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Tobacco-specific nitrosamines in *tuibur*

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ABSTRACT

Nicotine, not a carcinogen *per se*, is one of the most addictive substances known. Tobacco smoke contains a vast number of chemicals with important biological effects in disease processes. Tobacco use is a global epidemic and the adverse health conditions including cardio-vascular diseases, atherosclerosis, and cancer are the manifestations of sustained tobacco consumption. Cancer is a major public health burden in both developed and developing countries. Tuibur, considered as 'smokeless' tobacco product in Mizoram, is presumed as one of the "safe" nicotine delivery media. In this mini-review, few tobacco-specific carcinogens detected in tuibur solution and their potential effects on biological systems are discussed.

Key words: Tobacco, tuibur, nicotine, carcinogens, tobacco-specific nitrosamines.

INTRODUCTION

The International Agency for Research on Cancer (IARC) identified cigarette smoking as the major cause of cancer, prominently, at specific organ sites than any other human carcinogens. Tobacco use, including exposure to passive smoking or environmental tobacco smoke, has also been implicated globally as a causal or contributory agent in an ever-expanding list of cancers.¹ A carcinogen is any substance with the potential to cause cancer in living tissues. The exposure to a carcinogen can occur from the

inhalation, ingestion, or absorption of many different types of substances into human body.^{1,2} Carcinogenic species act on DNA, inducing chromosomal aberrations, and ultimately causing dangerous changes at the cellular level. The manifestations of carcinogens include a change in the rate of cell division, which increases the probability of abnormal DNA synthesis. This can lead to the development of cancer, a group of diseases involving abnormal cell growth, while evading the cell death processes along with the potential to metastasize, *viz.* spreads to other parts of the body.

In addition to and independent of the etiologic effects of tobacco carcinogens in numerous cancers, there is a growing literature on the direct and indirect effects of smoking on impairing

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treatment efficacy, enhancing toxicity and morbidity of the disease.¹

CHEMICAL SPECIES OF TOBACCO SMOKE

Cigarette smoke is a complex mixture of chemicals with thousands of compounds being generated from the incomplete combustion of tobacco. These chemical species are distributed between the gas phase and the particulate phase of microscopic particles/droplets (the “tar” phase) that constitute the smoke aerosol. The gas phase of tobacco smoke is oxidizing, while the tar phase is reducing.³ The gas as well as tar phases contain high concentrations of reactive oxygen species (ROS), including nitric oxide, peroxydinitrate, peroxydinitrate, and free radicals of organic compounds.⁴ The different thermal conditions along with the variations in the air flow through and around the tobacco mass during the smouldering combustion, in comparison to the high-temperature combustion during a puff, causes the side-stream and mainstream smoke composition to differ significantly, thus it can yield a variety of secondary metabolites along with combustion products of carbohydrates, amino acids, and lipids.

Tobacco smoke contains a vast number of chemicals with important biological effects in disease processes. Of more than 8,000 chemical species present in tobacco smoke, over 80 chemical species are known to be the putative causative factors of cancer (carcinogens)⁵ besides some of these chemical species may cause heart and lung diseases too, while few of them can be considered as cumulative poisons. It is important to note that many of the chemical species of tobacco smoke are potential participants in free radical-generating reactions in biological milieu.⁶

LIFESTYLE HABITS AND BIOLOGICAL EFFECTS

Nicotine itself is not carcinogenic, yet nicotine addiction is responsible for chronic tobacco use and the voluntary exposure to carcinogenic

substances. When a person smokes, nicotine is released as a gaseous aerosol which is rapidly absorbed in the lungs, transmitted through blood stream, crosses blood-brain barrier almost instantaneously and reaches brain within 20 seconds of absorption. When it is being chewed as ‘spit’ tobacco or sniffed as ‘dry’ snuff, nicotine is absorbed through mucous epithelial tissues in the buccal space or nasal space, respectively, which is relatively a slow mode of absorption of nicotine that eventually reaches brain.^{7,8} After reaching brain, as a mimic of neurotransmitter acetylcholine, nicotine binds with nicotinic acetylcholine receptors, stimulates the release of hormones and neurotransmitters. Thus, nicotine causes a strengthening of connections responsible for the production of dopamine in the ventral tegmental area of brain pleasure or reward centre (*Nucleus accumbens*). This strengthening leads to the release of dopamine.⁸ This is the ‘reward’ process, applied by the brain to enforce the addictive behavior and nicotine stimulates this process, encouraging repetitive nicotine intake.⁸

