Assessment of tobacco-specific nitrosamines in the tobacco and mainstream smoke of Bidi cigarettes

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Bidi cigarettes, or bidis, are a tobacco product that originated in India and have been gaining popularity in the USA during the past few years, particularly with adolescents. As with conventional cigarettes, tobacco and smoke from bidis contain chemical constituents including carcinogenic chemicals such as the tobacco-specific nitrosamines (TSNAs). To help better assess the potential public health risk associated with bidi cigarettes, we developed modern high throughput methods to accurately quantify TNSA levels in tobacco and mainstream cigarette smoke particulate. We determined the TNSA levels in the tobacco filler and mainstream smoke from 14 bidi cigarette brands. In the bidi tobacco filler, the 4-(methylisotosamino)-1-(3-pyridyl)-1-butanone (NNK) levels ranged from 0.09 to 0.85 ng/g, while N'-nitrosonornicotine (NNN) levels ranged from 0.15 to 1.44 ng/g. These amounts are comparable with those in typical American blended cigarettes. The levels of NNN in mainstream smoke from bidis ranged from 2.13 to 25.9 ng/cigarette, and NNN levels ranged from 8.56 to 62.3 ng/cigarette. The wide variation in the TNSA levels most probably reflects the hand-rolled nature of the bidi cigarettes, resulting in a product with less homogenous tobacco amount and a wider variation in overall cigarette construction quality. TNSA levels of bidis were comparable with those of conventional cigarettes, and bidis should not be considered a lower-risk alternative tobacco product. Our analytical findings concur with the previous biologic and biochemical evidence supporting epidemiologic studies linking bidi use with various cancers, especially oral cavity and lung cancers.

Introduction

Bidis are hand-rolled cigarettes consisting of a small amount of sun-cured oriental tobacco flakes rolled in a leaf from tendu, a broad-leaved tree native to India. Most bidi cigarettes are thinner than conventional cigarettes and their construction more closely resembles a miniature cigar. Bidi cigarettes sold in the USA are available with various flavorings, including vanilla, chocolate, strawberry, grape, root beer and others. High levels of flavor agents (1) are added, presumably to increase their appeal. Bidi cigarettes sold in India traditionally do not contain any flavor additives. Increased importation in the USA began in the 1970s, and bidi cigarettes are now available at a variety of retail outlets. In the last 10 years, use of bidi cigarettes has proliferated within middle school and high school students (2), although they were initially popular primarily among college students. Apart from ease of purchase and alluring packaging, many teenagers prefer to smoke bidi cigarettes because they perceive bidi smoking to be less harmful than traditional cigarette smoking. In fact, 44% of teenagers surveyed in a San Francisco study reported believing that smoking bidi cigarettes did not contribute to cancer (3). Many of them mistakenly thought bidi cigarettes were tobacco-free herbal cigarettes. Bidis’ wide availability, heavy use of flavoring agents and perception of lower risk than conventional cigarettes warranted an examination of the chemicals associated with smoking bidi cigarettes.

Previous studies have shown that bidis are quite different from conventional US cigarettes. The tobacco filler in a bidi cigarette can contain twice as much nicotine as that of a conventional cigarette (4). The bidi’s outer wrapper consists of a relatively low porosity tendu leaf. The lowered porosity probably contributes to its low combustibility, requiring bidi cigarettes to be smoked more rapidly than conventional cigarettes to prevent self-extinguishing. An increased puff frequency facilitates additional exposure of smoke constituents in the oral cavity, airway and lungs. In comparisons of US cigarettes smoked under standard Federal Trade Commission conditions (5) with bidis smoked at twice the puff frequency, bidis yielded five times more ‘tar’ and three times more nicotine and carbon monoxide than regular cigarettes (6). Previous chemical analysis of Indian bidi smoke condensate identified the presence of high levels of phenols, cresols, ammonia, hydrogen cyanide and polyaromatic hydrocarbons, including benzo[a]pyrene (7). A recently published study reported that selected flavor chemicals, with demonstrated toxic properties in laboratory animals or humans, are 10–2000 times higher in bidis than in conventional US cigarettes (1).

