Review Article

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Carcinogenicity of smokeless tobacco: Evidence from studies in humans & experimental animals

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A Working Group of the Monographs programme of the International Agency for Research on Cancer has classified smokeless tobacco as carcinogenic to humans (Group 1). This review article summarizes the data that support the evaluations of sufficient evidence in humans and in experimental animals for the carcinogenicity of smokeless tobacco whether used alone or with betel quid. It also identifies compounds of smokeless tobacco relevant to carcinogenicity (prominently tobacco-specific nitrosamines) and addiction (nicotine). The epidemiological evidence is summarized for oral cancer, other cancers associated with smokeless tobacco and oral potentially malignant lesions with a focus on analytical studies from the SEARO Region. Studies on cancer in experimental animals are summarized with a focus on studies applying smokeless tobacco products typical for the regions, such as *mishri* and *naswar*.

Key words Cancer bioassays - carcinogenicity - oral cancer - smokeless tobacco - tobacco-specific carcinogens

Introduction

The Monographs programme of the International Agency for Research on Cancer (IARC) seeks to identify the causes of human cancer. The objective of the programme is to systematically review and evaluate the published scientific literature on any agent suspected to be carcinogenic to humans. The carcinogenicity of smokeless tobacco (ST) was evaluated by four Working Groups convened by the IARC during the period from 1984 to 2009. The term smokeless tobacco implies the use of unburned tobacco either cured for chewing as tobacco leaves or as packaged commercial products for oral and nasal use. Products available for human use are listed in detail for each WHO Region in the IARC Monograph[1]. Based on the IARC evaluations there is sufficient evidence in humans and in experimental animals for carcinogenicity of smokeless tobacco whether used alone or with betel quid[1-4], leading to the overall evaluation that smokeless tobacco is carcinogenic to humans (Group 1). We present here the supportive global data on experimental studies and will focus the human cancer data on smokeless tobacco products as used in the SEARO Region.

Carcinogenic compounds in smokeless tobacco

The majority of ST products available in the markets are made from two species of the tobacco plant *Nicotiana tabacum* and *Nicotiana rustica*. ST is a heterogeneous product including a variety of chemicals

and multiple carcinogens have been identified in ST; their broad groups are listed: (*i*) Tobacco-specific nitrosamines (TSNA) (from tobacco alkaloids during curing, fermentation and ageing); (*ii*) N-nitrosamine acids (from amino acids present in tobacco leaves amenable to N-nitrosation); (*iii*) Volatile N-nitrosamines; (*iv*) Polycyclic aromatic hydrocarbons; (*v*) Aldehydes (formaldehyde, acetaldehyde, acrolein, crotonaldehyde); and (*vi*) Other carcinogenic compounds (mostly heavy metals: cadmium, uranium and polonium).

The TSNA are the most powerful and most abundant carcinogens in chewing tobacco, snuff and ST products. Both N'-nitrosonornicotine (NNN) and 4(methynitrosamino)-1-(3-pyridyl)-1-butanone(NNK) have strong carcinogenic effects, and there are dramatic variations in nitrosamine levels in ST globally[5]. From biochemical studies, it is clear that traditional products consumed in the Sudan[6] and most available commercial ST products in India have a high concentration of TSNA, particularly NNN and NNK[7]. A comparison of mean levels of carcinogens in traditional ST products obtained from India against some European products is shown in Table I[7]. Normal oral mucosa expresses all P450 cytochromes (1A2, 2A13, 3A4, 2A6, 2E1) that metabolize tobacco-associated nitrosamines, located in microsomes in the basal epithelium[5].

Table I. Mean levels of carcinogens in Indian smokeless tobacco (ST) products compared with a European product

Substance	Indian ST product concentration mean±SD	Swedish ST product (mean)
Total nicotine (mg/g wet wt)	10.0±1.8	8.34
Unprotonated nicotine (mg/g wet wt)	9.5±1.9	0.75
NNN (µg/g)	22.9±4.9	0.345
NNK (µg/g)	2.6±1.0	0.096
NNAL (µg/g)	3.1±1.5	0.013
NAT (µg/g)	6.8±2.5	0.248
NAB (µg/g)	8.4±2.9	0.021
Total TSNA	37.6±18.7	0.723