As a burning cigarette, the combustion of cured tobacco leaves under different thermal degradation conditions with the concomitant exposure to varying degree of oxygen concentrations, releases thousands of toxic gases and particulate matter, which are rapidly absorbed into the body. The organs involved in metabolism and/or excretion of metabolites also get doses of toxic substances. Therefore, the impact of tobacco is very widespread, causing multiple diseases and impairing many internal organs of the body. In addition, smoke from an unfiltered cigarette contains 5 billion particles per mm³ with the size ranging from 0.1-1.0 mm. When condensed, these particulate matter species form a sticky fluid like mass called *tar*.² Tar initiates the damage of respiratory system by paralyzing and destroying cilia irreversibly, ultimately destroys the alveoli or air sacs, diminishing the efficient absorption of oxygen and the release of carbon dioxide. This also causes the heart to beat faster and subsequently raises the blood pressure.

As of 2002, gastric cancer was the second most common cancer in the world based on global patterns of cancer incidence and mortality.⁹ An investigation was conducted to determine the quantity of cotinine and tobacco-specific nitrosamines (TSNA) present in human pancreatic juice by applying gas chromatography with mass spectrometric detection, among smokers and non-smokers who were exposed to smoke. This study has revealed that pancreatic juice is indeed exposed to TSNA which may be important contributors to pancreatic carcinogenesis in humans.¹⁰ Globally, it seems that the environmental carcinogens also exhibit important geographic differences in the incidence of gastric cancer, *viz.* tobacco and alcohol are the main causative factors involved in Europe and North America, while nutritional deficiencies (specifically of micronutrients) are thought to underlie the high risk in central Asia, China, and southern Africa, whilst interestingly chewing of tobacco (and betel) is important risk factor in the Indian subcontinent. In addition, factors such as pickled vegetables, nitrosamine rich foods, and mycotoxins may also be involved,⁹ while genetic predisposition may also explain the rather high rates of gastric cancer in Mizoram.¹¹

Northeast India is one of the most ethnically and culturally diverse regions of Asia and the home for more than 166 tribes. Indigenous people of the north-eastern region of India use soda (baking soda) or other alkaline preparations frequently as food additives. In Mizoram, for the preparation of *bai* (an alkaline 'soup' like preparation), normally soda is used as an additive. Kalakhar (an alkaline preparation), consumed in Assam, was implicated as a risk factor for esophageal cancer.¹² Frequent consumptions of *sa-um* ('processed' pork fat) was found to be associated with the risk of developing stomach cancer. This is a food material uniquely consumed in Mizoram. Dietary intake of total or saturated fat has been shown to be associated with stomach cancer.¹³ Processed pork fat (*sa um*), in addition to being a rich source of saturated fat, may form carcinogenic compounds during long storage, as in other preserved meats. In India, there is varia-

tion in the incidence of gastric carcinoma. The 2006-2008 report of National Cancer Registry Program of Indian Council of Medical Research (ICMR)¹⁴ reveals that in India, the highest incidence of gastric cancer was in Aizawl district followed by rest of Mizoram and Sikkim. Stomach cancer is the most frequent cancer in Mizoram, and the prevalence is high [age-adjusted rate (AAR), 39.1/10⁵ for men and 14.4/10⁵ for women], which is one of the highest incidence that was reported.¹⁵

In India, tobacco is mostly smoked or chewed but other forms of tobacco use are also prevalent. The prevalent smokeless tobacco products used in India are: khaini, mishiri, zarda, kiwam, pan-masala, gutkha, etc. There is sufficient evidence that smokeless tobacco causes oral and pancreatic cancer in humans and sufficient evidence of carcinogenicity from animal studies.¹⁶ Case-control studies have shown that chronic users of snuff have an about 50-fold increased risk for cancer of the gum and buccal mucosa compared to controls and that the risk increases with the duration, *i.e.* the number of years of snuff dipping. Low or lack of fruits and vegetables intake increases the risk for oral cancer among snuff dippers. Epidemiological studies from Asia have also reported a significantly increased risk for cancer of the mouth, oropharynx and oesophagus in chewers who consume tobacco alone or in combination with betel quid. Extracts of Indian chewing tobacco preparations are carcinogenic in laboratory animals and tobacco-specific nitrosamines (TSNAs), NNN, NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, a lung carcinogen), NAT (N-nitrosoanatabine) (Figure 1) have been found in the saliva of tobacco chewers in India.¹⁷