No studies have specifically addressed the health effects of bidi smoking in the USA. However, in India, where the use of tobacco products including bidi cigarettes is widespread, the adverse health effects from consuming and manufacturing bidis are better defined. Bidi cigarettes are the most common form of tobacco product in India. According to data collected by the World Health Organization, 675 billion bidis are produced and consumed each year in India, accounting for 40% of tobacco consumption (8). Increased cancer rates and chromosomal aberrations are seen in bidi rollers, who are exposed to toxic tobacco dust through nasopharyngeal and cutaneous routes (9–13). Bidi smoke condensate was reported to induce elevated frequency of micronucleated erythrocytes in the bone marrow of Swiss mice (14) than did conventional cigarette smoke condensate. Research from Menon and Bhide (15)

Abbreviations: GC, gas chromatography; MS, mass spectrometry; NNAL, 4-(methylisotosamino)-1-(3-pyridyl)-1-butanol; NNN, N'-nitrosonornicotine; TSNA, tobacco-specific nitrosamines.
showed that urine from bidi smokers was mutagenic, suggesting some of the conjugated or detoxified products could cause cancer. Yadav and Thakur (16) concluded that bidi smoke condensate is both clastogenic and genotoxic because a time-dose-effect relationship showed increased frequency of chromosomal aberrations and sister chromatid exchange. Additional reports from India have linked bidi smoking to an increase of cancers including those of the tongue, gums and floor of the mouth (17,18). Other malignancies associated with bidi consumption include laryngeal, esophageal, lung and liver cancer (19–21).

Tobacco products and cigarette smoke contain a variety of carcinogens (22). One of the major carcinogenic chemicals in tobacco and cigarette smoke is the tobacco-specific nitrosoamines (TSNAs). Of the seven TNSA found in the tobacco, N'-nitrosomorronicotine (NNN), 4-(methylnitrosamino)-1-(3-pyrridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyrridyl)-1-butanol (NNAL) are considered the most carcinogenic (23). NNK and its primary metabolite, NNN, are thought to be particularly important in the induction of adenocarcinoma, now the most common type of lung cancer among smokers in the USA (24). NNN induces benign and malignant tumors in the lung and in the upper aero digestive tract of mice, rats, hamsters and mink (25–28). NNN occurs in greater concentrations in cigarette smoke than any other esophageal carcinogen. Smoking causes esophageal cancer and is responsible for 70–80% of esophageal cancer deaths in the USA [23]. Numerous reviews have discussed aspects of TNSA formation, biochemistry, metabolism, carcinogenicity and relevance to human cancers (23,28–31).

Given that bidi cigarettes contain high levels of flavoring agents, presumably increasing their appeal, and are relatively new in the US, we believe the analysis of TSNAs in bidi tobacco and smoke is prudent. These results will assist in our understanding of tobacco-associated carcinogenesis by providing insights into the potential health risks associated with bidi cigarette. To the best of our knowledge, only one study has reported TNSA levels in both tobacco and smoke from four bidi cigarette brands (32). In that study, the bidi cigarettes were purchased in India and were unflavored, differing from the flavored bidi cigarette sold in the USA. Therefore, we measured the TNSA levels in both tobacco and smoke of selected flavored bidi cigarette brands. We analyzed 14 brands of bidi cigarettes and five brands of conventional cigarettes to provide representative TNSA ranges available in both the tobacco filler and mainstream cigarette smoke particulate.

Materials and methods

Cigarettes

Bidi cigarettes and conventional cigarettes were purchased at local retail outlets. Research cigarettes (IR4F and IR5F) were purchased from the University of Kentucky (Lexington, KY). Before smoking, all the cigarettes were conditioned at 22 °C and 60% humidity for at least 24 h. Because bidi cigarettes are hand-rolled, cigarettes vary considerably in length, diameter and weight. Standardized machine smoking protocols specify that the cigarettes are to be smoked to a specified butt length. Therefore, we chose bidi cigarettes of similar length from each brand to obtain more homogeneous sampling.

Apparatus

Mainstream cigarette smoke was generated using a Cerulean (Milton Keynes, UK) ASM 500 16-port smoking machine. Gas chromatography/mass spectrometry (GC/MS) analysis was carried out on an Agilent mass selective detector 5973N (Newark, DE) connected to an Agilent gas chromatograph 6890. Liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis was conducted using an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington, DE) coupled with an API 4000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, CA).