NNN, N′-nitrosonornicotine;
NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone;
NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol;
NAT, N′-nitrosoanatabine; NAB, N′-nitrosoanabasine;
TSNA, tobacco-specific N-nitrosamines; SD, standard deviation
Source: Ref. 7

Nicotine absorption from chewing tobacco

Studies that have measured plasma nicotine levels after administration of ST or cigarette smoking in volunteers have demonstrated that after a single exposure (7.9 g of ST) maximum plasma levels reached were equivalent for a single cigarette smoked and chewing a quid of ST[8]. Due to ST remaining in contact with oral mucosa for prolonged periods the plasma nicotine levels are sustained for prolonged periods, and the overall amount of nicotine absorbed was twice as high as that of a single cigarette[8]. Metabolites of tobacco, cotinine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) have been demonstrated to be significantly (P<0.001) higher in blood and urine of ST users than in smokers[9].

Human studies on smokeless tobacco and oral cancer

The majority of the studies assessing the carcinogenicity of smokeless tobacco in humans have been conducted in USA, Europe, India and Pakistan; one case-control study was conducted in Africa, in the Sudan[10]. The evidence for carcinogenicity of ST in humans arises from well conducted cohort and case-control studies. These studies have been comprehensively evaluated by the IARC[1,4]. A systematic review and meta-analysis examined all epidemiological studies conducted in South Asia published from 1989 to 2013 on smokeless tobacco and risk of oral cancer[11]. The search by these authors yielded 14 publications on smokeless tobacco and oral cancer[11]. Smokeless tobacco types used included chewing tobacco, *gutka* and *naswar*. In five publications, odds ratios (OR) for oral cancer were adjusted for tobacco smoking, alcohol consumption and other potential confounders. Adjusted OR ranged from 3.6 [confidence interval (CI), 2.5-5.6] to 8.3 (CI, 5.4-13). The meta-analytic OR for ST and oral cancer from all studies was 4.7 (CI, 3.1-7.1) and for adjusted studies, the OR was 4.3 (CI, 3.1-5.8). For Indian studies, the pooled estimate was slightly higher: OR was 4.8 (CI, 3.2-7.4). By examining the frequency and duration of use of the products, a dose-response was demonstrated. These data provide strong evidence of carcinogenicity of ST products used in India and other part of Asia[11].

Tobacco added to betel quid

Adding tobacco to betel quid is an age-old tradition in South Asia. Guha *et al*[12] in a meta-analysis examined the risk of betel quid with and without tobacco. In the Indian

subcontinent the meta-relative risk for oral/oropharyngeal cancer was 2.56 (95% CI, 2.00-3.28; 15 studies) for chewing betel quid without tobacco and 7.74 (95% CI, 5.38-11.13; 31 studies) for chewing betel quid with tobacco[12]. The estimated population attributable fraction for oral cancers attributable to betel quid chewing with tobacco was 49.5 per cent.

Smokeless tobacco and other cancers

There is evidence for increased risk of oesophageal and pancreatic cancer from smokeless tobacco, mainly from studies conducted in the USA and Nordic countries. For oesophageal cancer, five studies (1 from US and 4 from Nordic countries) reported an overall OR 1.8 (CI, 1.1-2.9). For pancreatic cancer, six studies (4 from US and 2 from Nordic countries) reported an overall OR 1.6 (CI, 1.1-2.2)[1]. There are no reported studies to estimate the risk of ST on these cancers in Asia.

Smokeless tobacco and oral potentially malignant disorders

Oral potentially malignant disorders (OPMDs) are conditions that precede the incidence of invasive cancers of the oral cavity. Among various OPMDs described in the literature[13], leukoplakia and erythroplakia are two conditions that are known to be associated with tobacco use. A recent systematic review[14] reported the meta OR (mOR) for any OPMD with the use of any ST product as 15.5 (95% CI, 9.9-24.2). Women had a higher risk, mOR=22.2 (95% CI, 9.1-54.1) compared to men, mOR=8.7 (95% CI, 2.1-34.8)[14]. Gupta *et al*[15] in an Indian cohort with nodular leukoplakia (mostly associated with ST use) followed up to 10 yr reported a malignant transformation rate of 16.2 per cent, with a relative risk of 3243.2.

