Mutagenesis vol. 19 no. 4 pp. 251–262, 2004 DOI: 10.1093/mutage/geh036

REVIEW

Alert for an epidemic of oral cancer due to use of the betel quid substitutes *gutkha* and *pan masala*: a review of agents and causative mechanisms

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In south-east Asia, Taiwan and Papua New Guinea, smoking, alcohol consumption and chewing of betel quid with or without tobacco or areca nut with or without tobacco are the predominant causes of oral cancer. In most areas, betel quid consists of a mixture of areca nut, slaked lime, catechu and several condiments according to taste, wrapped in a betel leaf. Almost all habitual chewers use tobacco with or without the betel quid. In the last few decades, small, attractive and inexpensive sachets of betel quid substitutes have become widely available. Aggressively advertised and marketed, often claimed to be safer products, they are consumed by the very young and old alike, particularly in India, but also among migrant populations from these areas world wide. The product is basically a flavoured and sweetened dry mixture of areca nut, catechu and slaked lime with tobacco (*gutkha*) or without tobacco (*pan masala*). These products have been strongly implicated in the recent increase in the incidence of oral submucous fibrosis, especially in the very young, even after a short period of use. This precancerous lesion, which has a high rate of malignant transformation, is extremely debilitating and has no known cure. The use of tobacco with lime, betel quid with tobacco, betel quid without tobacco and areca nut have been classified as carcinogenic to humans. As *gutkha* and *pan masala* are mixtures of several of these ingredients, their carcinogenic affect can be surmised. We review evidence that strongly supports causative mechanisms for genotoxicity and carcinogenicity of these substitute products. Although some recent curbs have been put on the manufacture and sale of these products, urgent action is needed to permanently ban *gutkha* and *pan masala*, together with the other established oral cancer-causing tobacco products. Further, education to reduce or eliminate home-made preparations needs to be accelerated.

Introduction

It has been estimated that, world wide, ~600 000 000 people chew areca nut (Nelson and Heischober, 1999). A causal association between tobacco and betel quid (BQ) chewing habits and oral mucosal diseases such as leukoplakia, oral submucous fibrosis and oral cancer has been established and heavy users have a significantly increased mortality rate. Oral cancer is the fifth most common cancer world wide (Parkin *et al*., 1993). A 2- to 3-fold increase in mortality has been recorded in eastern and central European countries in recent decades (Coleman *et al*., 1993) and upward trends in several other areas of Europe have been reported (Franceschi *et al*., 2000). Tobacco use has been estimated to account for 30% of the worldwide cancer burden. Tobacco smoking and heavy alcohol consumption are the main risk factors for oral cancer in the developed countries (La Vecchia *et al*., 1997), where over 80% of cases are attributable to these causes (Negri *et al*., 1993; Boyle *et al*., 1995).

Of the 390 000 oral and oro-pharyngeal cancers estimated to occur annually world wide, 58% occur in south and south-east Asia. In India there are 75 000–80 000 new cases of oral cancer each year and the incidence rates of cancers of the oral cavity in both males and females in all urban cancer registries are among the highest in the world. Age-standardized incidence rates per 100 000 population in India were estimated to be 12.8 in men and 7.5 in women (Ferlay *et al*., 2001). Time trend analysis of cancers at all sites for the period 1990–1996 showed a decrease in cancers of the oral cavity in Indian population-based registries (ICMR, 2001), but an increase in the incidence of mouth cancer was reported among those aged <50 yr between 1983–1987 and 1995 (Gupta, 1999b), consistent with the hypothesis of an increase in oral cancer among the young due to increased consumption of the alternative chewing products *gutkha* and *pan masala*. In this review we focus on these commercially available products and summarize what is known about their cancer-causing components and the mechanisms involved.

Description of betel quid

Chewing of BQ and areca nut is an ancient custom in several parts of south-east Asia, the south Pacific islands and Taiwan. This practice dates back several thousand years and is deeply entrenched in the culture of the population. A ceremonial gift of dried tobacco leaves given to Columbus by Native Americans in 1492 led to the introduction of tobacco into the rest of the world. It arrived in India in the 16th century; a sample was presented to the Emperor Akbar, who patronised smoking, rapidly spreading the habit in the sub-continent. An attempt to ban it in 1619 had little effect, as the revenues from tobacco were already considerable. BQ chewing was already a socially well accepted practice and the introduction of tobacco reinforced this practice. The BQ is a mixture of areca nut (*Areca catechu*), catechu (*Acacia catechu*) and slaked lime (calcium oxide and calcium hydroxide) wrapped in a betel leaf (*Piper betle*) (Figure 1A). Condiments, sweetening agents and spices may be added according to individual preferences. In India, most habitual chewers of BQ add tobacco. In some countries, such as Papua New Guinea and China, tobacco is not added. BQ chewing has been related mainly to oral, pharyngeal and oesophageal cancer (IARC, 1985, 2004).

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Commercial betel quid substitutes: *pan masala* and *gutkha* (Figure 1B)

Betel leaf is perishable and preparation of BQ is somewhat complex or requires visits to shops selling *pan*/BQ. With the emergence of commercial *pan masala* and *gutkha* about three decades ago, not only did the Indian market witness massive growth in the sales of smokeless tobacco and areca nut products, but also a huge worldwide export market developed. The packaging revolution has made these products portable, cheap and convenient, with the added advantage of a long shelf-life. Tobacco products which were usually consumed by a small section of the population are today part of the modern urban and rural lifestyle.

The web site (www.newindia.com/kothari/) of the first major manufacturer of *pan masala* and *gutkha* presents their strategy as '… to prepare convenient anytime, anywhere substitute for pan … give some respectability to a habit that was considered low in image by the genteel'. The product was put on the market in 1985 as 4 g sachets. Today sachets and bulk packages are produced and sold in India and exported to markets in the USA, Europe, the Middle East, Australia and many other countries.

Pan masala is basically a preparation of areca nut, catechu, cardamon, lime and a number of natural and artificial perfuming and flavouring materials. *Gutkha* is a variant of *pan masala*, in which in addition to these ingredients flavoured chewing tobacco is added. Both products are often sweetened to enhance the taste.