Tobacco smoking and use of smokeless tobacco such as khaini and tuibur are common among both the sexes in Mizoram. The high rates of stomach cancers in Mizoram may be attributed to relatively high prevalence of consumption of tobacco in various forms. Tobacco smoke contains various kinds of carcinogens including tobacco specific N-nitrosamine compounds, while nitrogen oxides/nitrate in tobacco

Tobacco-specific nitrosamines in tobacco

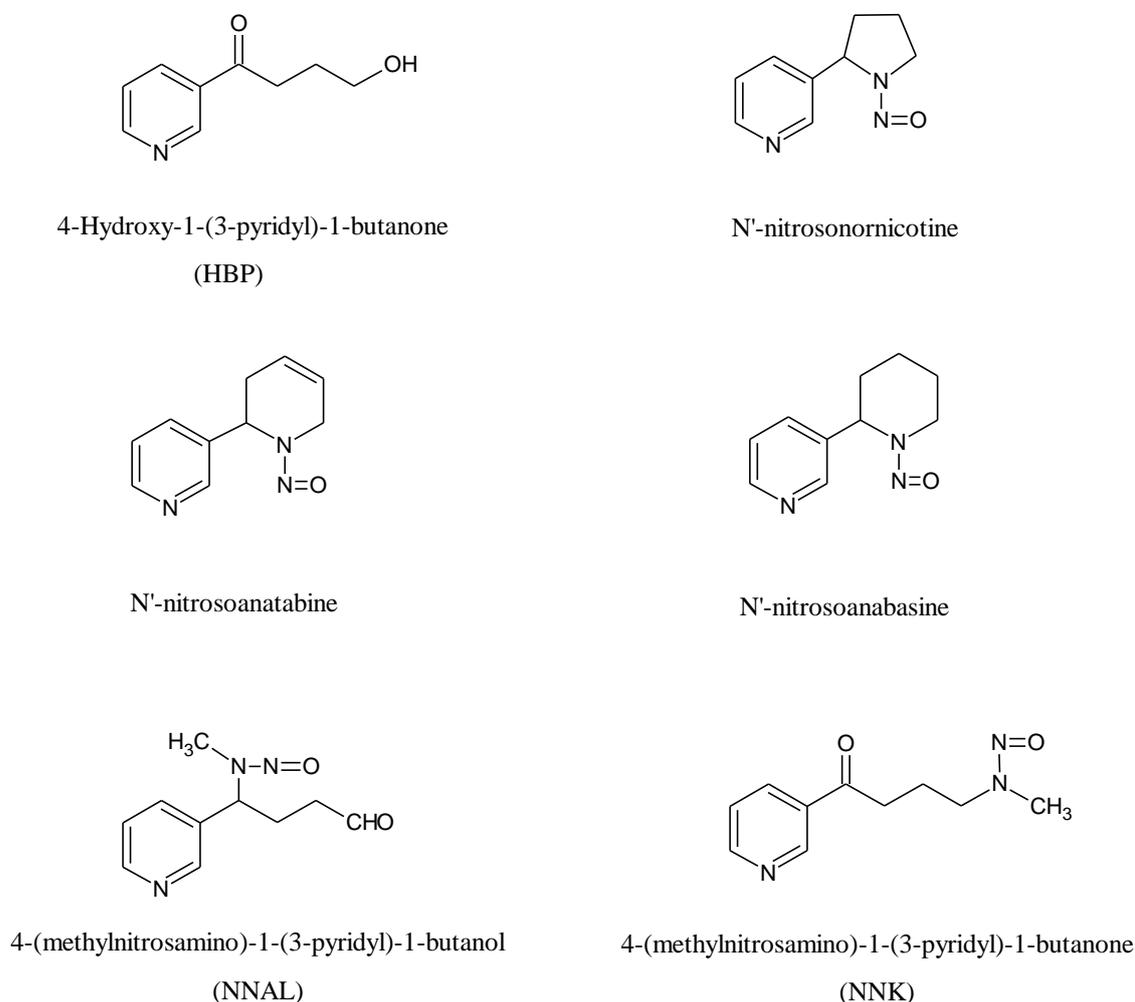


Figure 1. Various tobacco-specific nitrosamines (TSNAs) and its derivative, 4-hydroxy-1-(3-pyridyl)-1-butanone (HBP).