Reagents

The TNSA analytical standards, NNN, NNK, NNAL, N'-nitrosomorronicotine and N'-nitrosodimethylnitrate were purchased from Toronto Research Chemicals (Ontario, Canada). Trimethyl-[13C3] caffeine was purchased from Isotec (Miamisburg, OH). 2,2',3,3',4,4',5,5',6,6'-[13C6]-NNN and 1,2',3',4',5,6,6'-[13C6]-NNK were obtained from Cambridge Isotope Laboratories (Andover, MA). Ethyl acetate, methanol, acetone, and methanol and chloroform were obtained from Burdick & Jackson (Muskegon, MI). All other chemicals were of analytical grade and obtained through Sigma (St Louis, MO) unless otherwise indicated. Helium gas, supercritical fluid extraction (SFE)-grade carbon dioxide, industrial-grade liquid carbon dioxide and high-purity nitrogen gas were obtained from Air Products and Chemicals (Allentown, PA).

Analyses of TNSA in tobacco

The TNSA concentrations in bidi tobacco filler were determined using a previously described method (33). The filler was removed, weighed, ground, placed in a 3-ml SFE vessel, and spiked with 100 μl of an internal standard solution containing 10 μg/ml trimethyl-[13C3] caffeine solution. Although iso-topically labeled TSNAs potentially would have been a better choice for the internal standard, these compounds were not available at the time these measurements were made. After SFE extraction and solid phase extraction (SPE) purification, a 1-μl aliquot was analyzed by GC/MS using splitless injection. The GC inlet temperature was maintained at 250 °C. A J&W fused silica (Folsom, CA) DB-17MS (30 m × 0.25 mm i.d. × 0.25 μm film) chromatography column was used for analytic separation. The helium carrier gas was maintained at a flow of 40 cm/s. The GC oven temperature was initially held at 40 °C for 1 min, ramped at 20 °C/min to 160 °C and held for 1 min, increased by 4 °C/min to 200 °C, held for 1 min, increased by 15 °C/min to 260 °C, and held for 1 min. The post-run period was 20 min at 260 °C. The response of the ions of interest was determined in the selected-ion-monitoring mode. The quantification ions (m/z) for NNN, NAT, NAB, NNK and trimethyl-[13C3] caffeine were 177, 159, 161, 177 and 197.

Analyses of TNSA in mainstream smoke

Quantification of the TNSA levels in bidi mainstream smoke followed our previously established protocol (34). Conventional cigarettes are normally smoked as specified under the Federal Trade Commission (5) standard conditions with a 35-ml puff volume of 2-s puff duration at a frequency of one puff per min to a specified butt length of 23 mm or the length of the filter overwrap plus 3 mm. To prevent self-extinguishing of the lit bidi cigarettes, we increased the puff frequency to 2 puffs/min. Compared with conventional cigarettes, bidi have poorer combustibility because of the loosely packed tobacco filler and the low porosity of the tendu leaf wrapper. Conventional cigarettes composed of different style tobacco filler also were selected and smoked at 1 puff/min [normal Federal Trade Commission (FTC) regimen] and 2 puffs/min (bidi regimen) for comparison.

Mainstream smoke particulate was collected on Cambridge filter pads (CFP) pretreated with 40 mg of acetic acid to prevent artifact formation. After smoking a cigarette, each CFP was spiked with 20 μl of an internal standard solution, containing 1 μg/ml of 13C6-NNN and 1 μg/ml of 13C6-NNK, with subsequent extraction in methylene chloride for 45 min. The extract was evaporated to 500 μl. A one-step liquid extraction followed by SPE was used for analyte purification. The eluant from SPE was dried and reconstituted in 100 μl of a 5% methanol in 5 mM ammonium acetate solution. A 20-μl aliquot of this solution was used for subsequent LC/MS/MS analysis.

TSNA was separated from the smoke extract by reverse-phase high performance liquid chromatography with a Waters Xterra C18 MS column (4.6 × 50 mm i.d. × 5 mm) at 60 °C and equilibrated with 95% solvent A (5 mM ammonium acetate) and 5% solvent B (5 mM ammonium acetate in acetonitrile). A flow rate of 1 ml/min with a linear gradient was used as follows: 5% solvent B for 1 min, 5–50% solvent B over the course of 1 min, and 50% solvent B for 3 min. The total run time was 8 min including a 3-min equilibration time. TSNA analyses were ionized by the Turbospray ionization source operated in positive ion mode. Mass spectral data on precursor and product ions were collected in multiple-reaction monitoring mode.

