4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

(*a*) *Constituents of betel quid*

(i) *Areca nut*

Areca nut contains several alkaloids and tannins. Among the alkaloids, arecoline is most abundant, whereas arecaidine, guvacine and guvacoline occur in smaller quantities (see Figure 5).

Arecoline

Absorption after dermal application

Hayes *et al.* (1989) developed a gas chromatography–mass spectrometry (GC–MS) technique for quantitative analysis of arecoline in blood plasma, in the concentration range 1–50 ng/mL, which was used on plasma samples from healthy volunteers [number not given] who had received transdermal doses at 3 mg/h. The time–concentration profile showed a maximum plasma concentration of 4–5 ng/mL at 5–10 h after dermal application.

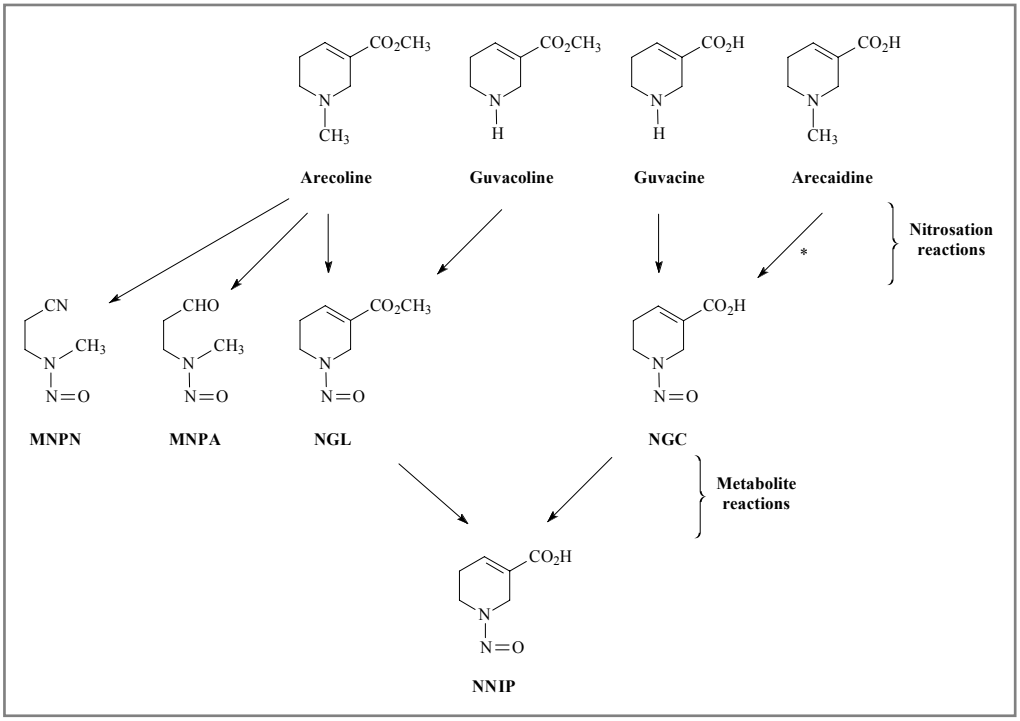
Absorption after application in the buccal cavity

Strickland *et al*. (2003) studied the influence of areca nut on energy, metabolism and hunger and also assessed the absorption of arecoline and its level in plasma among eight fasting men (20–29 years of age). Freshly dried areca nuts were pulverized and assayed for arecoline content (mean value, 0.17%); the powder was then suspended in bioadhesive gel and placed in the buccal cavity so as to deliver 0, 5, 10 or 20 mg arecoline. At 15, 155 and 365 min after placement, arecoline could be detected by GC–MS in blood plasma in amounts that increased with dose and time.

Polyphenols

Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites and are an integral part of both human and animal diets. There is a vast amount of literature on the metabolism of polyphenols in humans. Important polyphenolic constituents of areca nut are catechin, tannin, caffeic acid and ferulic acid. For detailed information on absorption, distribution, metabolism and excretion of these substances, the reader is referred to a number of review articles (Stich *et al.*, 1984a; Rosazza *et al.*, 1995; Bravo, 1998; Miyazawa, 2000; Scalbert *et al.*, 2002; Higdon & Frei, 2003).

Figure 5. Relationship of areca-nut alkaloids to areca-nut-derived nitrosamines (formed by nitrosation) and a urinary metabolite of *N*-nitrosoguvacoline and *N*-nitrosoguvacine



Adapted from Wenke & Hoffman (1983); Nair *et al.* (1985); Ohshima *et al.* (1989)
MNPN, 3-methylnitrosaminopropionitrile; MNPA, 3-methylnitrosaminopropionaldehyde; NGL, *N*-nitrosoguvacoline; NGC, *N*-nitrosoguvacine; NNIP, *N*-nitrosonipecotic acid
*It is likely that nitrosation of arecaidine would produce NGC but this has not been demonstrated.

Areca-nut-derived nitrosamines

The detection of areca-nut-derived nitrosamines in saliva and their formation during betel-quid chewing are discussed in Section 4.1.1(*a*) of this monograph; other data are reviewed in the monograph on areca-nut-derived nitrosamines.

(ii) *Betel leaf and betel inflorescence*

Safrole (IARC, 1976)

Chang, M.J.W. *et al.* (2002) developed a method to assess exposure to safrole based on HPLC analysis of its metabolites, dihydroxychavicol and eugenol (IARC, 1985b, 1987). The method was used to measure these compounds in 38 spot urine samples from Taiwanese betel-quid chewers and 115 samples from non-chewers. The urinary concentration of dihydroxychavicol was higher in non-chewers than in chewers, probably

because safrole exists in many common species such as ginger and black pepper that are frequently used in Taiwanese cooking. However, the chewers had a higher urinary concentration of eugenol.

(iii) *Tobacco*

The components of tobacco, as part of betel quid, will be reviewed in a forthcoming monograph on smokeless tobacco products (see also IARC, 2004).