smoke may also promote endogenous formation of various N-nitroso compounds including tobacco specific N-nitrosamines which have been linked to gastric tumorigenesis. On the basis of carcinogenicity data on animal models, human exposure data and mechanistic studies (metabolic activation), NNN and NNK together are classified as a human (Group 1) carcinogen by IARC.¹⁶ In addition, the evidence for the incidence of stomach cancer as a manifestation of *Helicobacter pylori* infection in stomach was considered sufficient by IARC to classify this bacte-

rium as carcinogenic species in humans. Its action is probably indirect by provoking gastritis, a precursor of gastric cancer.¹⁸ Diet also certainly plays an important role. Risk is increased by high intakes of some traditionally preserved salted foods, especially meats and pickles along with tobacco smoking.

IARC has also revealed that smoking is causally associated with cancer of the stomach.¹ In general, smokeless tobacco products are perceived as relatively less harmful than combusted tobacco products such as cigarettes. For smoke-

less tobacco products, a common factor undoubtedly capable of inducing and sustaining nicotine addiction, is delivering nicotine to the oral cavity and nasal passages rapidly, albeit some absorption occurs through the gastrointestinal tract from swallowed nicotine-infused saliva. Primarily, the nicotine concentrations in the smokeless products used vary by more than 100-fold.¹⁹ The plasma levels of nicotine and the speed of delivery usually depend on pH and buffering capacity: raising the oral pH into the alkaline range results in more rapid nicotine absorption through the buccal mucosa,²⁰ and hence, tuibur is designed indigenously in such a manner as it is alkaline in character.

TOBACCO-SPECIFIC NITROSAMINES (TSNAs)

Tobacco-specific N-nitrosamines occur in all commercially and non-commercially prepared tobacco products including a wide range of smoke and smokeless products. Sinha *et al.*, also reported that heavy metals such as cadmium, lead, nickel, arsenic and ethylene glycol along with anabasine, myosmine, anatabine, nitrate, mercury, selenium, chromium besides relatively high concentration levels of N-nitrosornicotine (NNN) were found in tuibur using stripping voltammetry.²¹ Unpublished data indicated that the identification of nornicotine in tuibur solution is significant²² as it is chemically similar to nicotine, but does not contain a methyl group. In addition, high performance liquid chromatograph methods coupled with mass spectrometry (HPLC-MS) analysis has indicated that the presence of a diverse range of carcinogenic species including NNN, NNK,



Figure 2. Demethylation of nicotine to nornicotine.

NAT.²² Nornicotine is a secondary tobacco alkaloid produced by the N-demethylation of nicotine (Figure 2). Tobacco-specific nitrosamines (TSNAs) are not usually present in freshly harvested green tobacco. It is worthwhile to note that factors which influence the concentration levels of TSNAs are the variety of tobacco plant, agro-climatic conditions (leading to nitrite accumulations) besides the duration and conditions of storage, whereas these carcinogenic species are formed during tobacco curing, processing, and storage by nitrosation of tobacco alkaloids.²³

²⁵ N-nitrosamines also occur in a wide variety of both food and non-food products, but the amount of TSNAs in all tobacco products exceed the levels of other N-nitrosamines in other commercial products by several orders of magnitude.²⁶ Interestingly, the highest levels of TSNAs were found to be present in smokeless tobacco products. These compounds are also present in side-stream smoke also known as second-hand tobacco smoke. The degree of exposure to TSNAs depends not only on the levels of these compounds in tobacco products or smoke, but also on the manner in which the products are used.¹⁶ The levels of NNK and NNN in unburnt tobacco contribute significantly to and are correlated with the levels in smoke. It is also known that NNK and NNN levels vary significantly with tobacco type, independent of the effects of the heat source used in curing, being higher in air-cured, processed burley tobacco than in flue-cured bright tobacco.²⁷

