ENDOGENOUS NITROSATION AS A CONTRIBUTOR TO CANCER RISK IN TOBACCO USERS

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Introduction

Endogenous formation of carcinogenic *N*-nitroso compounds from secondary amines and other precursors is an important risk factor for cancer risk in humans. Investigation of the endogenous nitrosation processes and the approaches to their prevention has been, and remains, a key research focus of academician Gh. Duca and several of his former and current advisees. This important field first attracted me to the studies of human carcinogenesis when I was an undergraduate student in the Department of Industrial and Ecological Chemistry. I started working under the mentorship of Professor M. Gonta, also a former advisee to Gh. Duca and a contributor to this monograph. Later, during graduate studies, Gh. Duca advised me in studies of tobacco-specific *N*-nitrosamines as part of my PhD dissertation. The fascinating research and the high standards for research that were seeded in me during these years led to a lifelong interest in tobacco carcinogenesis and commitment to conducting rigorous research that can lead to the development of preventive measures to reduce the deadly toll of tobacco use.

The historical outline of research on nitrosamines and endogenous nitrosation under advising and mentorship of academician Gh. Duca is summarized in the chapter titled "Procese de epurare a apelor reziduale de polutanti greu biodegradabili", written by M. Gonta and co-authors. This research program resulted in many important findings and publications, produced a cadre of successful researchers, and spurred new areas of scientific investigations. In this chapter, I will provide an overview of research on endogenous nitrosation in tobacco users that evolved during my postgraduate career as an independent researcher. As a testament to continuous importance of this field to public health, this research and relevant studies have been supported by grants from the U.S. National Institutes of Health and have been published in high ranking peer-reviewed scientific journals. I will always be grateful for the inspiration and guidance I received from academician Gh. Duca and the colleagues at the Department of Industrial and Ecological Chemistry, which laid foundation to my current research program in global tobacco control.

N'-nitrosonornicotine: a key carcinogen in tobacco products

N'-nitrosonornicotine (NNN, Figure 1) is one of the most carcinogenic of the commonly occurring tobacco-specific nitrosamines (TSNA), and is believed to play an important role in the induction by tobacco products of cancers of the esophagus and oral cavity (reviewed in [1]).

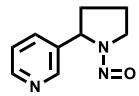


Figure 1. Structure of N'-nitrosonornicotine (NNN)

In laboratory animals, NNN causes oral and esophageal tumors in rats, respiratory tumors in mice and hamsters, and nasal cavity tumors in rats and mink.[1] Oral swabbing with a mixture of NNN and the related tobacco nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) caused oral cavity tumors in rats.[2] Furthermore, in a recent study we recently demonstrated that the treatment of rats with NNN in drinking water causes oral tumors in the absence of NNK (Table 1).[3]

Table 1.

Number of tumors in the oral cavity and esophagus of rats treated with NNN in drinking water for 17-19 months [a].

Group (number of rats)	Number of tumors in target tissues (number of tumors per rat)	
	Oral cavity	Esophagus
Drinking water control (12)	0	0
NNN, 28 ppm in drinking water (22)	99 (8.3)	153 (13)

[a] *Adopted from the Balbo et al.*[3]. *In that study, (S)-NNN, (R)-NNN, and racemic NNN were used to treat rats. We here present numbers for racemic NNN.*

The relevance of these animal data to humans is supported by our recent finding of the strong relationship between NNN exposure in smokers and risk of esophageal cancer in a prospective cohort,[4] as well as by the epidemiological evidence that smokeless tobacco products contaminated with high levels of NNN cause oral cancer.[5-7] In addition, we recently showed that smokers with head and neck squamous cell carcinoma have elevated levels of urinary biomarkers of exposure to NNN.[8] Based on mechanistic and laboratory animal data, as well as epidemiological evidence, IARC classified NNN together with NNK as carcinogenic to humans (Group 1).[9] Prevention of human exposure to this carcinogen in those smokeless tobacco users who are unwilling or unable to quit tobacco use is critical.