Experimental studies in animals

In general, *in vivo* experimental studies in animals were conducted in mouse, rat and hamster, with ST applied topically on the skin, oral mucosa or vaginal mucosa. Some studies reported oral administration of ST in drinking water or by gavage, by subcutaneous administration, injected in to surgically created canals in the lower lip or by implantation of ST pellets. Hamster pouch provides a means of retaining ST in contact with the pouch (oral) mucosa for longer periods. Early studies reported before 1985 had various limitations[2], but new *in vivo* studies[4] had improved methodology and substantial improvements with rigorous analysis of data. Two reviews on these studies are also available[16,17] in the literature.

ST preparations used in experimental studies included Indian smokeless tobacco mixtures, US and Scandinavian snuff, *bidi* tobacco, *mishri* and *naswar*. Having revisited a large volume of experimental studies included in the IARC evaluation in 2007[4], studies with small group sizes and those with inconclusive data were excluded. Studies conducted using the US or Swedish snuff were also excluded. Several studies reporting on Indian ST that produced positive data are summarized below.

Tobacco and *mishri*

Hamster

Rao[18] administered 1 mg of lypophilized aqueous tobacco extract in 0.05 ml water twice daily for six months to the oral cavity in a group of 20 female Syrian golden hamsters. The control group was sham treated with water applications only. Squamous cell papillomas and/ or carcinomas occurred in 3 of 17 treated animals compared to none in 10 sham-treated animals. The findings were however, not statistically significant.

Mouse

The potential carcinogenic effect of intravesicular implantation of paraffin pellets that contained alkaloid-free tobacco was demonstrated by Randeria[19] in a Swiss mouse model. Among C17 mice 2 of 12 developed transitional-cell tumours of the bladder and one female mouse developed a myosarcoma of the cervix with metastasis to the kidney. No tumours were found in the control group.

The carcinogenic effect of a diet containing 10 per cent brown or black *mishri* given for 20 months was examined in four groups of eight week old Swiss mice[20] compared with a group consuming standard diet. The incidence of forestomach papillomas was significantly (P<0.001) higher than in both male and female control mice.

In a parallel experiment, Kulkarni *et al*[21] tested the carcinogenic or promoting effect of brown and black *mishri* by skin application on hairy and hairless Swiss mice. *Mishri* preparations of 1 or 2.5 mg were applied up to 24 months both after 7,12-dimethylbenz[a] anthracene (DMBA) initiation and without initiation. Table II shows the results when treated with various concentrations of *mishri* in the two groups of mice. Promotion with brown or black *mishri* extract significantly (P<0.05) increased the total tumour incidence in Swiss mice but not in Swiss bare mice[22]. Application of *mishri* extracts alone to the skin of

Table II. Tumour incidence in groups of 8 wk old hairy and bare Swiss mice following repeated treatment with *mishri* with or without DMBA initiation

Initiation	Agent	Swiss mice	Tumour yield	
			Male	Female
	Acetone 20 µl	Bare mice	1/15 papilloma (5%)	
	1 mg black *mishri*	Bare mice	6/21 papillomas, 1/21 skin carcinoma (33%)	5/24 papillomas (21%)
	2.5 mg black *mishri*	Bare mice	6/17 papillomas (35%)	5/23 papillomas (22%)
200 nmol DMBA		Hairy mice	0/30 papillomas	
200 nmol DMBA	2.5 mg brown *mishri*	Hairy mice	4/30 papillomas (P<0.05)	
200 nmol DMBA	2.5 mg black *mishri*	Hairy mice	4/29 papilloma (P<0.05)	
200 nmol DMBA		Bare mice	9/21 papillomas 2/21 carcinomas	
50 nmol DMBA		Bare mice	7/17 papillomas 2/17 carcinomas	
200 nmol DMBA	1 mg black *mishri*	Bare mice	8/20 papillomas 2/20 carcinomas	
50 nmol DMBA	2.5 mg black *mishri*	Bare mice	7/16 papillomas 4/16 carcinomas	

DMBA, 7,12-dimethylbenz[a] anthracene
Source: Adapted from Ref. 22

male and female Swiss bare mice induced papillomas (Table II).