(*b*) *Biomarkers of constituents of betel quid*

(i) *Detection of* N-*Nitrosamines in saliva*

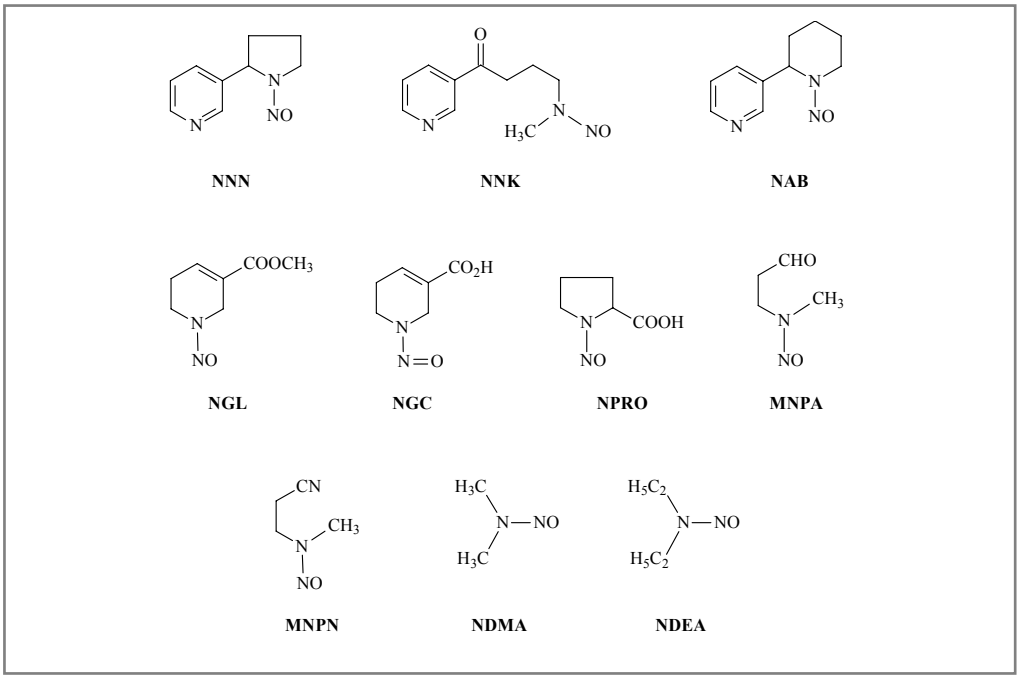
Carcinogens derived from tobacco and areca nut have been detected in the saliva (mixture of saliva with macerated betel-quid ingredients produced while chewing) of users of these products. The tobacco-specific nitrosamines, *N*′-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) (NNK) and *N*′-nitrosoanabasine (NAB) (see Figure 6 for the structures), as well as the volatile nitrosamines, *N*-nitrosodimethylamine and *N*-nitrosodiethylamine, were detected in the saliva of chewers of betel quid with tobacco. These volatile nitrosamines are probably also tobacco-derived. Three areca-nut-derived nitrosamines, *N*-nitrosoguvacoline (NGL), *N*-nitrosoguvacine (NGC) and 3-methylnitrosaminopropionitrile (MNPN), a rodent carcinogen (Figure 6), were detected in the saliva of chewers of betel quid with or without tobacco (Table 70).

The highest levels of tobacco-specific nitrosamines were detected in samples collected from India (Bhide *et al*., 1986), whereas the highest levels of areca-nut-derived nitrosamines (NGL) were found in the sediment of saliva collected from Taiwanese betel-quid chewers (Stich *et al*., 1986). NNN, NNK and NGL were reported in more than one study (Wenke *et al*., 1984; Nair *et al*., 1985; Bhide *et al*., 1986; Nair *et al*., 1986; Stich *et al*., 1986), whereas NAB (Bhide *et al*., 1986), MNPN (Prokopczyk *et al*., 1987), NGC (Nair, J. *et al.*, 1985), and the volatile nitrosamines (Bhide *et al*., 1986) were reported in single studies. One study that especially looked for MNPN failed to demonstrate its presence (Stich *et al*., 1986). Concentrations of NGL in the saliva obtained from Taiwanese chewers were higher than those in saliva from Indian chewers (Stich *et al.*, 1986).

Volatile nitrosamines and tobacco-specific nitrosamines found in the saliva of chewers could result from the leaching of those present in tobacco or could be formed endogenously during chewing from abundant precursors (see Section 4.1.1(*b*)(ii)). Areca-nut-derived nitrosamines have not been reported to be components of areca nut or betel quid, except on one instance in which the presence of some NGC was noted. Hence their occurrence in the saliva of betel-quid chewers is most probably due to their formation during chewing.

In-vitro nitrosation of betel quid with nitrite and thiocyanate at neutral pH for 1 h generated NGL (Nair *et al*., 1985). Nitrosation of arecoline at neutral pH yielded approximately four times more NGL than at acidic or alkaline pH (Wang & Peng, 1996).

Figure 6. Structures of nitrosamines



NNN, *N*′-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAB, *N*′-nitrosoanabasine; NGL, *N*-nitrosoguvacoline; NGC, *N*-nitrosoguvacine; MNPN, 3-methylnitrosaminopropionitrile; NDMA, *N*-nitrosodimethylamine; NDEA, *N*-nitrosodiethylamine; NPRO, *N*-nitrosoproline; MNPA, 3-methylnitrosaminopropionaldehyde

(ii) *Formation of* N-*nitroso compounds in the oral cavity*

Areca nut and tobacco contain secondary and tertiary amines that can be nitrosated in the saliva during the chewing of betel quid when they react with available nitrite in the presence of nitrosation catalysts such as thiocyanate. Microgram per millilitre levels of nitrite and thiocyanate have been reported in the saliva of chewers of betel quid (Nair *et al*., 1985; Nair, J. *et al.*, 1987). Using a modified *N*-nitrosoproline (NPRO) test (Ohshima & Bartsch, 1981), whereby 100 mg L-proline were added to the betel quid and saliva was collected from each subject 20 min later, it was clearly shown that NPRO (a non-carcinogenic compound) is formed during the chewing of betel quid with and without tobacco, although the extent of increased nitrosation varies among individual subjects (Nair, J. *et al*., 1987).

The role of poor oral hygiene in the formation of *N*-nitroso compounds was investigated using the NPRO assay. Endogenous nitrosation is significantly higher in subjects with poor oral hygiene (determined by dental plaque) compared with those with good oral hygiene (Nair *et al*., 1996). This implies that, on the basis of the availability of nitrosa-

Table 70. Levels (range, ng/mL) of nitrosamines detected in saliva of chewers of betel quid with tobacco (BQ + T) and without tobacco (BQ)

Nitrosamine	BQ + T (*n*)	BQ (*n*)	Reference
Volatile			
NDMA	0–35.5 (17)	NR	Bhide *et al*. (1986)
NDEA	0–5.6 (17)	NR	Bhide *et al*. (1986)
Tobacco-specific			
NNN	1.2–38 (6)	ND (5)	Wenke *et al*. (1984)
	1.6–14.7 (12)	ND (12)	Nair *et al*. (1985)
	3–85.7 (17)	NR	Bhide *et al*. (1986)
	4.9–48.6 (10)	NR	Nair, J. *et al*. (1987)
NNK	1–2.3 (6)	ND (5)	Wenke *et al*. (1984)
	0–2.3 (12)	ND (12)	Nair *et al*. (1985)
	0–14.3 (17)	NR	Bhide *et al*. (1986)
	0–9.4 (10)	NR	Nair, J. *et al*. (1987)
NAB	0–40[a] (17)	NR	Bhide *et al*. (1986)
Areca nut-specific			
NGL	4.3–350 (5)	2.2–9.5 (5)	Wenke *et al*. (1984)
	0–7.1 (12)	0–5.9 (12)	Nair, J. *et al*. (1985)
	NR	0–142 (11)	Stich *et al*. (1986)
	3.1–23.5 (10)	0.6–8.8 (10)	Nair, J. *et al*. (1987)
NGC	0–30.4 (6)	0–26.6 (6)	Nair *et al*. (1985)
MNPN	NR	0.5–11.4 (10)	Prokopczyk *et al*. (1987)