Endogenous formation of NNN via nitrosation of nornicotine

Nornicotine is a tobacco alkaloid structurally related to nicotine (Figure 2). It normally constitutes less than 5% of total tobacco alkaloids (for comparison, nicotine comprises about 95% of total tobacco alkaloid content).[10] While nornicotine itself is not carcinogenic, its nitrosation in tobacco (during its curing, processing, and storage) or endogenously (in laboratory animals or humans) leads to the formation of NNN. Previous work, which included the dissertation by Dr. Porubin, has shown that the treatment of F344 rats with nornicotine and sodium nitrite resulted in endogenous formation of NNN.[11, 12]

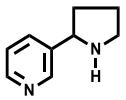
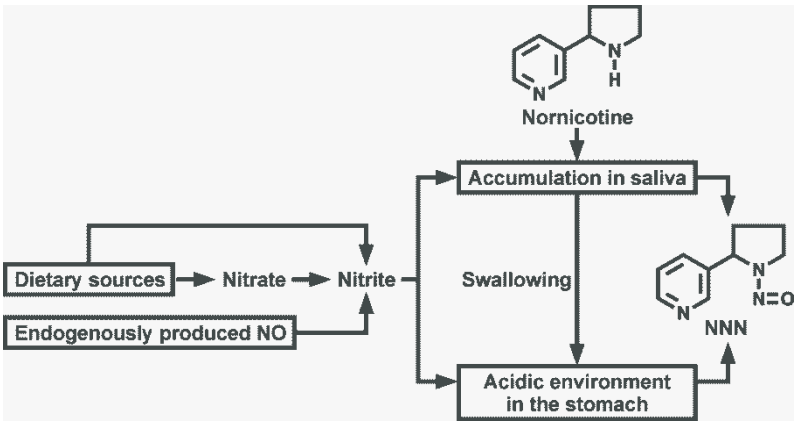


Figure 2. Structure of nornicotine

In humans, endogenous formation of *N*-nitrosamines through the reaction of dietary precursors with nitrosating agents supplied by diet has been convincingly demonstrated in multiple studies.[13-16] Furthermore, our studies reported occasional significant increases in urinary biomarkers of exposure to NNN in some users of oral nicotine replacement therapy (NRT) products such as nicotine gum or lozenge, compared with baseline smoking levels in the same subjects.[17, 18] Abstinence from smoking during NRT use in these individuals was biochemically confirmed, and exposure to NNN was assessed via the measurement of urinary total NNN, the sum of free and glucuronidated NNN.[19] We hypothesized that the observed increases in urinary total NNN were due to endogenous nitrosation of nornicotine that is metabolically formed from nicotine or originally present in NRT products. Even though nicotine is also a NNN precursor, and we determined that the amounts of nornicotine in nicotine gum and lozenge are only approximately 0.2% of nicotine content in these products, nornicotine is more likely than nicotine to be responsible for the observed endogenous formation of NNN in NRT users. This is because as a secondary amine, nornicotine can be nitrosated to form NNN much more efficiently than nicotine.[20]



Scheme 1. Hypothesized pathways of endogenous NNN formation from nornicotine (adapted from [18])

The hypothesized pathway of endogenous NNN formation from nornicotine is outlined in Scheme 1 above. Dietary nitrate is absorbed from the small intestine into the bloodstream, and about 25% of nitrate in the blood is taken up by the salivary glands and secreted into the oral cavity.[21] As a consequence, the concentration of nitrate in saliva is approximately 10-20 times higher than that in circulating blood.[22, 23] Nitrate reducing bacteria of the tongue convert 10-90 % salivary nitrate to nitrite, leading to high salivary concentrations of nitrite.[14, 24, 25] When nitrite-containing saliva encounters acidic gastric juice, nitrous acid and nitrosating species, including N_2O_3 and NO^+, are formed.[25] Nitrosonium cation (NO^+) also reacts with thiocyanate, which is highly concentrated in saliva via the same mechanism as nitrate,[26] to form the particularly potent nitrosating agent NOSCN.[25, 27] These nitrosating species are capable of reacting with a number of dietary amines to form *N*-nitroso compounds. It has been demonstrated that nicotine concentrates in saliva,[28] and it is possible that nornicotine could also be concentrated in the saliva of people exposed to this alkaloid. After saliva containing nornicotine and nitrite is swallowed, the stomach provides highly favorable conditions for nitrosation.[20, 29] Furthermore, nitrosation reactions can be catalysed by bacteria at neutral pH,[30] and thus may take place elsewhere in the body, for example in the oral cavity or urinary bladder. In support of this notion, we also demonstrated that NNN can be formed from nornicotine in human saliva without deliberate addition of any other substance, while nitrosation of nicotine in saliva was minimal.[31] In that study, we used saliva samples collected from nonsmokers to which we added deuterium-labeled nicotine and nornicotine. This allowed us to specifically identify NNN as formed from these precursors added to saliva prior to incubation, thus eliminating concerns that NNN measured in saliva of our nonsmoking volunteers could have other sources, for example exposure to secondhand smoke. Nitrosation of nicotine during saliva incubation experiments was minimal, which is in agreement with kinetic studies showing that nornicotine is nitrosated to form NNN much more efficiently than

nicotine [20]. These results also suggest that nornicotine, and not nicotine, is the major precursor of endogenously synthesized NNN in users of oral NRT products, as observed in our previous studies.