Promoted by a slick, high profile advertising campaign and aggressive marketing, *pan masala* and *gutkha* have become very popular with all sections of Indian society, including school children. For most children, teenagers and women, cigarette smoking still remains taboo in India. These alternative tobacco products are often advertised as being safer than conventional cigarettes, leading to a much higher frequency of use, so that these younger chewers constitute an alarming *avant garde* for a new epidemic of oral cancer. Further, these habits and preparations have spread to Europe and the USA, wherever there are Asian migrant communities.

Although the actual prevalence of this habit is unknown, its popularity can be gauged by commercial estimates valuing the Indian market for *pan masala* and *gutkha* at several hundred million US dollars (Gupta, 1999a). These products are typically consumed throughout the day. A number of small surveys conducted in schools and colleges in several states of India have shown that 13–50% of students chew *pan masala* and *gutkha* on a regular basis (Gupta and Ray, 2003). A large proportion of migrant ethnic groups resident in the UK practice various chewing habits (Warnakulasuriya *et al*., 2002); population studies conducted among Asian ethnic groups in the UK suggest that chewing habits are prevalent in 14–15% of 11–15 yr old children, with *pan masala* having the highest average frequency of use (Farrand *et al*., 2001). Areca nut chewing is an addictive habit (Chu, 2001) and evidence from the UK shows that the use of *pan masala* and *gutkha* is also addictive (Winstock, 2002).

Oral cancer and precancerous conditions

Oral cancer, a malignancy of the lip, mouth or tongue, is predominantly a squamous cell carcinoma. The prognosis is poor and severe functional and cosmetic defects accompany its

Fig. 1. Traditional betel quid chewing ingredients (**A**) and the commercial examples of betel quid chewing substitutes, *pan masala* and *gutkha* (**B**).

treatment. As an early sign of damage to the oral mucosa, chewers of BQ with or without tobacco often develop clinically visible whitish (leukoplakia) or reddish (erythroplakia) lesions and/or stiffening of the oral mucosa and oral submucous fibrosis (OSF). All these well-established precancerous lesions are easily diagnosed and present an important indicator of oral cancer risk. Some 2–12% of these lesions have been reported to turn malignant over several years. The malignant transformation of non-homogeneous lesions involving erythroplakia and nodular leukoplakia is particularly high, reportedly ranging from 15 to 40% depending upon the time period (Sankaranarayanan *et al*., 1997). Almost every BQ/tobacco chewing-related oral malignancy is preceded by a clinically distinct premalignant stage at the site of cancer development (WHO, 1984; Gupta *et al*., 1989; Murti *et al*., 1995).

OSF is predominantly caused by the use of areca nut (Murti *et al*., 1995). Besides being regarded as a precancerous condition, it is a seriously debilitating and progressive disease. Marked by stiffening of the oral mucosa and development of fibrous bands, loss of elasticity of the mucosa results in a progressive restriction of mouth opening. Affected users experience a burning sensation of the oral mucosa, occasional mucosal ulceration, a peculiar marble-like blanching of the mucosa and palpable fibrous bands of the buccal mucosa, soft palate and lips. OSF does not regress and there is no known cure.

In recent years, studies in India, China, south-east Asia and South Africa and on Asian migrants in the UK have shown a clear link between areca nut chewing and OSF. Several case–control studies in India have shown a high risk for OSF among

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areca nut chewers; over 70% of the cases were under 35 yr old (Gupta and Ray, 2003). Several studies have reported relative risks of from 29 to 154 for developing OSF due to chewing of areca nut (Sinor *et al*., 1990; Maher *et al*., 1994; Gupta *et al*., 1998; Hazare *et al*., 1998). A dose–response relationship has been suggested by an increasing relative risk with increasing frequency of areca nut chewing (Sinor *et al*., 1990; Hazare *et al*., 1998; Lee *et al*., 2003).

Oral cancer was hitherto considered a disease of the elderly, appearing after several decades of the causal lifestyle habits. Although no epidemiological studies on *pan masala* or *gutkha* have yet been reported, several surveys showing an increase in the incidence of OSF attributed to their use, especially among youngsters, portend an epidemic of oral cancer. As with tobacco and areca nut, the addictive nature of *pan masala* and *gutkha* results in a high frequency of chewing. A relative risk of 489 has been reported for OSF in *pan masala* chewers compared with non-users (Hazare *et al*., 1998). In a survey of 236 consecutive cases of OSF and 221 matched control subjects, chewing of areca nut, BQ or *pan masala* was directly related to OSF. *Pan masala* was chewed by a comparatively younger age group and was associated with OSF changes earlier than areca nut or BQ chewing. Moreover, the frequency of chewing rather than the total duration of the habit was directly correlated with OSF (Shah and Sharma, 1998). In a clinico-pathological study in current chewers, chewers of *pan masala* or *gutkha* presented with OSF after a significantly shorter duration of the habit (2.7 ± 0.6 yr) than BQ chewers (8.6 ± 2.3 yr) (Babu *et al*., 1996a). Symptoms of cancer appeared at an early age in youngsters (Babu *et al*., 1996b).

Oesophageal subepithelial fibrosis, an extension of oral submucosal fibrosis, was seen more frequently in patients who had consumed *pan masala*, *gutka*, areca nut, tobacco or a combination of some or all of these, with or without betel leaf, for ⩾5 yr than in those consuming these products for a shorter period (91 versus 46%, $P < 0.001$), suggesting that submucosal fibrosis is not a disease confined to the oral cavity, but that the oesophagus may also be involved in about two-thirds of patients. (Misra *et al*., 1998). *Mawa*, a preparation similar to *gutkha*, containing tobacco, lime and areca nut slivers, has also been linked to OSF, oral cancer and oesophageal cancer. A study carried out in the Bhavnagar district in India, where chewing of *mawa* has mushroomed in recent years, showed a corresponding increase in OSF (Gupta *et al*., 1998).

A malignant transformation rate of 7.6% in an Indian cohort over a period of 17 yr has been reported (Murti *et al*., 1985). Based on three new oral cancer cases arising among 25 OSF cases and four new cases among 10 145 persons in an 8 yr follow-up, the relative risk of malignant transformation of OSF was reported to be 397 compared with lesion-free controls with tobacco habits (Gupta *et al*., 1989). In Pakistan, the malignant transformation rate was reported to be 19 times higher (95% CI 4.2–87.7) in patients with OSF than in subjects without any lesion (Merchant *et al*., 2000).