[a] Detected in only three samples

NDMA, *N*-nitrosodimethylamine; NR, not reported; NDEA, *N*-nitrosodiethylamine; NNN, *N*′-nitrosonornicotine; ND, not detected; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAB, *N*′-nitrosoanabasine; NGL, *N*-nitrosoguvacoline; NGC, *N*-nitrosoguvacine; MNPN, 3-(methylnitrosamino)propionitrile

table amines from areca nut and tobacco, there is more extensive formation of nitrosamines in subjects with poor oral hygiene if they chew tobacco. The enhanced endogenous nitrosation in subjects with poor oral hygiene may be due to the increased conversion of nitrate to nitrite or bacterial enzyme-mediated formation of nitrosamines, or both (Calmels *et al.*, 1988). Increased formation of nitrite and nitric oxide (NO) in the mouth has been reported during the formation of dental plaque (Carossa *et al*., 2001).

(iii) *Products of endogenous nitrosation*

Endogenous nitrosation in betel-quid chewers is significant. Many chewers swallow the quid that contains precursors of nitrosamines (nitrosatable amines, nitrite); these are then subjected to the acidic pH of the stomach, which is a more favourable condition for the nitrosation reaction of many secondary and tertiary amines. Urinary levels of NPRO,

a marker for endogenous nitrosation, were ~6.5-fold higher in chewers of betel quid with tobacco following ingestion of L-proline compared with chewers not ingesting proline (mean, 0.28 versus 1.86 μg/24 h; $n = 5$). In groups with no chewing habit, this increase was only marginal (mean, 0.32 versus 0.55 μg/24 h; $n = 4$) (Nair *et al*., 1986). In another study, chewers of betel quid without tobacco also showed a significant ~2.4-fold increase in urinary excretion of NPRO following ingestion of L-proline compared with no ingestion of proline (2.60 versus 6.31 μg/24 h, $n = 8$), and the group with no chewing habit again had a marginal increase in NPRO excretion (1.92 versus 2.54 μg/24 h; $n = 9$). An increase in mean NPRO excretion was also measured in 6-h urine (μmol/mol creatinine) of chewers of betel quid with tobacco (2.2 versus 3.6; $n = 15$) and betel quid without tobacco (1.9 versus 4.7; $n = 18$) and only a marginal increase in those with no chewing habit (2.9 versus 3.3; $n = 18$) (Chakradeo *et al.*, 1994). The above studies clearly demonstrate the potential of gastric nitrosation in betel-quid chewers. Using the NPRO assay, Stich *et al.* (1983) showed that the polyphenolic fractions of areca nut inhibited endogenous nitrosation in one male and one female volunteer following ingestion of sodium nitrate and L-proline.

(iv) *Formation of reactive oxygen species in the oral cavity*

Direct evidence that reactive oxygen species such as the hydroxyl radical ($HO^{\bullet}$) are generated in the oral cavity during betel-quid chewing was provided by measuring the formation of *ortho*- and *meta*-tyrosines from L-phenylalanine in human saliva by means of high-performance liquid chromatography (HPLC)-fluorescence detection (Nair *et al*., 1995; see Section 4.1.2 for in-vitro studies). Saliva from five Indian volunteers collected after chewing betel quid (consisting of betel leaf, areca nut, catechu and slaked lime but no tobacco) contained high concentrations of *para*-tyrosine, but no appreciable amounts of *ortho*- or *meta*-tyrosine. Saliva samples from the same subjects collected after chewing betel quid complemented with 20 mg phenylalanine contained 1010–3000 nM *ortho*-tyrosine and 1110–3140 nM *meta*-tyrosine. These levels were significantly higher ($p < 0.005$) than those detected in the saliva of subjects who kept L-phenylalanine in the oral cavity without betel quid (*ortho*-tyrosine, 14–70 nM; *meta*-tyrosine, 10–35 nM). These studies clearly demonstrate that the $HO^{\bullet}$ radical is formed in the human oral cavity during betel-quid chewing and could contribute to the genetic damage observed in the oral epithelial cells of chewers. Using the same method, formation of the $HO^{\bullet}$ radical was monitored in Taiwanese subjects who chewed unripe areca nut and lime with either *Piper betle* inflorescence ($n = 9$) or betel leaf ($n = 9$) (Chen *et al*., 2002). *ortho*- and *meta*-Tyrosines were detected at levels that were ~5–14 times lower than those detected in Indian chewers (Nair *et al*., 1995), which is probably due to differences in betel-quid ingredients.

4.1.2 *Experimental systems*

(*a*) *Areca nut/arecoline*

(i) *Metabolism*

In rats, arecoline is de-esterified in the liver to arecaidine, and both arecoline and arecaidine are excreted as the mercapturic acid, *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (Boyland & Nery, 1969).

According to Nery (1971), the metabolism of arecoline may be complex, with up to five major metabolic products being released from three different routes of metabolism: arecoline 1-oxide, arecaidine 1-oxide, arecaidine, *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine and an unidentified product.

Arecoline is rapidly metabolized in both liver and kidney (essentially 100% and approximately 87%, respectively). Arecoline was not metabolized by either blood or brain homogenates to any significant degree. Using various enzymatic inhibitors, any significant involvement of monoamine oxidase or hepatic microsomal oxidative enzymes in the metabolism of arecoline was ruled out. However, the specific carboxylesterase inhibitors tri-*ortho*-tolylphosphate and tetraisopropyl pyrophosphoramide completely blocked arecoline metabolism in the liver homogenate. It was therefore suggested that a carboxylesterase (EC 3.1.1.1) is primarily responsible for the metabolism of arecoline in mice (Patterson & Kosh, 1993). The short half-life of arecoline has been attributed to the rapid in-vivo enzymatic hydrolysis of the ester functionality (Saunders *et al.*, 1988) to form the carboxylic acid derivative arecaidine which appears to be a major metabolic product of arecoline in mice (Nieshulz & Schmersahl, 1968).

(ii) *Influence of areca nut/arecoline on drug-metabolizing enzymes and cellular antioxidant profiles*

In humans: in-vitro studies

Jeng *et al*. (1996) studied the effect of arecoline on the intracellular antioxidant profile of glutathione (GSH) in human fibroblasts *in vitro*. At concentrations of 0.4 mM and 1 mM, arecoline depleted about 26% and 45% of GSH, respectively, after 2 h of incubation; control cells maintained their original GSH levels during the incubation period.

Chang, Y.C. *et al*. (1998) demonstrated that arecoline-induced cytotoxicity in human buccal fibroblasts *in vitro* could be inhibited by exposure to GSH. This cytotoxicity could also be inhibited by exposure to *N*-acetyl-L-cysteine, a glutathione synthesis precursor, and by exogenous addition of carboxylesterase (Jeng *et al*., 1999a).