It is important to note that endogenous nitrosation of nornicotine in individual tobacco users may be greatly affected by various dietary and host factors. For example, certain dietary constituents, such as ascorbic acid and vitamin E have been shown to inhibit endogenous nitrosation in humans, while some phenolic compounds can both inhibit and catalyze *N*-nitroso compound formation, depending on pH, the nature of the nitrosated amine, and the relative concentrations of nitrite and phenolics.[32] The time of tobacco product use relative to the time of food consumption is also important. It has been demonstrated that the concentration of nitrite in saliva increases 2-5-fold for several hours after the ingestion of nitrate-containing food.[25] Moreover, tobacco users are significantly more susceptible to multiple bacterial infections, including periodontitis and *H. pylori*, and these infections can lead to increased levels of nitrite in oral cavity and stomach.[33, 34] For example, the use of antiseptic mouthwash was shown to reduce endogenous nitrosamine formation by 62-74%.[35] In our study of nornicotine nitrosation in human saliva, we observed a significant variation in the extent of NNN formation in saliva samples collected from different individuals and incubated with the same amount of nornicotine,[31] which further supports the role of host factors in endogenous nitrosation.

Implications for users of smokeless tobacco products

Endogenous formation of NNN is particularly relevant to smokeless tobacco use which involves keeping nornicotine- and nitrate-containing tobacco in the mouth for prolonged periods of time, creating favourable conditions for NNN synthesis. Moreover, smokeless tobacco contains much higher levels of nornicotine than nicotine gum or lozenge for which endogenous nitrosation was observed.[18] Smokeless products also contain considerable amounts of nitrate[11] which can be converted by the nitrate-reducing oral microflora to nitrite during and after product use.[14, 24, 25] Indeed, some earlier studies indicated that additional amounts of NNN could be formed in saliva of smokeless tobacco users.[36] Therefore, endogenous NNN formation is likely to be more extensive in smokeless tobacco users than in the users of NRT products.

Table 2.

Nornicotine content in some U.S. smokeless products[37]

Product	Nornicotine, mg/g product (wet weight)[a]
Taboka Original	0.903
Marlboro Snus Rich	0.394
Camel Snus Original	0.243
General Snus	0.115
Copenhagen Snuff	0.111
Copenhagen Long Cut	0.068

Skoal Long Cut	0.104
Kodiak Wintergreen	0.074

[a] *In the published manuscript, nornicotine levels are reported per dry weight*[37]

Given its role as the NNN precursor and a possible contributor to the addictiveness of tobacco,[36-39] nornicotine content in smokeless tobacco products is highly important for the characterization of their carcinogenic and addictive potential. We previously showed that nornicotine levels vary substantially across smokeless products marketed in the USA.[37] The levels of nornicotine ranged from 0.068 mg/g to 0.111 mg/g product in several samples of traditional moist snuff, and up to 0.903 mg/g product in some novel smokeless spitless products marketed to smokers[37] (Table 2). It is known that the levels of nornicotine in tobacco products depend on a variety of factors, including tobacco type, growing conditions, and processing techniques.[38] Moreover, constituent levels within the same brand of tobacco products may vary over time due to changes in tobacco blending or other manufacturing modifications.[39-41]

It is important to note that nicotine metabolism to nornicotine may also contribute to the endogenously formed NNN.[11] However, nornicotine is only a minor nicotine metabolite: in a study that included 12 individuals, it accounted for about 0.41±0.12% of total nicotine dose.[42] In our study mentioned above, nornicotine levels in various smokeless products ranged from 0.6 to 5.1% of nicotine content in the same products.[37] Therefore, smokeless users' immediate exposure to nornicotine extracted from different tobacco products is expected to be higher and more variable than that from the metabolically formed nornicotine.