Carcinogens in *pan masala* and *gutkha* ingredients

The main carcinogens in *pan masala* and *gutkha* are derived from their ingredients areca nut, lime, catechu and tobacco. Although carcinogens present in *pan masala* or *gutkha* have not been systemically analysed, studies of the ingredients and their mixtures provide indications of the carcinogenic potential of these commercial products (Table I).

Table I. Major carcinogenic and genotoxic agents in *pan masala* and *gutkha*

Products	Ingredients	Genotoxic agents/carcinogens[a]
Gutkha	Tobacco	NNN, NNK
	Areca nut	arecoline, MNPN
	Areca nut + lime	ROS
	Catechu + lime	ROS
Pan masala	Areca nut	Arecoline, MNPN
	Areca nut + lime	ROS
	Catechu + lime	ROS

NNN, *N*′-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MNPN, 3-(methylnitrosamino)propionitrile; ROS, reactive oxygen species, $O^{-\bullet}$, H_2O_2, $OH^{\bullet}$.
[a]For structures and pathways see Figures 2 and 3.

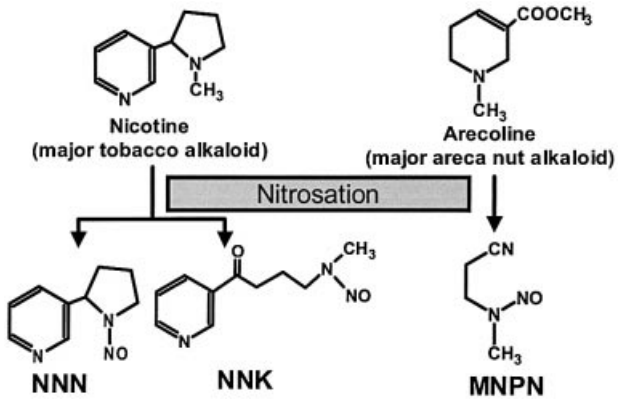

Fig. 2. Carcinogenic nitrosamines that could be derived from major ingredients of *pan masala* (areca nut) and *gutkha* (areca nut and tobacco). NNN, *N*′-nitrosonornicotine, which could also be formed from the minor tobacco alkaloid nornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MNPN, 3-(methylnitrosamino)proprionitrile.

Several carcinogens are derived from tobacco but also from areca nut (Hoffmann *et al*., 1994). Chewing of tobacco with BQ results in high exposure to carcinogenic tobacco-specific nitrosamines (TSNAs), to ~1000 μg/day (Nair *et al*., 1999), compared with ~20 μg/day in smokers (Hoffmann and Hecht, 1985), as well as leading to exposure to nitrosamines derived from areca nut alkaloids (Figure 2). The carcinogenic TSNAs *N*′-nitrosonornicotine (NNN), 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosoanabasine (NAB), as well as the volatile nitrosamines *N*-nitrosodimethylamine and *N*-nitrosodiethylamine, have been detected in the saliva of chewers of BQ with tobacco (Wenke *et al*., 1984; Nair *et al*., 1985, 1987a; Bhide *et al*., 1986).

TSNAs undergo metabolic activation by cytochrome P450s and other enzymes. NNK, a major carcinogenic TSNA, is activated by either methylene hydroxylation to generate an intermediate that decomposes to a DNA-methylating agent, resulting in the formation of 7-methylguanine, O^6-methylguanine (O^6-MeG) and O^4-methylthymidine in DNA or via methyl hydroxylation to form bulky pyridyloxobutyl DNA adducts. NNK is also converted metabolically to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, which can also be activated by α-hydroxylation to yield methyl and pyridylhydroxybutyl adducts in DNA (Hecht, 2003). 2′-Hydroxylation of NNN, another important TSNA, can give rise to the same

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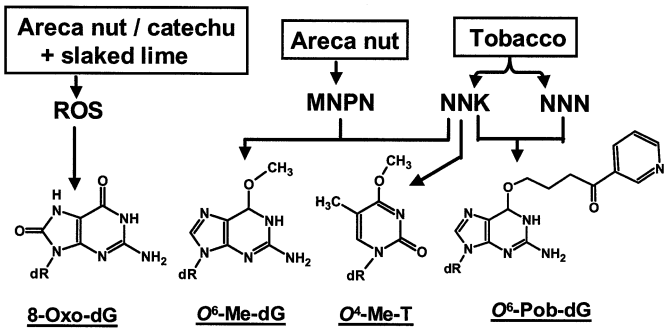

Fig. 3. Miscoding DNA adducts derived from genotoxic agents that are present or formed from major ingredients of *pan masala* and *gutkha*. 8-oxo-dG, 8-oxodeoxyguanosine; O^6-Me-dG, O^6-methyldeoxyguanosine, O^4-Me-T, O^4-methylthymidine; O^6-Pob-dG, O^6-[4-oxo-4-(3-pyridyl)butyl]-deoxyguanosine.

intermediate as is formed by methyl hydroxylation of NNK, resulting in pyridyloxobutylation of DNA (Figure 3).

The areca nut-specific nitrosamines (ASNAs) *N*-nitrosoguvacoline (NG) (Wenke *et al*., 1984; Nair *et al*., 1985, 1987a; Stich *et al*., 1986) and the carcinogenic 3-(methyl-*N*-nitrosamino)propionitrile (MNPN) (Prokopczyk *et al*., 1987) were also detected in the saliva of chewers of BQ without tobacco (Table II). ASNAs were not detected in BQ containing areca nut. Nitrosation of BQ with nitrate and thiocyanate *in vitro* at neutral pH resulted in the formation of NG (Nair *et al*., 1985). Nitrosation of arecoline at neutral pH yielded approximately four times more NG than at acidic or alkaline pH (Wang and Peng, 1996). Hence the reported presence of ASNAs in the saliva of BQ chewers could arise from their formation during chewing of BQ. The highest levels of an ASNA (NG) were found in the sediment of saliva collected from Taiwanese BQ chewers (Stich *et al*., 1986), whereas the highest levels of TSNAs have been found in saliva samples collected in India (Bhide *et al*., 1986).