Chang, Y.-C. *et al*. (2001a) studied the effect of arecoline on glutathione *S*-transferase (GST) activity and lipid peroxidation in cultured human buccal mucosal fibroblasts. At concentrations higher than 50 μg/mL, arecoline significantly decreased GST activity in a dose-dependent manner. At concentrations of 100 and 400 μg/mL, arecoline reduced GST activity by approximately 21% and 46%, respectively, during a 24-h incubation period. However, it did not increase lipid peroxidation at any dose tested in this assay system. The adverse effect of arecoline on GST may increase the risk for oral cancer due to other

chemical carcinogens such as tobacco products. Exposure to areca-nut extract and arecoline also gives rise to GSH depletion and mitochondrial dysfunction of oral keratinocyte KB epithelial cells (Chang, M.C. *et al*., 2001).

COX2, an inducible enzyme responsible for prostaglandin (PGE_2 and 6-keto-$PGF_{1\alpha}$) synthesis, plays an important role in certain inflammatory diseases and carcinogenesis. Areca-nut extract induced COX2 mRNA and protein expression and PGE_2 and 6-keto-$PGF_{1\alpha}$ in primary human gingival keratinocytes (Jeng *et al*., 2000); it was suggested that this stimulation of PGE_2 production could partly result from the up-regulation of COX2 mRNA expression.

Tsai *et al*. (2003) studied the influence of arecoline on the expression of COX2 in human buccal mucosal fibroblasts incubated for 2 h with different doses of arecoline (10–160 μg/mL) *in vitro*. COX2 mRNA increased approximately 1.5- and 2.7-fold after exposure to 20 and 160 μg/mL arecoline, respectively, and a peak level was induced by 80 μg/mL. In addition, pretreatment with the GSH precursor, 2-oxothioazolidine-4-carboxylic acid, led to a decrease in the induction of COX2 mRNA by arecoline, and the GSH synthesis inhibitor, buthionine sulfoximine, led to an increase, suggesting that regulation of COX2 expression induced by arecoline is critically dependent on cellular glutathione concentration.

Areca-nut extract (50–150 μg/mL) slightly enhanced the activity of COX (also called prostaglandin-endoperoxidase synthase (PHS)) in the human oral carcinoma cell line, OEC-M1, but inhibited its activity in KB cells (> 50 μg/mL) after 24 h of exposure (Yang *et al*., 2002).

In animals: in-vivo studies

Mice given areca nut in the diet (0.25, 0.5 or 1.0%) for 5 or 36 weeks had significant increases in hepatic levels of cytochrome P450, cytochrome b5, malondialdehyde and GST, whereas hepatic content of sulfhydryl groups (-SH) was depressed (Singh & Rao, 1995a). Mice that had been on this diet for 45 days were supplemented during the last 10 days with mace (*Myristica fragans*), mustard seed (*Brassica niger*), garlic (*Allium sativum* L.) or butylated hydroxyanisole (BHA) — known chemopreventive agents; the levels of GST and -SH content normally induced by these agents were found to be decreased by areca nut. Conversely, levels of cytochrome P450 and cytochrome b5 normally induced by these agents were augmented following ingestion of an areca-nut diet (Singh & Rao, 1993a,b,c, 1995b,c).

Singh *et al.* (1996, 1997, 2000) studied the modulatory influence of intraperitoneal administration of arecoline on the chemopreventive efficacy of phytic acid, clocimum oil or chlorophyllin in lactating and suckling neonate mice. Phytic acid increased the hepatic levels of GST and -SH content in both lactating dams and suckling neonates whereas supplementation with arecoline inhibited these levels. Conversely, phytic acid elevated the levels of cytochrome P450 and cytochrome b5 in dams, and supplementation with arecoline further increased these elevated levels (Singh *et al*., 1997). Clocimum oil raised the hepatic levels of GST, -SH, cytochrome b5 and cytochrome P450 in lactating dams

and suckling neonates; treatment with arecoline depressed the phase II enzyme and -SH content level but further raised the phase I enzyme levels (Singh *et al*., 2000). Arecoline treatment inhibited the levels of chlorophyllin-induced hepatic GST and -SH content in lactating females. Arecoline, whether given alone or concomitantly with chlorophyllin, elevated the levels of hepatic cytochrome b5 and cytochrome P450 to the same significance level compared to the untreated lactating dams (Singh *et al*., 1996).

(iii) *Effect of arecoline on TIMP and MMP*

Chang, Y.-C. *et al*. (2002a) hypothesized that oral submucous fibrosis is caused by increased or altered de-novo synthesis and deposition of extracellular matrix and/or altered fibrolysis, which, if unbalanced, may result in this fibrosis during betel-quid chewing. These authors attempted to assess the role of tissue inhibitors of metalloproteinases (TIMPs) and matrix metalloproteinases (MMPs) in the pathogenesis of OSF associated with chewing betel quid. Biopsy specimens were taken from buccal mucosal fibroblasts (BMFs) of six healthy individuals with no chewing habit and OSF of 10 male patients with betel-quid chewing habits. These biopsy specimens were used for preparation of fibroblast cultures and expression of TIMP-1 and production of MMP-2, which is the main gelatinolytic proteinase secreted by the human buccal mucosal fibroblasts, and were analysed by Western blot and gelatin zymography, respectively; OSF specimens were found to have higher TIMP-1 expression than BMFs. In-vitro exposure of human buccal mucosal fibroblasts to arecoline resulted in elevated TIMP-1 expression at concentrations below 20 µg/mL in a dose-dependent manner and inhibited MMP-2 secretion and production at higher concentrations (40 µg/mL), reducing MMP-2 activity by ~54% at 80 µg/mL. Arecoline acted not only as an inhibitor of gelatinolytic activity of MMP-2 but also as a stimulator for TIMP-1 activity, altering the balance between these two processes in favour of matrix stability. These synergistic effects may contribute to the accumulation of extracellular matrix components in betel quid-associated oral submucous fibrosis.

(*b*) *Betel leaf/hydroxychavicol/eugenol*

(i) *Metabolism*

Hydroxychavicol is a major phenolic compound in the leaf as well as the inflorescence of *Piper betle*. During its metabolism, an *ortho*-quinone is produced, which subsequently induces the production of reactive oxygen species via redox cycling (Iverson *et al*., 1995; Krol & Bolton, 1997). The reactive metabolites of hydroxychavicol such as quinones, quinone methide and imine methide enter into conjugation reactions with the reduced GSH (Iverson *et al*., 1995; Krol & Bolton, 1997; Nikolic *et al*., 1999). A similar pattern of metabolism was shown for eugenol (Bolton *et al.*, 1992; Thompson *et al.*, 1993).