Endogenous formation of NNN in users of electronic cigarettes

Electronic cigarettes (e-cigarettes) offer a promise of a less harmful alternative to smoking tobacco cigarettes. Compared to more than 7,000 constituents that have been identified in tobacco and cigarette smoke, the number of constituents that may be present in e-liquid and e-cigarette aerosol is relatively small. Furthermore, the levels of harmful constituents that have been measured in some e-cigarette aerosols are generally much lower when compared to cigarette smoke.[43] However, emerging data including our preliminary studies suggest that e-cigarette use may pose certain health risks. For example, aerosol of e-cigarettes operated at high voltage may contain substantial amounts of formaldehyde, a genotoxic agent that has been classified by IARC as carcinogenic to humans.[44] In addition, existing research suggests that e-cigarette users may experience elevated oxidative damage and inflammation.

While the reported levels of NNN are extremely low in e-cigarettes, our recentdata show that it can be found in saliva of e-cigarette users.[45] Salivary NNN and urinary tobacco biomarkers, including total NNN, were analyzed in 20 e-cigarette users (various e-cigarette brands, 3 to 36 months reported duration of exclusive e-cigarette use) and compared to smokers and nonsmokers. The geometric mean of NNN in saliva of e-cigarette users was 2.60 pg/mL, ranging from non-quantifiable (LOQ) to 76.0 pg/mL, while in smokers, salivary NNN ranged from LOQ to 739.0 pg/mL.

Approximately 80% of smokers had salivary NNN in the range of levels found in e-cigarette users (Figure 3). Consistent with a previous report,[46] very low levels of urinary total NNN were present in only 5 out of 20 e-cigarette users (ranging from 0.001 to 0.01 pmol/mL urine), and only trace levels of NNN were found in e-cigarette liquids. Together, our findings demonstrate that NNN is formed in the oral cavity of e- cigarette users.

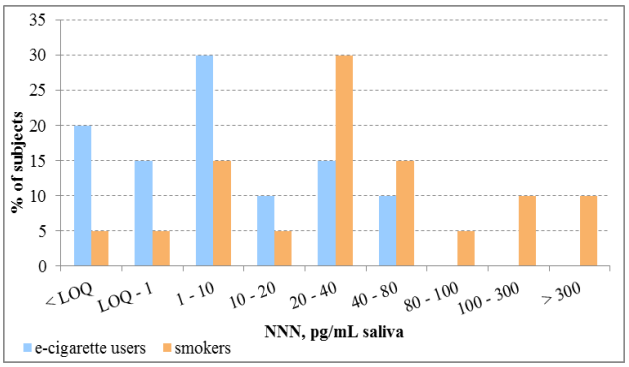


Figure 3. Distribution of salivary NNN in e-cigarette users and smokers.[45]

Better assessment of such exposures and the resulting macromolecular alterations are critically important in characterizing an electronic cigarette device and can provide key insights into the potential long-term health effects of e-cigarette use. Given that popularity of e-cigarettes has grown exponentially around the world, particularly among the youth and cigarette smokers,[47-49] such research is highly significant.

Summary and future directions

This chapter illustrates just one example of the continuous evolution and branching out of a scientific direction fostered by academician Gh. Duca. The research outlined here is ongoing and is expected to lead to the development of preventive measures and inform tobacco product regulation, and therefore have a substantial impact on public health. In addition, this research may lead to the development of new tools for identifying individuals who ate elevated risk for developing cancer due to tobacco product use. Current efforts in my research group include the development of novel biomarkers of biological effects induced by exposures to NNN and other tobacco carcinogens, such as DNA adducts and oral microbiome. I am looking forward to leveraging these cutting-edge tools to pursue innovative collaborative efforts between my current home institution, University of Minnesota, and my colleagues at Moldova State University.

Acknowledgements

The studies summarized here were supported by the NIH grants, including CA-81301, DA-13333, CA-180880, CA-129534, and CA-144034. Analyses of tobacco products and biomarkers were carried out in the Analytical Biochemistry Shared Resource of the Masonic Cancer Center, University of Minnesota, supported in part by

Grant CA-77598 from the National Cancer Institute. I would like to thank my Moldovan colleagues Maria Gonta and Diana Porubin for outstanding contributions to some of the described projects. Special gratitude to my U.S. mentor Dr. Stephen Hecht for his help and guidance over the years of work at the University of Minnesota, to Dr. Dorothy Hatsukami for her mentorship and friendship, and to all U.S. colleagues who contributed to these studies. And last but not least, to academician Gheorghe Duca for his leadership and support at the initial stages of my career as a scientist.

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