Formation of *N*-nitroso compounds in the oral cavity

Volatile nitrosamines and tobacco-specific nitrosamines in the saliva of chewers are derived from leached-out preformed nitrosamines present in tobacco, but can also be formed endogenously from abundant precursors during chewing. Secondary and tertiary amines present in areca nut and tobacco can be nitrosated during BQ chewing when they react with available nitrite in the presence of catalysts such as thiocyanate (Nair *et al*., 1985, 1987a). Using a modified *N*-nitrosoproline (NPRO) test (Ohshima and Bartsch, 1981), it was clearly shown that NPRO, a marker of endogenous nitrosation, is formed during chewing of BQ with or without tobacco (Nair *et al*., 1987a). Further, nitrosation was significantly more extensive in subjects with poor oral hygiene, as determined by dental plaque, compared with those with good oral hygiene (Nair *et al*., 1996). The enhanced nitrosation in subjects with poor oral hygiene may be due to greater conversion of nitrate to nitrite and bacterial enzyme-mediated formation of nitrosamines or both (Calmels *et al*., 1996; Ziebarth *et al*., 1997). Elevated levels of nitrite and nitrate reductase activity have been reported in the saliva of Indian chewers of BQ with tobacco (Murdia *et al*., 1982). There is increased nitric oxide and nitrite formation in subjects during deposition of dental plaque (Carossa *et al*., 2001). Thus, in view of the availability of nitrosatable amines from areca nut and tobacco, increased formation of nitrosamines might be expected in the oral cavity of BQ, tobacco, *pan masala* and *gutkha* chewers with poor oral hygiene.

Table II. Carcinogenic tobacco- and areca nut-specific nitrosamines detected in saliva of chewers of betel quid with and without tobacco

Carcinogenic agent	BQT (range, ng/ml)	BQ (range, ng/ml)	Reference
Tobacco-specific nitrosamines			
NNN	1.2–3.8	NR	Wenke *et al*. (1984)
	1.6–14.7	NR	Nair *et al*. (1985)
	3.0–85.7	NR	Bhide *et al*. (1986)
	4.9–48.6	NR	Nair *et al*. (1987a)
NNK	1–2.3	NR	Wenke *et al*. (1984)
	0–2.3	NR	Nair *et al*. (1985)
	0–14.3	NR	Bhide *et al*. (1986)
	0–9.4	NR	Nair *et al*. (1987a)
Areca nut-specific nitrosamines			
MNPN	NR	0.5–11.4	Prokopczyk *et al*. (1987

NNN, *N*′-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MNPN, 3-(methylnitrosamino)propionitrile; BQ, betel quid; BQT, betel quid with tobacco; NR, not reported.

Endogenous nitrosation in BQ chewers

Many chewers swallow the quid that contains precursors of nitrosamines. The acidic pH of the stomach would favour the nitrosation of secondary and tertiary amines in the quid. Urinary levels of NPRO were 4- to 6.5-fold higher in chewers of BQ with or without tobacco following ingestion of L-proline compared with non-chewers (Nair *et al*., 1986; Chakradeo *et al*., 1994). Detection of NG and its metabolite *N*-nitrosonipecotic acid in the urine of Syrian hamsters fed areca nut and nitrite (Ernst *et al*., 1987; Ohshima *et al*., 1989) also supports the notion that exposure to carcinogenic nitrosamines formed by endogenous nitrosation is likely to be higher in BQ chewers who swallow the quid.

Reactive oxygen species

Reactive oxygen species (ROS), implicated in multistage carcinogenesis, are generated in substantial amounts in the oral cavity during chewing (Nair *et al*., 1992, 1995). Nair *et al*. (1987b) first demonstrated that aqueous extracts of areca nut and catechu were capable of generating superoxide anion and hydrogen peroxide at pH > 9.5. The areca nut-induced production of ROS was enhanced by Fe^{2+}, Fe^{3+} and Cu^{2+}, but inhibited by Mn^{2+}. These results show the importance of pH for the formation of ROS that is likely to occur due to autoxidation, redox cycling via quinone/semiquinone radical- and iron-catalysed Haber–Weiss and Fenton reactions (Figure 4).

When calf thymus DNA was incubated with an aqueous extract of areca nut under alkaline conditions, 8-oxodeoxyguanosine (8-oxo-dG) was formed, and more so in the presence of Fe^{2+} and Fe^{3+}. The presence of $Ca(OH)_2$ in slaked lime leads to alkaline conditions in the oral cavity, favouring ROS generation. Slaked lime used by chewers was collected in a region of Papua New Guinea where the incidence of oral cancer is high (Nair *et al*., 1990). In 25 lime samples, the free calcium hydroxide content and pH were highly correlated with the generation of ROS from areca nut extract *in vitro* and DNA damage *in vitro* measured as 8-oxo-dG. Fe^{2+} and Mg^{2+} levels in

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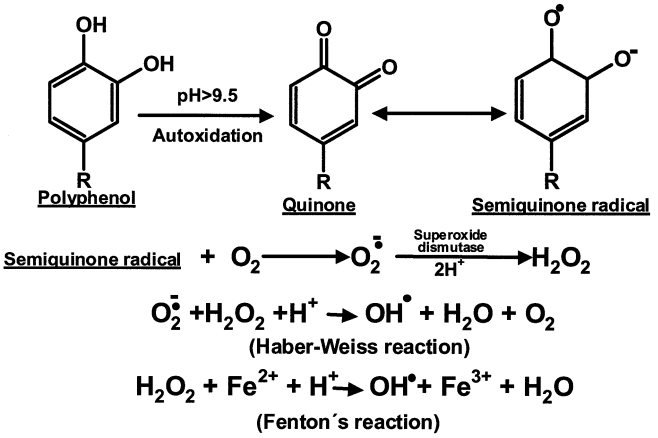

Fig. 4. Pathways of formation of superoxide anion, hydroxy radical and hydrogen peroxide from areca nut and catechu in the presence of slaked lime, which are the major ingredients of *pan masala* and *gutkha*

the lime samples were too low to modify the formation of ROS, but hydrogen peroxide formation was almost entirely inhibited by addition of Mg^{2+} to the reaction mixture. These results suggest that the calcium hydroxide content of lime in the presence of areca nut is a major factor responsible for the formation of ROS which cause oxidative damage in the DNA of buccal mucosa cells of BQ chewers. Decreasing the slaked lime content of BQ should therefore reduce its toxicity. Extracts of tender areca nut caused more 8-oxo-dG formation in DNA than ripe areca nut extract when incubated with Fe^{2+} under alkaline conditions (Liu *et al*., 1996).