(ii) *Influence on pro-oxidant and antioxidant mechanisms*

Chang, M.C. *et al*. (2002a) studied the effects of hydroxychavicol, which, like many phenolic antioxidants, can show pro-oxidant properties on oral KB carcinoma cells

in vitro. Hydroxychavicol induced the intracellular production of reactive oxygen species at concentrations higher than 0.1 mM and depleted GSH. This compound acts as an antioxidant at low concentrations, whereas at high concentrations (> 0.1 mM), it may elicit intracellular accumulation of ROS and changes in redox status.

Choudhary and Kale (2002) evaluated the antioxidant properties of betel-leaf extract *in vitro* and *in vivo*. Male Swiss albino mice (7–8 weeks old) were given 1, 5 and 10 mg/kg bw betel-leaf extract orally for 2 weeks. The activity of superoxide dismutase (SOD) in liver increased in a dose-dependent manner. However, at 5- and 10-mg/kg dose levels, catalase activity was inhibited.

Panda and Kar (1998) studied the effect of betel-leaf extract (0.10, 0.40, 0.80 and 2.0 g/kg bw per day for 15 days) on the activities of catalase and SOD in the thyroid of male Swiss albino mice. Higher doses decreased the activities of catalase and SOD while increasing the level of lipid peroxidation. However, these effects were reversed with lower doses of the extract.

In a study to investigate its anticarcinogenic effect on tobacco carcinogens, 8-week-old inbred male Swiss albino mice were treated with betel-leaf extract (2.5 mg per mouse per day in drinking-water) for 10–16 months (13 mice) or 17–22 months (six mice). The concentrations of vitamin A and vitamin C (both strong antioxidants) in the liver were increased (Padma *et al*., 1989a).

Jeng *et al*. (2002) showed that an aqueous extract of betel-leaf components contained potent scavengers of hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$) and was also an inhibitor of xanthine oxidase in a dose-dependent manner. In the presence of 10 and 20 μg/mL betel-leaf extract, about 95% of $O_2^{\bullet -}$ radicals were scavenged. Similarly, 20, 100 and 200 μg/mL betel-leaf extract inhibited $HO^{\bullet}$ by 47, 76 and 88%, respectively. The extract also effectively prevented $HO^{\bullet}$-induced DNA breaks on PUC18 plasmid DNA.

Patel and Rajorihia (1979) demonstrated that betel leaf has an antioxidative property that is much more effective than that of either BHA or butylated hydroxytoluene (IARC, 1986) *in vitro*.

(*c*) *Formation of nitrosamines*

(i) *Endogenous formation of areca-nut-derived nitrosamines in animals: in-vivo studies*

Ohshima *et al*. (1989) identified *N*-nitrosonipecotic acid (NNIP) (see Figure 5 for structure) as a major urinary metabolite of the areca-nut-derived nitrosamines, NGL and NGC, in BDIV rats. In a previous study, NNIP was detected in the urine of Syrian hamsters that were fed with powdered areca nut and sodium nitrite in the diet. Urine collected from control animals fed only areca nut or nitrite alone did not contain any NNIP (Ernst *et al*., 1987). The authors concluded that the origin of this nitrosamine metabolite was from the endogenously formed areca-nut-derived nitrosamines, NGL and/or NGC.

(ii) *Formation of nitrosamines from arecoline and from betel quid: in-vitro studies*

In-vitro experiments with nitrite and the areca-nut alkaloid arecoline have shown the formation of at least three nitrosamines: NGL, MNPN and 3-methylnitrosaminopropionaldehyde (MNPA) (Wenke & Hoffmann, 1983). Nitrosation of arecoline at neutral pH yielded approximately four times more NGL than at acidic and alkaline pH (Wang & Peng, 1996). In-vitro nitrosation of betel quid without tobacco with nitrite and thiocyanate at neutral pH generated NGL, whereas similar nitrosation of betel quid with tobacco yielded NGL to a lesser extent and NGC (Nair *et al*., 1985). The structure of the alkaloids and the nitrosamines derived from these alkaloids are given in Figure 5.

When the nitrosation reaction of betel quid with tobacco was carried out at acidic pH, formation of NNN and *N*′-nitrosoanatabine (NAT) was observed in addition to the formation of areca-nut-derived nitrosamines. NNK, MNPN and MNPA were not detected in any of the betel-quid samples nitrosated *in vitro* (Nair *et al*., 1985). In-vitro nitrosation of betel quid with added L-proline yielded NPRO; addition of ascorbic acid with proline reduced the formation of NPRO in only one of three betel-quid samples tested (Nair, J. *et al*., 1987). *N*-Nitrosopiperidine, *N*-nitrosopyrrolidine and *N*-nitrosomorpholine were detected in nitrite-treated aqueous extract of *Piper betle* L. fruit (Chen *et al*., 1984).

(iii) *Modulation of nitrosamine formation by polyphenols from betel-quid ingredients*

Polyphenols have been shown to inhibit or enhance the nitrosation of amines (Pignatelli *et al*., 1982; Walker *et al*., 1982). Wang and Peng (1996) investigated the effect of crude phenolic extracts from areca fruit (areca nut) and the inflorescence and leaf of *Piper betle* on the formation of NGL from arecoline. They observed that low concentrations (< 60 mg/300 mL [< 0.2 mg/mL]) of polyphenolic extract from areca fruit and all tested concentrations of phenolics from *Piper betle* (up to ~530 mg/300 mL [~1.8 mg/mL]) inhibited the formation of NGL from arecoline, whereas higher concentrations of areca phenolics (> 260 mg/300 mL [> 0.86 mg/mL]) enhanced the formation of NGL. Hydroxychavicol, a polyphenol from betel leaf, has been shown to scavenge nitrite, thereby reducing the formation of *N*-methyl-*N*-nitrosourea, the mutagenic reaction product of nitrite plus methylurea (Nagabhushan *et al*., 1989).