FORMATION OF TSNAs

The aerial nitrosylation of nornicotine during the combustion of tobacco is causing the concomitant generation of group I carcinogens (Figure. 1), NNN, NNK and/or NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol).²³ In animal studies, due to administration of nitrite and secondary amines together, easily the formation of nitrosamines has occurred.²⁸ Other studies have demonstrated that tobacco nitrate levels are potential contributors to the smoke levels of NNK and NNN. The puffing regimen

neoplastic lesions,⁴¹ while NNN and NNK induce benign and malignant tumours in mice, rats and hamsters. Both polyaromatic hydrocarbons (PAH) and nitrosamines have been causally related to oral cancer, whilst NNK is the most potent carcinogen among the tobacco-specific nitrosamines. Animal studies have demonstrated that NNK induces lung tumours in mice, while causing nasal cavity, tracheal, and lung tumours in hamsters besides nasal cavity, lung, and liver tumours in rats. NNN and NNK also induce carcinogenesis by causing DNA adducts and mutations as well as promoting tumour growth through receptor-mediated effects.^{40,42} Nitrosamines are indeed the scourge of nicotine consumption, i.e. in contrast to nicotine, NNK and NNN may also induce cancer cell growth through neuronal nicotinic acetylcholine receptors (nAChR). Moreover, it has been demonstrated that NNK can stimulate the growth of pulmonary adenocarcinoma through β -AR followed by COX-2 over expression. It has been further demonstrated that antagonist blocking of β -AR reversed the nicotine-induced cellular proliferation. It has been shown that NNK can promote β -AR mediated transactivation of EGFR followed by ERK1 & ERK2 phosphorylation leading to an increased proliferation in pancreatic cancer cells.⁴³ NNK is also implicated to induce endogenous insulin-like growth factor receptor (IGFR) leading to development of lung tumors.

Many carcinogens (including PAHs and TSNA) undergo a series of metabolic reactions more akin to the physiological processes associated with drug metabolism, catalysed by enzymes of liver, yielding chemical species that are excreted more readily. Based on currently available data from previous studies of metabolism as well as structure-mutagenicity and structure-carcinogenicity correlation studies, α -carbon hydroxylation appears to be the major pathway of metabolic activation of NNK and NNN.^{24,44,45} Initially, NNK in tobacco smoke is a procarcinogen that requires metabolic activation to exert its carcinogenic effects. An etiological role of NNK in the induction of lung cancer is due to the acti-

vation of NNK, catalysed by enzymes of the cytochrome pigment (CYP) multigene family. Besides the CYP family, NNK can also be activated by other metabolic enzymes, like myeloperoxidase (MPO) and epoxide hydrolase (EPHX1).⁴⁶⁻⁴⁸

The initial metabolic processes usually involve oxidative (phase I) enzymes, the most important of which include the microsomal cytochrome P₄₅₀ isozymes. The cytochrome P₄₅₀ enzymes (particularly CYP2D6 and CYP1A1) are responsible for the formation of highly reactive compounds that serve as substrates for inactivating (phase II) enzymes.⁴⁰ Phase II enzymes convert these highly reactive intermediates to inactive, water-soluble conjugates such as glucuronides that are more readily eliminated from the body, essentially by the kidneys. Reactive metabolites formed by phase I enzymes that are not appropriately inactivated may eventually form a covalent (irreversible) bond to cellular DNA leading to the formation of DNA adducts. These bulky DNA adducts, if not repaired by normal cellular homeostatic processes, can lead to the miscoding and permanent cellular mutations. In some cases, cells with damaged DNA undergo apoptosis or programmed cellular destruction, arresting the development of cancer, whereas permanent mutations may lead to activation of oncogenes and inactivation of tumor suppressor genes, resulting in unregulated cellular growth and ultimately the development of cancer.⁴⁰

NNK can be activated by two different routes, the oxidative path and the reductive path. In the oxidative metabolism, NNK undergoes α -hydroxylation catalyzed by cytochrome P₄₅₀. This reaction can be carried out by two pathways namely by α -methylhydroxylation or by α -methylenehydroxylation.²³ Both pathways generate the carcinogenic, metabolized isoform of NNK, known as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (Figure 1). In the reductive metabolism, NNK undergoes either a carbonyl reduction or a pyridine N-oxidation, both producing NNAL.⁴⁹ From a lifetime study in rats, it was found that NNAL was equally as effective as NNK in inducing lung tumours.⁵⁰ In

male rats, an earlier study demonstrated that NNAL, which was administered in the drinking-water, induced adenomas, adenocarcinomas and adenosquamous carcinomas of the lung besides benign and malignant pancreatic tumours. Other studies in female mice, intraperitoneal injection of NNAL induced lung adenomas, adenocarcinomas were also observed.¹⁶ These animal studies have conclusively demonstrated that NNK is an organ-specific and strong carcinogen, irrespective of the route of administration.