Hydroxyl radicals ($OH^{\bullet}$) were shown to be generated *in vitro* using L-phenylalanine as substrate together with some ingredients of BQ and *pan masala*. Therefore, the formation of *o*- and *m*-tyrosine from L-phenylalanine can be measured as a marker of $OH^{\bullet}$ generation. Both *o*- and *m*-tyrosine were formed *in vitro* in the presence of extracts of areca nut and/or catechu, transition metal ions (Cu^{2+} and Fe^{2+}) and alkaline pH (slaked lime or sodium carbonate). Omission of any of these ingredients from the reaction mixture significantly reduced the yield of tyrosines. Scavengers of $OH^{\bullet}$ such as ethanol, D-mannitol and dimethylsulphoxide inhibited the hydroxylation of phenylalanine in a dose-dependent manner (Nair *et al*., 1995).

Direct evidence for the generation of ROS in the oral cavity during BQ chewing was obtained by measuring *o*- and *m*-tyrosine formation from L-phenylalanine in human saliva by HPLC with fluorescence detection (Nair *et al*., 1995). The tyrosine levels were significantly higher than in saliva of subjects who kept phenylalanine in the oral cavity without BQ. These studies demonstrated that $OH^{\bullet}$ are formed in the oral cavity of BQ chewers and probably implicated in the genetic damage observed in oral mucosal cells of chewers. By the same method, $OH^{\cdot}$ formation was monitored in Taiwanese subjects chewing tender areca nut and lime with either *Piper betle* inflorescence or betel leaf (Chen *et al*., 2002). Levels of *o*- and *m*-tyrosine were increased but were lower than those detected in Indian chewers, perhaps due to differences in the BQ ingredients.

Iron and copper are the transition metal ions that are involved in the catalytic process of ROS generation. Copper content in various BQ ingredients has been reported to range from 3 to 108 μg/g in areca nut and from 8 to 53 μg/g in *pan masala* (Trivedy *et al*., 1997; Ridge *et al*., 2001; Zaidi *et al*., 2002). The iron levels measured were 75 ng/g in areca nut, 132 ng/g in betel leaf, 5.2 ng/g in catechu and 22–256 ng/g in slaked lime samples (Nair *et al*., 1990; Zaidi *et al*., 2002). *In vitro*, not only was areca nut-induced ROS production enhanced by Fe^{2+}, Fe^{3+} and Cu^{2} (Nair *et al*., 1987b), but formation of 8-oxo-dG in calf thymus DNA was also increased in the presence of Fe^{2+} and Fe^{3+} (Nair *et al*., 1990, 1995). Significant superoxide anion production, assayed by cytochrome c reduction and lipid peroxidation by formation of thiobarbituric acid-reactive substances, was demonstrated in normal human oral keratinocytes following exposure to commercially available *gutkha* and *pan masala* extracts (Bagchi *et al*., 2002). Thus, some of the cytotoxic effects of these chewing products appear to be mediated through production of ROS.

Genotoxicity and mutagenicity of *pan masala* and *gutkha* ingredients

The mutagenic, clastogenic and carcinogenic properties of areca nut, the major constituent of *pan masala*, have been extensively studied in a variety of experimental systems (Jeng *et al*., 2001). Areca nut contains 5–40% polyphenols and several alkaloids including arecoline, arecaidine, guvacine and guvacoline. Arecoline, the most important areca nut alkaloid, is present at 1% of the dry weight and has been shown to be genotoxic (Dave *et al*., 1992a). Areca nut extract was mutagenic to *Salmonella typhimurium* strains in the presence and absence of an exogenous metabolic activation system (Shirname *et al*., 1983, 1984). Exposure to aqueous areca nut extract induced mitotic gene conversion at pH > 10 (Rosin *et al*., 2002). Recently, areca nut chewing has been classified as carcinogenic to humans (IARC, 2004).

Pan masala and *gutkha* have been reported to be genotoxic and mutagenic in several short-term assays. Aqueous extracts of various brands of *pan masala* were mutagenic in *S.typhimurium* strains (Polasa *et al*., 1993). Aqueous extracts of both *pan masala* and *gutkha* induced chromosomal aberrations, sister chromatid exchange and micronucleated cells in Chinese hamster ovary cells in the presence or absence of an exogenous metabolic system, although metabolic activation markedly inhibited the chromosome damaging effect, implicating the presence of direct-acting mutagens (Dave *et al*., 1991). A significant dose-dependent increase in sister chromatid exchange was observed in bone marrow cells of Swiss albino mice injected i.p. with *pan masala* suspensions. Higher doses caused significant delay in cell cycle progression of bone marrow cells (Mukherjee and Giri, 1991).

Oral feeding of *pan masala* caused significantly elevated frequencies of sperm head abnormalities and chromosomal aberrations in male mice, indicating its clastogenic potency (Mukherjee *et al*., 1991). Chronic feeding of *pan masala* impaired liver function in rats, as indicated by changes in marker enzyme activities and decreased organ weights of the gonads and brain (Sarma *et al*., 1992). *Pan masala* reduced testis weight in mice and enhanced the frequency of morphological abnormalities in mouse sperm (Kumar *et al*., 2003). *Pan masala* applied to the palate and cheek mucosa of albino Wistar rats resulted in keratosis, thickening of the submucosal collagen, an inflammatory reaction and changes in tissue vasculature, similar to those observed in oral submucous fibrosis and leukoplakia in humans (Khrime *et al*., 1991).

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Gutkha and *pan masala* have been shown to be carcinogenic in experimental animals, causing tumours in various organs. *Pan masala* acts as a tumour promoter in mice (Ramchandani *et al*., 1998). Mice fed *pan masala* developed tumours of the lung, liver, stomach and testis (Bhisey *et al*., 1999). Swiss mice fed *gutkha* or *pan masala* in the diet developed tumours affecting various organs such as lung, stomach, liver, testis, ovary and adrenal gland, *gutkha* being more potent than *pan masala* (Nigam *et al*., 2001).

Catechu, another constituent of pan masala, has mutagenic (Stich *et al*., 1983) and clastogenic activity (Giri *et al*., 1988), while lime is known to cause irritation and hyperplasia of the oral mucosa (Dunham *et al*., 1966).