(*d*) *Formation of reactive oxygen species from betel-quid ingredients*

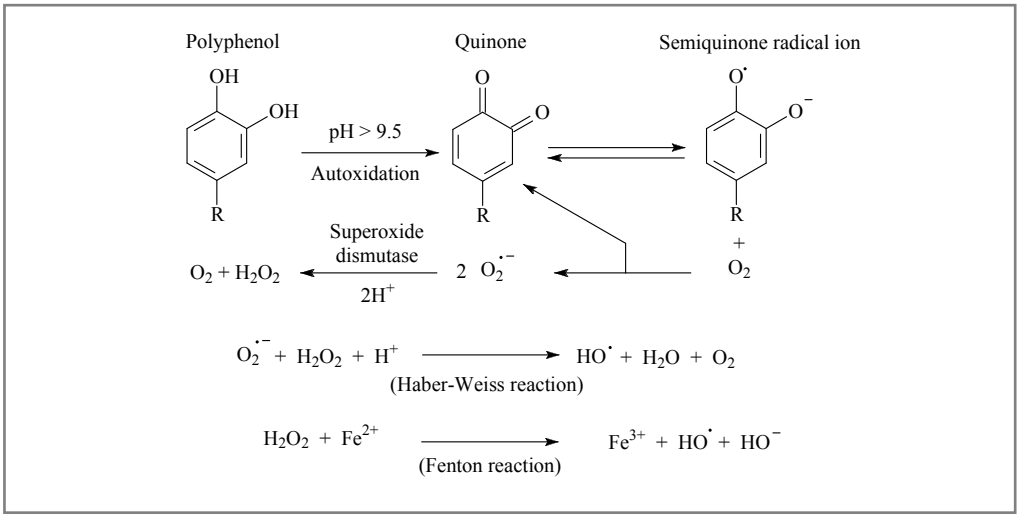
Cell-free systems

Several polyphenols including the tannin fraction of areca nut have been shown to be genotoxic at alkaline pH in *Saccharomyces cerevisiae* (Rosin, 1984). The genotoxicity of polyphenols was postulated to be attributable to the generation of H_2O_2 and free radicals as a result of rapid autoxidation under alkaline pH. The generation of superoxide anion ($O_2^{\bullet-}$) and H_2O_2 from aqueous extracts of areca nut and catechu at pH greater than 9.5 was demonstrated by Nair, U.J. *et al*. (1987) using a chemiluminescence technique. Of the various areca-nut extracts, the catechin fraction, at alkaline pH, was shown to be the most

active producer of reactive oxygen species. The formation of $O_2^{\bullet-}$ was enhanced by Fe^{2+}, Fe^{3+}, Mg^{2+} and Cu^{2+} but inhibited by Mn^{2+} and higher concentrations of Mg^{2+} and Cu^{2+}. Tobacco extract failed to generate reactive oxygen species under similar conditions but showed formation of $O_2^{\bullet-}$ only in the presence of metal ions. Saliva inhibited both $O_2^{\bullet-}$ and H_2O_2 formation from betel-quid ingredients. Upon incubation of DNA at alkaline pH with areca-nut extract or catechu and Fe^{3+}, 8-OH-deoxyguanosine (8-OHdG) was formed as quantified by HPLC-electrochemical detection (Nair, U.J. *et al*., 1987).

Using 25 samples of slaked lime from Papua New Guinea, Nair *et al*. (1990) demonstrated that the free calcium hydroxide content and pH of slaked lime samples (≥ 9.5) were highly correlated with the generation of reactive oxygen species from areca-nut extract and DNA damage *in vitro*, as measured as 8-OHdG. The Fe^{2+} and Mg^{2+} levels in the lime samples were too low to modify the formation of reactive oxygen species, but the formation of 8-OHdG in DNA was enhanced by the addition of high concentrations of Fe^{2+} and the formation of H_2O_2 was almost entirely inhibited by adding Mg^{2+} to the reaction mixture. These results show the importance of pH for the formation of reactive oxygen species that is likely to occur via autoxidation of polyphenols, redox cycling via quinone semiquinone radicals and iron-catalysed Haber-Weiss and Fenton reactions (Figure 7).

Figure 7. Scheme for the formation of reactive oxygen species from polyphenols present in the betel-quid ingredients, areca nut and catechu



Adapted from Nair *et al.* (1996)

To determine whether hydroxyl radical ($HO^{\bullet}$) is generated while chewing betel quid, in-vitro studies were performed using L-phenylalanine with different reaction conditions. The formation of *ortho*- and *meta*-tyrosine from L-phenylalanine was measured as a

marker of $OH^{\bullet}$ radical generation. Both *ortho*- and *meta*-tyrosine were formed *in vitro* in the presence of extracts of areca nut and/or catechu, transition metal ions such as Cu^{2+} and Fe^{2+} and alkaline pH due to slaked lime or sodium carbonate. The omission of any of these ingredients from the reaction mixture significantly reduced the yield of tyrosines. $HO^{\bullet}$ scavengers such as ethanol, D-mannitol and dimethylsulfoxide inhibited phenylalanine oxidation in a dose-dependent fashion (Nair *et al*., 1995).

Iron and copper are involved in the catalysis of reactive oxygen species generation. Copper contents in various betel-quid ingredients are reported to be in the range 3–188 μg/g (areca nut) and 8–53 μg/g (*pan masala*) (Trivedy *et al*., 1997; Ridge *et al*., 2001), whereas the iron contents were 75 μg/g (areca nut), 171 μg/g (betel leaf), 5.2 mg/g (catechu), 190 μg/g (slaked lime) (Zaidi *et al*., 2002) and 22–526 μg/L [89.6–2100 μg/g] (slaked lime) (Nair *et al*., 1990).

In the presence of $FeCl_2$ (1–1000 μM) and under alkaline conditions, the reaction of tender (1 mg/mL) and ripe (1 mg/mL) areca-nut extracts with herring sperm DNA for 60 min produced more 8-OHdG than without iron (Liu *et al.*, 1996).

Cultured cell systems

Significant amounts of superoxide anion production (determined by the cytochrome c reduction assay) and lipid peroxidation (formation of thiobarbituric acid-reactive substances) were demonstrated in normal human oral keratinocytes treated with commercially available *gutka* and *pan masala* with or without saccharin (Bagchi *et al*., 2002).

Exposure of Chinese hamster ovary (CHO)-K1 cells to ripe areca-nut extract (0.05–0.4 mg/mL) for 18 h induced a 1.2–2.8-fold increase in 8-OHdG formation. Tender areca-nut extract induced only a slight increase in 8-OHdG formation ($p > 0.05$). A 3.5-h exposure of CHO-K1 cells to areca nut produced marked formation of reactive oxygen species (hydrogen peroxide), as indicated by an increase in intracellular dichlorofluorescein fluorescence (Liu *et al.*, 1996).

(*e*) *Antioxidant effects*

Lei *et al*. (2003) directly evaluated the antioxidative properties of aqueous extract of *Piper betle* inflorescence. It was an effective scavenger of reactive oxygen species, with a 50% inhibitory concentration (IC_{50}) of 80, 28 and 73 μg/mL towards H_2O_2, superoxide and hydroxyl radicals, respectively.

Aqueous extract of *Piper betle* leaf was also shown to be a potent scavenger towards reactive oxygen species such as hydroxyl radicals (> 15 μg/mL), superoxide radicals (> 10 μg/mL) and a xanthine oxidase inhibitor (> 50 μg/mL). It inhibited the rabbit platelet aggregation induced by arachidonic acid and collagen, possibly because of its antioxidative property and its inhibition of cyclooxygenase. *Piper betle* leaf extract also inhibited thromoxane B_2 and prostaglandin-D_2 production in platelet aggregates induced by collagen and arachidonic acid (Jeng *et al*., 2002; Lei *et al.*, 2003), but had no effect on areca-nut-induced platelet aggregation (see Section 4.2.2(*b*)(iii)).

4.2 Physiological and toxic effects

4.2.1 *Humans*

(*a*) *Psychopharmacological effects*

Areca nut is the fourth most commonly used psychoactive substance in the world after tobacco, alcohol and caffeine-containing beverages (Sullivan & Hagen, 2002). It is described as a popular pleasure-giving substance in South Asia (Schneider, 1986) and chewed for its psychostimulating effects (Norton, 1998). Areca-nut chewing is reported to have varied and widespread, predominantly stimulant, effects (Chu, 2001, 2002).