A further study in female mice showed that intraperitoneal injection of NAB (N-nitrosoanabasine) induced lung adenomas. While, a previous study in rats also showed that NAB, which was administered in the drinking-water, induced oesophageal carcinomas and/or papillomas in males and females, whereas NAT is inactive when tested in rats.¹⁶ There is limited evidence in experimental animals for the carcinogenicity of NAB and inadequate evidence in experimental animals for the carcinogenicity of NAT. NAB and NAT (Figure 1) are, therefore, yet to be classified with respect to their putative carcinogenic effects in humans.¹⁶ NNK, (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) (Figure 1) is another key ingredient in various tobacco products as it is a tobacco specific product and it plays an important role in carcinogenesis. NNK is a known mutagen as it causes a lot of polymorphisms in the human genome. Furthermore, NNK induced gene polymorphisms in cells that involve in cell growth, proliferation and differentiation. NNK also plays a very important role in gene silencing, modification, and functional disruption which cause the early development of carcinogenesis.⁴¹ Virtually all commercial tobacco products contain NNN and NNK as they are always occurring together. But, there is a great variation in the concentration levels of these compounds in smoke as well as smokeless tobacco products which is mainly due to the difference in tobacco types used for various tobacco products, in agricultural practices, curing and processing methods, and in the concomitant manufacturing processes.^{16,25}

The environmental exposure and human dis-

ease pathophysiology can be better unfolded by understanding the genetic-epigenetic interplay. Epigenetic markers integrate the effect of environmental events on genome, and its interaction with an individual's genetic background, without altering the genome sequence and thus may be useful for understanding the impact of environmental exposures in carcinogenesis.⁵¹ Further studies are needed to integrate genetic and epigenetic analyses, which in turn will facilitate to identify suitable epigenetic biomarker(s) that may serve as potential "first hits" for tumorigenesis. As epigenomic alterations can be reversed, a better understanding in epigenetic network will be not only important for prognosis but also for the ultimate remediation. Cancer is a multi-factorial disorder and most of the cancers are caused by sporadic mutations, occurring due to spontaneous genetic events, environmental events or interaction between genetic and environmental factors. Tumor biomarkers are alterations in metabolites that are ultimate attributes of this dynamic process that may reflect the level of the neoplastic process by modulating the level of endogenous metabolites relative to that of normal cells. Therefore, alteration of cancer biomarker profile, at genetic and epigenetic level must be explored in the population chronically exposed to "tuibur solution".

CONCLUSION

In summary, use of 'smokeless' tobacco in the liquid form as tuibur is common among both sexes of Mizoram and found to be a significantly higher risk factor albeit the excess risk is largely confined to long-term consumers. Relatively high prevalence of the consumption of tuibur in Mizoram may have contributed to the high rates of stomach cancer.^{15,52} Development of cancer for an individual occurs during the most productive years, as the ramifications of lifestyle habits, it can have profound delirious psychological and economic ramification. Nevertheless, misconceptions about the benefits of using tuibur are widespread. Much more work is needed to explore and understand the role and biochemical

mechanisms of exposure to the putative carcinogen/pro-carcinogen constituents of tuibur. As tuibur is the derivative of tobacco smoke, the presence of tobacco specific N-nitrosamines and the concomitant risk of stomach cancer due to its consumption cannot be ruled out. Further experimental studies are warranted to confirm the risks of consumption of tuibur in Mizoram. The detection of carcinogen-DNA adducts and tobacco specific compounds such as metabolites of nicotine and tobacco specific N-nitrosamine compounds in biological matrices would provide the additional supporting link between consumption of tuibur and incidences of various kinds of cancer in Mizoram. Therefore, alteration of cancer biomarker profile, at genetic and epigenetic level must be explored in the population chronically exposed to “tuibur solution”.

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