Genotoxicity in humans

The frequency of micronucleated cells was measured to assess genotoxic damage in BQ chewers. Significantly elevated frequencies of exfoliated human oral mucosal cells were observed in chewers of BQ with tobacco (4.83/1000 cells) and of tobacco with lime (5.20/1000 cells) compared with the control group (2.59/1000 cells). In addition, chromosome breaks have been reported in oral exfoliated cells in chewers of BQ with or without tobacco. Micronucleus formation has been observed in precancerous lesions of the oral cavity of chewers (Nair *et al*., 1991).

Sister chromatid exchange and chromosome aberrations were examined in peripheral blood lymphocytes and the frequency of micronucleated cells was scored in exfoliated buccal mucosa cells of *pan masala* and *gutkha* consumers. All three cytogenetic end-points showed a statistically significant increase among the habit groups as compared with the controls (Dave *et al*., 1991; Desai *et al*., 1996).

Healthy individuals and OSF patients from several parts of India who were regularly using either areca nut alone, *mava* or tobacco with lime were investigated. Compared with 'no chewing habit' healthy controls, all the habit groups, irrespective of their type of chewing, had significantly higher frequencies of micronucleated cells in exfoliated oral mucosal cells (Kayal *et al*., 1993). The frequencies of sister chromatid exchanges and chromosome aberrations in peripheral blood lymphocytes and the percentage of micronucleated cells in exfoliated cells of buccal mucosa were significantly increased among areca nut chewing controls, OSF and oral cancer patients compared with those of non-chewing controls (Dave *et al*., 1992b).

Areca nut and oral submucous fibrosis (OSF)

There is conclusive evidence for the role of areca nut as the major risk factor in the development of OSF, but the mechanisms by which this occurs are not fully understood. *In vitro* studies with cultured fibroblasts have shown that areca nut alkaloids such as arecoline and its hydrolysed product arecaidine stimulate proliferation and collagen synthesis in a dose-dependent manner (Canniff and Harvey, 1981; Harvey *et al*., 1986), higher concentrations being cytotoxic (van Wyk *et al*., 1994; Jeng *et al*., 1996). Flavonoids, catechins and tannins in areca nuts cause collagen fibres to crosslink, making them less susceptible to collagenase (Scutt *et al*., 1987). This can cause increased fibrosis due to increased collagen production and decreased collagen breakdown. OSF is irreversible and persists even after cessation of the chewing habit, suggesting that components of the areca nut initiate OSF and then affect gene expression in the fibroblasts, which then produce greater amounts of normal collagen (Meghji *et al*., 1987; de Waal *et al*., 1997). In OSF patients with a habit of chewing areca nut or *pan masala*, a significant increase in total serum protein was observed with lower levels of ascorbate and iron, which are used in collagen synthesis. The total tissue collagen content increased significantly in patients with advanced disease and with progression of the disease, leading to hypomobility of the tongue, lips, cheeks, soft palate and faucial pillars (Anuradha and Devi, 1993).

Copper appears to play a significant role in the pathogenesis of OSF. Considerable amounts of copper have been found in areca nut products (Trivedy *et al*., 1997) and copper salts significantly increased the production of collagen by oral fibroblasts *in vitro* (Trivedy *et al*., 2001). Areca nut chewing for up to 20 min releases significant amounts of soluble copper into the saliva (Trivedy *et al*., 1999) and mucosal biopsies taken from OSF subjects had a higher copper concentration than those from controls (Trivedy *et al*., 2000). Activity of the copper-dependent enzyme lysyl oxidase was increased in fibroblasts cultured from OSF (Ma *et al*., 1995). Copper was found to up-regulate collagen production in oral fibroblasts (Trivedy *et al*., 1999), indicating that the increased tissue copper may increase the activity of lysyl oxidase, which catalyses the crosslinking of collagens and elastin and is implicated in the pathogenesis of OSF.

OSF is a collagen-related disorder induced by cumulative exposure to BQ/areca nut chewing. Specific genotype combinations of six collagen-related genes situated on different chromosomes (collagen 1A1 and 1A2, collagenase-1, transforming growth factor β1, lysyl oxidase and cystatin C) were associated with risk for OSF in a low exposure group, while a different configuration was associated with risk in a high exposure group of OSF patients in Taiwan (Chiu *et al*., 2002). Tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) are the major gelatinolytic proteinases secreted by human mucosal fibroblasts. Arecoline treatment alters the balance in favour of matrix stability by elevating TIMP-1 expression and inhibiting MMP-2 activity, which could lead to development of fibrosis in chewers (Chang *et al*., 2002).

Possible mechanisms of carcinogenicity of *gutkha* and *pan masala*

The salient points of the postulated mechanism of oral premalignant lesions and oral carcinoma development due to *gutkha* and *pan masala* are summarized in Figure 5.

Pan masala and *gutkha* have been shown to be clastogenic and carcinogenic in animal studies and a battery of *in vitro* test systems, the tobacco-containing *gutkha* being more potent. Increased cytogenetic damage has been observed in peripheral blood lymphocytes and exfoliated buccal mucosal cells of *pan masala* chewers. These genotoxic effects are most likely caused by tobacco- and areca nut-specific nitrosamines (Figure 2) and ROS generated by areca nut and catechu polyphenols and slaked lime (Figure 4).

Gutkha and *pan masala* are dry products and one can assume that the ROS concentration will increase in the oral cavity of chewers as soon as the areca nut and catechu polyphenols together with slaked lime dissolve in the saliva, similar to the reaction observed *in vitro* (Nair *et al*., 1987b). This could result in the formation of high levels of ROS close to the buccal