'Betel-nut' (areca-nut) psychosis was originally described about 25 years ago in Papua New Guineans by Burton-Bradley (1977). He described how traditional healers challenged victims with so-called betel nut to induce insanity as a part of their diagnostic strategy. Psychosis, although rare, was described in predisposed people following abrupt cessation of the habit (Burton-Bradley, 1978). In Melanesia, other authors have also reported areca nut as producing altered status of consciousness and being capable of inducing intoxication (Cawte, 1985). More recently, so-called betelmania was reported among Cambodian refugee women living in the USA who were addicted to areca nut (Pickwell *et al*., 1994).

Habituation and addiction to areca nut in Papua New Guineans has been reported (Burton-Bradley, 1978; Talonu, 1989). More recently, regular, daily use of areca nut at high frequency — on average 17.3 portions a day — was reported among aborigines of Taiwan (Yang, Y.-H. *et al.*, 2001). Winstock *et al*. (2000) described findings consistent with a dependence syndrome related to areca-nut use among regular users in the Gujarat community in the United Kingdom. Reported effects of areca-nut chewing were relaxation, improved concentration, mild lifting of the mood and enhanced satisfaction after eating. Withdrawal symptoms on trying to quit the habit were mood swings, anxiety, irritability, reduced concentration, sleep disturbance and craving for the nut. The mean severity of dependence score was 7.3 (range, 1–12), which is equivalent to problematic use of amphetamines. The majority of the users reported the development of tolerance to the effects of the nut.

In a preliminary study conducted in a hospital population in Sri Lanka, a higher proportion of patients diagnosed with schizophrenia chewed betel quid compared with control subjects, and the frequency of chewing betel quid was also higher among these patients (Kuruppuarachchi & Williams, 2003).

(*b*) *Effects on oral hard and soft tissues*

A growing body of evidence over the last five decades from epidemiological and experimental studies has shown that areca nut, even when consumed in the absence of tobacco or slaked lime, may have potentially harmful effects on the oral cavity (Trivedy *et al*., 1999a).

(i) *Effects on hard tissues*

Dental attrition

The main effects of areca on the hard tissues are on the teeth. The habitual chewing of areca nut may result in severe tooth wear involving incisal and occlusal tooth surfaces, particularly the enamel covering. The loss of enamel exposes the underlying dentine which, as it is softer than enamel, wears away at an increased rate. The exposure of dentine may also result in dentinal sensitivity. The degree of attrition is dependent upon several factors, which include the consistency (hardness) of the areca nut, the frequency of chewing and the duration of the habit. Root fractures have also been demonstrated in chronic areca-nut chewers, which are probably a consequence of the increased masticatory load, and excessive and repetitive masticatory stress applied on teeth during chewing (Yeh, 1997; Gao *et al*., 2001).

Extrinsic staining of teeth in the form of black or brown surface discoloration due to areca-nut deposits is often observed among areca-nut chewers, particularly when good oral hygiene prophylaxis is lacking and where regular dental care is minimal.

Dental caries

It has been suggested that areca-nut chewing may confer protection against dental caries. Epidemiological studies carried out in South-East Asia suggest that the prevalence of dental caries in areca-nut chewers is lower than that in non-chewers (Möller *et al*., 1977; Schamschula *et al*., 1977; Nigam & Srivastava, 1990). Some investigators, however, have shown that there is no difference in the prevalence of dental caries between areca-nut chewers and non-chewers in other Asian populations (Reichart & Gehring, 1984; Williams *et al*., 1996). Although little is known about the cariostatic properties of areca nut, it has been suggested that the stain that often coats the surface of the teeth may act as a protective varnish (Howden, 1984).

(ii) *Effects on soft tissues*

Gingivae and the periodontium

A higher prevalence of gingivitis was recently reported among chewers of betel quid with tobacco (Amarasena *et al*., 2003).

Ling *et al.* (2001) found that the levels of two periodontal pathogens, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, are higher in betel-quid chewers who show a higher gingival index (an indicator of gingival inflammation) than in non-chewers.

It has been shown that loss of periodontal attachment and calculus formation is greater in areca-nut chewers (Ånerud *et al*., 1991). [The Working Group noted that it is difficult to interpret these studies, as there are several confounding variables such as the level of oral hygiene, dietary factors, general health and dental status, and especially tobacco smoking, which may have a significant influence on periodontal status. It is therefore difficult to ascertain the biological effects of chewing areca nut on periodontal health.]

Oral submucous fibrosis

There has been recent interest in the role of copper in the pathogenesis of oral submucous fibrosis, and elevated copper concentrations have been found in products containing areca nut in comparison with other nut-based snacks (Trivedy *et al*., 1997). Chewing areca nut for up to 20 min releases significant amounts of soluble copper into the oral cavity (Trivedy *et al*., 1999b), and mucosal biopsies taken from oral submucous fibrosis patients contain higher concentrations of copper than those taken from healthy controls (Trivedy *et al*., 2000). This has led to the hypothesis that higher tissue concentrations of copper may increase the activity of the enzyme lysyl oxidase, an extracellular copper-dependent enzyme that catalyses the cross-linking of elastine and soluble collagen to form insoluble collagen in the extracellular matrix. Lysyl oxidase is implicated in the pathogenesis of several fibrotic disorders including oral submucous fibrosis (Ma *et al*., 1995; Trivedy *et al*., 1999c). Further support for this theory comes from in-vitro studies showing that inorganic copper salts significantly increase the production of collagen by oral fibroblasts (Trivedy *et al*., 2001).

As described in Section 4.1.2(*a*)(iii), Chang, Y.-C. *et al*. (2002a) hypothesized that oral submucous fibrosis is probably the consequence of a disturbance in the homeostatic equilibrium between synthesis and degradation of extracellular matrix and/or altered fibrolysis which may result in this fibrosis during betel-quid chewing, and attempted to assess the role of tissue inhibitors of metalloproteinase (TIMP) in the pathogenesis of this disease. Biopsy specimens of buccal mucosal fibroblasts were taken from six healthy individuals with no chewing habit and oral submucous fibrosis tissue from 10 men who chewed betel quid, and were used to prepare fibroblast cultures for the analysis of TIMP-1 expression by Western blot. Oral submucous fibrosis specimens were found to have higher TIMP-1 expression than fibroblasts derived from normal buccal mucosa. In this study, arecoline was found to increase TIMP-1 expression and to inhibit matrix metalloproteinases (MMP) in human buccal mucosal fibroblasts *in vitro*.