Results

The ranges of NNN, NNK, NAT and NAB in the tobacco filler from 14 bidi cigarette brands and selected conventional cigarette brands were quantitatively determined (Table I). Comparing the NNN and NNK levels from the bidi filler by a two-tailed, two-sample unequal variance t-test, we found that the bidi
tobacco filler contained no statistically significant difference for NNK ($P = 0.13$) and NNN ($P = 0.15$) between two manufacturers. Levels of NNK ranged from 0.09 to 0.85 μg/g, and NNN ranged from 0.15 μg/g up to 1.44 μg/g. The highest level of NNK was observed in one of the Darshan chocolate-flavored cigarettes at 0.85 μg/g. The highest levels of NNN, 1.44 μg/g, were measured in Darshan’s chocolate- and vanilla-flavored bidi cigarettes. The NNN and NNK levels in the bidi filler generally are comparable with those in the American blended US brand cigarette filler. The flue-cured (Virginia style) cigarette brand has much lower TSNA levels.

The mainstream smoke TSNA concentrations from bidi cigarettes and selected conventional cigarettes were determined in triplicate (Table II). Like the TSNA levels measured in the tobacco filler, no statistically significant difference for mainstream smoke levels of NNK ($P = 0.23$) and NNN ($P = 0.24$) was observed between two manufacturers. The average concentration of NNK in the mainstream bidi smoke from all 14 brands was 10.5 ng/cigarette. The NNN levels ranged from 8.56 to 112 ng per bidi cigarette, with an average of 37.5 ng. The highest levels of both NNK and NNN in mainstream smoke were from the Shiv Sagar regular brand that contained an average of 22.0 ng NNK per cigarette and 90.7 ng NNN per cigarette. Comparing the NNN and NNK levels in mainstream smoke from bidi and conventional cigarettes smoked at 2 puffs/min indicated that the average TSNA levels in bidis were lower than American-blended style cigarettes but higher than flue-cured (Virginia style) cigarettes.

### Discussion

Formation of TSNA is primarily a post-harvest event for all *Nicotiana tabacum* types of tobacco including flue-cured bright, sun-cured oriental and air-cured burley tobacco. The TSNA levels we observed in bidi filler (Table I) containing sun-cured oriental tobacco generally were comparable with conventional American blended cigarettes (Table I) containing a mixture of bright, burley, oriental, tobacco plant stems and reconstituted tobacco. Burley tobacco is known to have a higher TSNA and nicotine content than that in oriental tobacco, primarily because of its high nitrate content (35). The comparable TSNA level between bidi and conventional American blended cigarettes, together with the high nicotine content in bidi filler (4), suggest that bidi tobacco is a specific cultivar of oriental tobacco, which differs considerably from the typical oriental tobacco used for conventional American blended cigarettes. Fischer *et al.* found that overall TSNA levels are typically highest in burley tobacco, followed by Virginia and oriental tobacco (36). In the present study, much lower TSNA levels were observed in the tobacco filler from the cigarettes that contained only flue-cured (Virginia style) tobacco. Factors influencing the TSNA distribution in tobacco filler could include tobacco plant variety, cultivation practices, nitrate fertilizer use and curing techniques.

Measuring TSNA levels in mainstream smoke, rather than in the cigarette filler, directly estimates a smoker’s carcinogenic exposure. As with the tobacco filler, the NNN content in mainstream smoke is higher than NNK in most cigarettes except the flue-cured (Virginia style) cigarette brand. Although the average mainstream smoke deliveries of NNN and NNK were fairly consistent among bidi cigarette brands, TSNA levels...
varied significantly from cigarette to cigarette (Table II). Variation in NNK levels, calculated as coefficient of variance (CV), ranged from 5.6% in Shiv sagar cinnamon-flavored cigarette to 62% in Darshan clove-flavored cigarette while variation of NNN ranged from 14% in Darshan cinnamon-flavored cigarette to 58% in Darshan clove-flavored cigarette. We postulate that such variations are a consequence of the hand-rolled nature with limited quality control during bidi cigarette production. We measured bidi tobacco filler from 60 individual bidi cigarettes and found that it ranged from 0.01 to 0.42 g/cigarette with an average value of 0.2 g. Total weights of unburned bidi cigarettes in this study ranged from 0.34 to 0.61 g. The level of variation is significantly higher than in conventional cigarettes, which vary in weight usually <10%. The variations in mass result from bidi-to-bidi variation in the tobacco filler amount and from the amount of tendu leaf used. The tendu leaf wrapper usually comprises at least half of the bidi cigarette’s mass. The average levels of NNK and NNN in bidi mainstream smoke are lower than in the conventional domestic full-flavored cigarettes but much higher than in the flue-cured (Virginia style) cigarette. Conventional cigarettes contain more tobacco filler (~0.7 g) than bidi cigarettes (~0.2 g). When normalized per gram of tobacco smoked, the mainstream smoke deliveries of the total amount of NNK and NNN from bidis and conventional domestic cigarettes are nearly equal.