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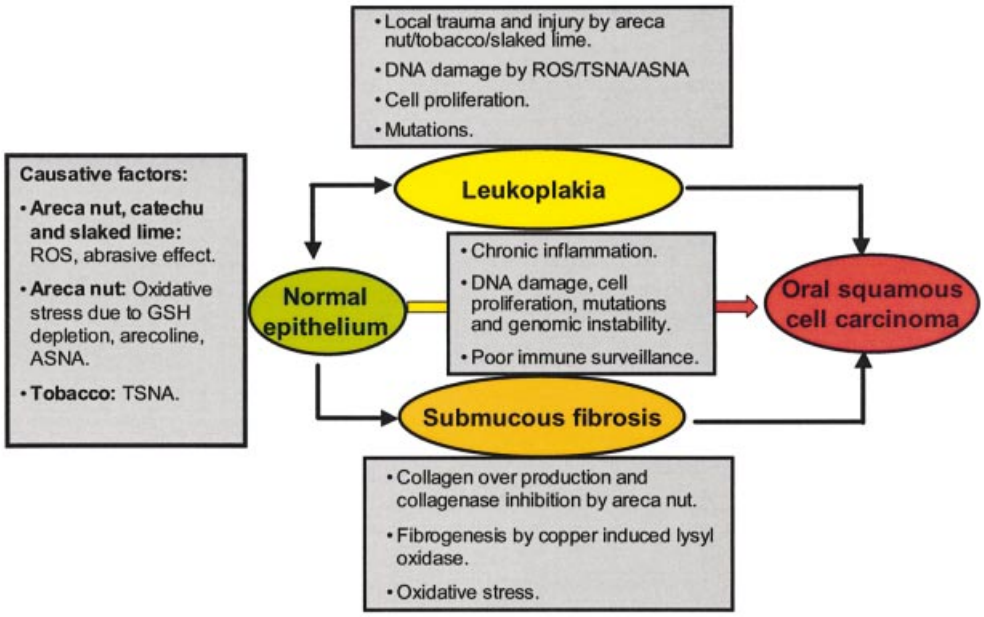

Fig. 5. Postulated causative factors and mechanisms implicated in the induction of leukoplakia/submucous fibrosis and oral squamous cell carcinoma due to *pan masala* and *gutkha* use.

mucosa, overwhelming the protective enzymes and thus causing direct damage to the tissue. High copper and iron content in these products would further add to the load through their action as catalysts via the Haber–Weiss and Fenton reactions. This has been demonstrated in primary human oral keratinocytes, where *pan masala* induced superoxide radical production and lipid peroxidation.

Areca nut chewing is known to cause local trauma and injury to the oral mucosa due to its abrasive nature. This could be more severe in users of *pan masala* and *gutkha* due to their fine particulate nature, with the high probability of particle adhesion to the traumatized mucosa, leading to morphological changes and membrane damage. Areca nut, present in these mixtures, can disturb collagen homeostasis, cause crosslinks and accelerate the onset of OSF, a collagen-related disorder, in habitual chewers. This continuous local irritation by *pan masala*, *gutkha* or areca nut can lead to injury-related chronic inflammation, oxidative stress and cytokine production. Oxidative stress and subsequent ROS generation can induce cell proliferation, cell senescence or apoptosis, depending upon the level of ROS production. During chronic exposure, these events can lead to preneoplastic lesions in the oral cavity and subsequently to malignancy.

Pan masala and *gutkha*, developed and marketed as BQ substitutes, are mixtures of the individual components of the BQ but without the betel leaf. The fresh betel leaf itself has been shown to be non-genotoxic and contains several compounds such as chlorophyll, chavicol and hydroxychavicol, thought to be possible chemoprotective agents (Amonkar *et al*., 1986; Nagabhushan *et al*., 1987, 1989; Bhide *et al*., 1994). Betel leaf extracts act as potent scavengers of ROS (Jeng *et al*., 2002) and have antimutagenic and anticarcinogenic activity against the TSNAs NNN and NNK (Amonkar *et al*., 1989; Padma *et al*., 1989a,b).

Depletion of the cellular antioxidant glutathione and reduced glutathione *S*-transferase activity has been demonstrated in arecoline-treated cultured human oral keratinocytes and fibroblasts (Jeng *et al*., 1996; Chang *et al*., 2001). Reduced glutathione content and enhanced CYP450 activity, which was observed in the liver of mice treated with areca nut (Singh and Rao, 1995), could cause increased oxidative metabolism of carcinogens and reduced detoxification. Glutathione depletion leads to increased oxidative stress that can cause DNA damage and trigger several response signals implicated in the carcinogenic process. Glutathione *S*-transferases M1 and T1 are enzymes known to detoxify ROS, lipid peroxidation products and tobacco-derived carcinogens that have been found in the saliva of BQ/tobacco chewers. Null genotypes for *GSTM1* and *GSTT1* increase the risk of developing leukoplakia in chewers (Nair *et al*., 1999). So far, no similar studies on gene–environment interactions in *pan masala*, *gutkha* or areca nut chewers have been reported.

A key initiating step in the carcinogenic process is the formation of DNA adducts. Some miscoding DNA adducts that could be formed by use of *pan masala* or *gutkha* are shown in Figure 4. Persistence of these adducts during DNA replication can cause miscoding, leading to mutations and derangement of cellular growth control processes. The tobacco-specific nitrosamines NNN and NNK induce miscoding DNA adducts, including O^6-pyridyloxobutyl and O^6-MeG adducts (Hecht, 2003), that could initiate the tumourigenic process in the oral cavity of BQ/tobacco and *gutkha* chewers. The areca nut-specific nitrosamine MNPN also forms O^6-MeG, which causes G→A transitions following DNA replication (Horsfall *et al*., 1990). In Thai betel chewers who neither smoked nor drank alcohol, such G→A transition mutations were observed exclusively in the *p53* gene (Thongsuksai *et al*., 2003). Moreover, BQ extracts also inhibit the DNA repair activity of O^6-methylguanine-DNA methyltransferase in buccal mucosal tissue and cell cultures *in vitro* (Liu *et al*., 1997). Generation of ROS from polyphenols can oxidize DNA bases, e.g. deoxyguanosine to yield 8-oxo-dG (inducing G→T transversions), promoting the tumourigenic process in the oral cavity.

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Generation of prostaglandins and overexpression of cyclooxygenase 2 (COX-2) have been implicated in several human cancers. Areca nut extract-enhanced COX-2 expression and prostaglandin production in cultured human gingival keratinocytes and human buccal mucosa fibroblasts (Jeng *et al*., 2000). Up-regulation of COX-2 expression was also observed in human submucous fibrosis tissue samples (Tsai *et al*., 2003). Chewers of BQ could have impaired immune surveillance, in view of the inhibition by arecoline of both humoral and cell-mediated immune responses in mice (Shahabuddin *et al*., 1980).

