNN, both of which are listed on the IARC tobacco smoke carcinogen list (4), the other six carcinogenic TSNAs should be examined to understand the various interactions between constituents. Additional analyses on samples of similar tobacco with differing nitrate levels would allow a more complete characterization of the influence of nitrate on other smoke constituents. Therefore, we must not overgeneralize our results because limited tobacco samples were tested.

Current evidence strongly suggest that the high mainstream smoke TSNA levels from cigarettes made with burley tobacco can be lowered without substantially raising mainstream smoke PAH levels by reducing burley content. Through careful selection of tobacco types, curing practices, reducing nitrate fertilizer usage and nitrate in the reconstituted tobacco, and optimizing free radical scavenger chemical processes, it is technically feasible to concurrently lower the mainstream smoke levels of both PAHs and TSNAs. Whether such reductions would reduce the health risk associated with smoking is unknown.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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