The mainstream smoke TSNA levels could originate as a combination of direct transfer from the tobacco or pyro-synthesis during the smoking process. Adams et al. (37) spiked conventional cigarettes with ^14C nicotine to the cigarettes before smoking and observed that 63–74% of NNK in mainstream smoke was formed by pyro-synthesis during the smoking process. Moldoveanu et al. (38) studied TSNA pyro-synthesis on a small number of cigarettes by addition of isotopically labeled nicotine into filler before smoking and found 9.1–62% for NNK and 4.5–54% for NNN in the mainstream smoke originated from pyro-synthesis and the percentage depended on the preformed TSNA levels in the tobacco. Therefore, the considerable variation in TSNA mainstream smoke deliveries for certain bidi cigarettes could be attributed to a combination of factors, including heterogenous tobacco filler content, wrapper leakage, tobacco quality and nitrate content.

Our data show that bidi cigarettes deliver comparable TSNAs to conventional cigarettes when smoked using a standardized machine smoking protocol. However, people smoke bidi cigarettes differently from conventional cigarettes. An assessment of bidi smoker practices in Bombay found that the average puff frequency ranged from 4 to 5 puffs/min (39). This is significantly faster than either the Federal Trade Commission smoking regimen of 1 (5) or 2 puffs/min used in the Massachusetts smoking protocol (40), which mimics more intense cigarette smoking patterns associated with modern ‘lower-delivery’ conventional cigarettes. Previous studies have reported that a more rapid puff frequency significantly increases the TSNA yields in the mainstream smoke (41). Smoking more rapidly would undoubtedly result in higher TSNA mainstream smoke deliveries, and those values could be significantly higher than we measured.

Conventional cigarette deliveries of NNN and NNK increase considerably when the puff frequency increases from 1 to 2 puffs/min (Table II). Other results from our laboratory indicate that TSNA levels in the mainstream smoke do not necessarily parallel tar delivery, although tar has been used widely as a surrogate measure for carcinogenic compounds in tobacco smoke (42). Therefore, TSNA levels in the mainstream smoke should be considered as an additional risk factor for cancer as suggested by Hoffmann et al. in 1982 (43). Reductions in tar and nicotine levels in conventional cigarettes that began in the 1960s have not resulted in fewer lung cancers because, as the smoke deliveries were reduced, smokers apparently compensated by increasing puff frequency and depth of inhalation (44,45). The increased puff frequency and deeper inhalation by smokers of low-delivery cigarettes contributes to higher deliveries of TSNAs and other carcinogenic compounds. Such additional exposure may have contributed to the rise in adenocarcinoma of the lung among cigarette smokers (46,47). Therefore, it may not be appropriate to focus exclusively on decreasing tar delivery. Reduction of TSNAs appears feasible because of improvements in tobacco curing and advances in filtration (48) for conventional cigarettes.

Quitting smoking and avoiding exposure to environmental tobacco smoke are the only established means to significantly reduce the risk associated with exposure to TSNAs and other harmful chemicals in tobacco smoke. The Surgeon General’s health warnings should be included on the bidi packaging to help dissuade the misconception that bidi cigarettes are somehow more ‘natural’ products and therefore less hazardous. Additional action is needed to counter the popularity of bidi cigarette smoking, especially among young people and others who may experiment with bidis. Additional research, consumer education and public advocacy are critically needed to help reduce the health consequences of bidi smoking.

In summary, our data clearly show that TSNA levels in mainstream bidi cigarette smoke are comparable with those from conventional cigarettes. The analytical findings agree with other studies that tobacco products, including bidis, contain carcinogenic compounds, which are of concern in the formation of cancer in users of these products. Most importantly, the growing body of scientific evidence in the tobacco research field has advanced our understanding of the process of tobacco-associated carcinogenesis, and such findings will help provide needed information to researchers in their quest for effective prevention of cancer from the use of tobacco products.

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