A genetic progression model for head and neck squamous cell carcinoma (HNSCC) to explain the field cancerization theory has been proposed, by which an entire epithelial surface is primed for neoplastic changes following prolonged carcinogen exposure, leading to focal areas that progress at different rates towards invasive cancer (Califano *et al*., 1996; Oh and Mao, 1997). Microsatellite analysis in HNSCC for allelic loss at 10 major chromosome loci demonstrated that the spectrum of chromosomal deletions progressively increases at each histopathological step from benign hyperplasia to dysplasia to carcinoma *in situ* to invasive cancer (Califano *et al*., 1996). The most common gains in BQ and/or tobacco chewing associated oral cancers are on chromosomes 8p, 9p, 9q, 11q, 17q and 20q and the most frequent losses are in chromosome arms 3p (genes *FHIT* and *RARB*), 4q, 5q, 9q and 18q (Mahale and Saranath, 2000; Lin *et al*., 2002b; Pai *et al*., 2002).

HNSCCs that develop in patients from India frequently have abnormalities of *ras* oncogenes, including mutations, loss of heterozygosity (H-*ras*) and amplification (K- and N-*ras*), in contrast to the low prevalence of mutations in these genes in the same malignancies from developed countries. A high incidence of H-*ras* mutations (35%) has been reported (Saranath *et al*., 1991). The *p53* tumour-suppressor gene is found in mutated form in many common human cancers, but in India *p53* mutations are infrequent in BQ-associated oral premalignant lesions and squamous cell carcinomas (Heinzel *et al*., 1996; Ralhan *et al*., 2001; Saranath *et al*., 1991). Although *p53* mutations have been reported in 43% of oral cancers in BQ chewers from Sri Lanka, several subjects were also smokers (Chiba *et al*., 1998). On the other hand, a high frequency of p53 protein overexpression was reported in premalignant and malignant oral lesions of Indian patients who were heavy consumers of betel, areca nut and tobacco (Ranasinghe *et al*., 1993b; Kaur *et al*., 1994; Kuttan *et al*., 1995; Pillay *et al*., 2003). This effect could be used as a marker to identify lesions that are more likely to progress to malignancy. However, a lack of correlation between p53 protein expression and mutations (Ranasinghe *et al*., 1993a) suggests that other mechanisms are involved in oral tumourigenesis in BQ chewers. Interactions of p53 with other cellular proteins such as murine double minute 2 (MDM2), 70 kDa heat shock protein (HSP70) and/or E6 protein of human papilloma virus (HPVE6) have been identified in some of these lesions (Agarwal *et al*., 1999; Ralhan *et al*., 2000; Nagpal *et al*., 2002; Pande *et al*., 2002).

Gutkha and *pan masala* are marketed as substitutes for several prevalent chewing habits. Compelling evidence has led to a classification of oral use of tobacco mixed with lime (khaini) (group 1) and BQ containing tobacco (group 1) as carcinogenic to humans (IARC, 1985). Recently, chewing BQ without tobacco (group 1) and areca nut (group 1) have also been found to be carcinogenic to humans (IARC, 2004).

Overall, although specific studies on these comparatively recent BQ substitutes are lacking, an association and similarity of mechanisms can be convincingly projected from the large body of data on chewing tobacco, areca nut and BQ to hold true for the commercial preparations *pan masala* and *gutkha* and sound a red alert for their carcinogenic potential to humans. In addition, the submucous fibrosis observed after short use of these preparations, especially in the very young, points to a high susceptibility to an irreversible and debilitating disease.

Perspectives

Banning of *gutkha* and *pan masala* has been strongly advocated by oncologists as a preventive measure to reduce oral cavity cancers. Recently, a number of States in India have banned the manufacture and sale of both products and this should reduce the incidence rate. Similar regulations regarding other health-impairing tobacco products which have been on the market for centuries, together with cigarettes and bidis (an indigenous smoking product), should also be reinforced.

However, for those who are addicted to these products or are already affected by premalignant lesions, educational interventions to encourage stopping the habit are essential. Additionally, chemopreventive interventions are being explored. Retinoids, NSAIDS and green tea are among the promising agents (Garewal, 1994; IUSHNCC, 1997; Papadimitrakopoulou and Hong, 1997; Lin *et al*., 2002a). Although a large percentage of lesions did respond to treatment, recurrence after terminating the chemopreventive regime was also observed (Sankaranarayanan *et al*., 1997), perhaps due in part to continuation of the addictive habit.

As with all cancers, early diagnosis is important for successful treatment of oral cancer, as its prognosis is still very poor. There is, nowadays, a strong drive to apply proteomics technology to molecular diagnosis of cancer. Expression profiling of tumour tissues, molecular classification of tumours and identification of markers to allow early detection, sensitive diagnosis and effective treatment are now being explored for oral cancers. Genes with significant differences in expression levels between normal, dysplastic and tumour samples have been reported and this should help in better understanding the progression of oral squamous cell carcinoma (Kuo *et al*., 2002; Leethanakul *et al*., 2003).

DNA aneuploidy in oral leukoplakia in Caucasian tobacco users has been found to signal a very high risk for subsequent development of oral squamous cell carcinomas and associated mortality (Sudbo and Reith, 2003; Sudbo *et al*., 2004). A risk assessment model to predict progression of premalignant lesions that includes histology and a score combining chromosomal polysomy, p53 expression and loss of heterozygosity on 3p or 9p has also been described (Lee *et al*., 2000; Rosin *et al*., 2002). Once diagnosed, these premalignant lesions could be treated at a much earlier stage by chemopreventive agents, surgery, chemotherapy and/or intense radiotherapy to prevent new lesions and premalignant lesions from progressing to invasive cancer.

Conclusions

Gutkha and *pan masala* have flooded the Indian market as cheap and convenient BQ substitutes and become popular across all age groups wherever this habit is practised. There is sufficient evidence that chewing of tobacco with lime, BQ with tobacco, BQ without tobacco and areca nut are carcinogenic in

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humans (IARC, 1985, 2004). These evaluations in conjunction with the available evidence on the BQ substitutes *gutkha* and *pan masala* implicates them as potent carcinogenic mixtures that can cause oral cancer. Additionally, these products are addictive and enhance the early appearance of OSF, especially so in young users who could be more susceptible to the disease. Although recently some curbs have been put on the manufacture and sale of these products, urgent action needs be taken to permanently ban *gutkha* and *pan masala*, together with the other well-established oral cancer-causing tobacco products. Finally, as the consequences of these habits are significant and likely to intensify in the future, an emphasis on education aimed at reducing or eliminating the use of these products as well as home-made preparations should be accelerated.

Acknowledgement

The authors thank Dr J.Cheney for editing the manuscript.

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Received on February 4, 2004; revised and accepted on April 28, 2004

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