Rat

In a study by Kulkarni *et al*[22], 121 weaning male Sprague Dawley rats were divided into two groups and 60 were fed diets containing shark liver oil (labelled as vitamin A sufficient) and 61 without shark liver oil (labelled as vitamin A deficient). In each group, half received a tobacco extract dissolved in dimethylsulphoxide (DMSO) by gavage five times per week for 21 months, and the remaining rats received DMSO only. Among the vitamin A sufficient rats receiving the tobacco extract 6 of 29 developed various tumours, and among the vitamin A deficient group 29 of 31 had one or more tumours. The proportion of tumour bearing rats was significantly higher in the tobacco extract treated group compared with controls[23].

Naswar

Four different experiments were reported on hamster by application of *naswar* to the cheek pouch or skin. *Naswar* as a dry powder was applied to the left cheek pouch of Syrian hamsters (28 females and 33 males) for life. Another group received *naswar* as a 50 per cent suspension in sunflower oil. None of the hamsters developed tumours at the site of application. Of the 64 treated hamsters, 13 developed tumours in various organs and among 110 untreated hamsters two developed tumours[24]. In a further experiment, *naswar* was applied to the cheek pouch as a dry powder (mean 53.8±2.5 g) or as a 50 per cent suspension in refined sunflower oil. *Naswar* was administered throughout the life. No tumours were found at the site of application, and 26 of 138 hamsters developed tumours at various sites[25].

A suspension of *naswar* was topically applied to the skin of 60 hamsters. None developed tumours at the site of application. Of the surviving hamsters, three of the nine animals developed neoplasms. In the untreated controls two of 45 surviving hamsters developed tumours[24].

Naswar as a promoting agent was tested by the same authors[25]. A group 30 Syrian hamsters received a single application of 0.1 mg DMBA as a 0.1 per cent solution of benzene in the cheek pouch and another 30 had additional treatment of *naswar* as a dry powder applied

to the cheek pouch. Six of 11 animals who received DMBA and *naswar* developed various tumours[25].

These various animal experiments using chewing tobacco or ST products available in India and in South Asia provided evidence that ST products were carcinogenic in experimental animals, and data from these studies contributed to the overall IARC evaluation of the carcinogenicity of ST (Group 1).

Conclusion

There is no safe form of tobacco, and both smoked and smokeless tobacco are carcinogenic to humans. As summarized here, the evidence for increased risk for oral cancer among people consuming ST products in South Asia is based on case-control and cohort studies mostly conducted in India and Pakistan. Dose-response data demonstrate a higher risk with increased frequency and duration of ST use and further support causal inference. This evidence is further corroborated by studies conducted in the USA reporting increased risks of mouth cancer[1].

Based on a meta-analysis of regional cancer epidemiological studies and the prevalence of use of ST it has been estimated that approximately 50 per cent of oral cancers in India are attributable to ST use[12] (Table III). Based on the estimated annual incidence of oral cancer in India, this would amount to approximately 35,000 cancers each year[26,27]. The use of ST is the major cause of oral cancer in South Asia, and therefore, oral cancer is largely preventable. This demands heightened public health action in the Region to increase the public awareness of the dangers of ST use.

Governments and professional bodies should consider the translation of this knowledge on ST to public health action. In this context, it is important to consider how ST products can be regulated in the WHO member countries using the existing Framework Convention on Tobacco Control (FCTC) recommendations. Overall, of the 181 member countries, 52 Parties have adopted and implemented policy or regulation specific to ST[28]. Several articles of the FCTC are of relevance for regulation of ST in the WHO member countries in the SEARO Region. In India and neighbouring regions, *gutka* (ST mixed with areca nut) is the most abundantly consumed commercially packaged smokeless tobacco product. *Gutka* has now been banned in some States in India, but a more vigorous implementation is necessary[29]. It is essential to include programmes that create awareness about effects of smokeless tobacco on health and sustain surveillance levels on ST use in the Region.

Table III. Chewing of betel quid (BQ) with tobacco and oral cancer, meta-relative risk (mRR) [95% confidence interval (CI)] by gender from Indian studies and population attributable fraction (PAF)

	Both	Men	Women
mRR	7.9	4.9	23.1
95% CI	4.1-15.1	3.9-6.2	5.9-89.6
Prevalence of chewing (%)	14.2	20.6	7.8
PAF%	49.5	44.7	63.2
Incident oral cancer cases/yr	69,820	45,445	24,375
Number of cases attributable to BQ chewing	34,528	20,303	15,416

Source: Adapted with permission from Ref. 12

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