Cyclooxygenase (COX), an inducible enzyme responsible for prostaglandin synthesis, plays an important role in certain inflammatory diseases and carcinogenesis. Tsai *et al*. (2003) studied the influence of betel-quid (areca-nut) chewing on the expression of COX2 in six normal buccal mucosa specimens from persons who did not chew betel quid and in 15 oral submucous fibrosis specimens from betel-quid chewers; of the latter, 10 showed moderate fibrosis and five showed severe fibrosis. Immunohistochemical analysis showed that COX2 expression was significantly higher in oral submucous fibrosis specimens than in buccal mucosal fibroblasts. Strong immunostaining for COX2 was detected in epithelial cells, fibroblasts and inflammatory cells.

(*c*) *Effects on various physiological systems*

(i) *General physiological effects*

Dose-dependent increases in energy expenditure (varying with basal metabolism) and decreases in carbohydrate utilization (independent of variation in insulin secretion),

together with increased hunger, were seen in a human volunteer when gels of betel extract were applied to the buccal sulcus after fasting. However, suppression of appetite after food intake was enhanced (Strickland & Duffield, 1997; Strickland *et al*., 2003). The four areca alkaloids have anti-muscarinic effects on smooth muscle and bind to γ-aminobutyric acid (GABA) receptors that are thought to contain chloride channels. These are similar to acetylcholine receptors and are found in many organs in the body, including the brain and pancreatic islets. Areca alkaloids can, therefore, be predicted to have widespread physiological effects (for a review, see Boucher & Mannan, 2002).

(ii) *Effects on the nervous and cardiovascular systems*

Areca nut contains a number of psychoactive alkaloids, one of which is arecoline, and affects the parasympathetic nervous system in intolerant users, inducing salivation and sweating. More importantly, areca-nut chewing also activates a sympathoadrenal response (Chu, 2002). Areca-nut chewing has been shown to cause a significant elevation in plasma concentration of adrenaline and noradrenaline (Chu, 1995a), but the sites of activation have not been defined. Chu (2001) hypothesized that these actions on the nervous system may be both central and peripheral. Arecoline readily crosses the blood–brain barrier (Asthana *et al*., 1996) and increases brain levels of acetylcholine in animals by 150–250% (Shannon *et al*., 1994). The areca-nut alkaloids, arecaidine and guvacine, are reported to be inhibitors of GABA-uptake (Johnston *et al*., 1975; Lodge *et al*., 1977). Thus, in addition to its stimulant effects, areca nut contributes to relaxation through its anxiolytic effects. This mixture of effects is common in other misused substances and supports earlier anecdotal reports claiming desirable effects by users. Arecoline and betel-quid use increase occipital α activity and generalized β activity on electroencephalograms (EEG), with reduction in θ activity, suggesting that areca alkaloids increase cerebral arousal and relaxation (Joseph & Sitaram, 1990; Chu, 1994). Short-term elevation in heart rate following administration of betel quid has been observed, indicating peripheral stimulation, but visual information processing is unaltered by areca nut (Frewer, 1990).

Betel quid reduces the severity of both positive and negative symptoms in schizophrenia but extrapyramidal symptoms (marked stiffness, tremor and distressing akathisia) are occasionally aggravated during heavy use in those on neuroleptic medication (Dealh, 1989). It is a risk factor for the movement disorder, Meige's syndrome (Behari *et al*., 2000). Betel-quid chewing increases central sympathetic activity in humans, leading to increased heart rate and increased blood flow through the common and external carotid arteries and facial-flush sensation (Chu, 2002; Lin, S.-K. *et al*., 2002).

Palpitations, sweating and facial flushing with sensations of skin warmth are early effects of areca chewing and skin temperature increases by 0.5–2 °C (Chu, 1993). Heart rates increase with or without habituation, but systolic blood pressure increases only in novice users (Chu, 1993). Betel leaves activate the sympathetic nervous system and increase the secretion of adrenal medullary catecholamine. Moderate areca-nut intake may activate the sympathetic nervous system and high doses activate both the sympathetic and parasympathetic nerves experimentally and *in vivo*. The effect on parasympathetic nerves

reduces the RR interval variation (RRIV) as recorded on electrocardiograms of heavy areca-nut users during rest and hyperventilation (Chu, 1995b). In the presence of coronary artery disease, this could, with vasoconstriction caused by areca nut, increase the risk of heart attack, although few case reports suggest this possibility (Hung & Deng, 1998; Deng *et al*., 2001). Elevated serum homocysteine is a risk factor for heart disease common among British Bangladeshis (Alfthan *et al*., 1997; Obeid *et al*., 1998) and increases in homocysteine levels related to smoking and areca-nut use were found to be as large as those related to folate deficiency in a study of Bangladeshis in the United Kingdom (Obeid *et al*., 1998).

Arterial plaque instability and fibrosis: role of matrix metalloproteinases

Instability of arterial plaque is a major causal factor in myocardial infarction. It is associated with increases in MMP enzyme activity in active plaque, which destroys collagen and weakens the interstitium. High levels of circulating MMP-2 and MMP-9 are markers of coronary events (Galis *et al*., 1994; Fabunmi, 1998; Kai *et al*., 1998). Circulating TIMP-1 increases with the use of betel quid, independent of increases related to MMP-9 and other risk factors (Timms *et al*., 2002a). It is not yet known whether the balance of these effects contribute to the risk for coronary heart disease or heart attack in areca-nut users.

Increases in TIMP-1 disproportionate to those in MMP-9, reported in areca-nut users, could contribute to the pathogenesis of diseases in which increased fibrosis is a feature, such as cirrhosis of the liver and hypertensive and left ventricular hypertrophy (Burt, 1993; Timms *et al*., 1998, 2002a,b). Arecoline itself produces dose-dependent increases in TIMP-1 expression in human buccal fibroblasts together with inhibition of secretion of MMP-2 (Chang, Y.-C. *et al*., 2002a), a phenomenon relevant to the risk for oral submucous fibrosis (see Section 4.1.2(*a*)(iii)).

(iii) *Effects on the respiratory system*

Arecoline has been known since 1912 to induce contraction of the bronchial muscles (Trendelenburg, 1912). Sufferers of asthma report worsening of their symptoms with areca-nut chewing: over 50% of those giving up the habit do so because of this effect. Minor reductions in forced expiratory volume in 1 s (FEV_1) are seen in non-asthmatics who chew areca nut. FEV_1 can improve by up to 10% in asthmatics using areca nut but reductions of ~22% are seen in those asthmatics who report worse asthma after chewing (Kiyingi, 1991; Kiyingi & Saweri, 1994). Arecoline causes dose-related constriction of bronchial smooth muscle *in vitro* and bronchoconstriction in most of the asthmatics studied who were not betel-quid users, some non-asthmatic controls and in betel-quid users with asthma. Reduction of FEV_1 (by up to 30%) may last several hours (Taylor *et al.*, 1992).

(iv) *Effects on the gastrointestinal system*

Users report that betel quid soothes the digestion and avoids constipation; colonic smooth muscle is stimulated through its muscarinic effects. Peptic ulceration, however, is increased in chewers of betel quid [with tobacco] (Ahmed *et al*., 1993). Chewers secrete more saliva on chemical stimulation, diluting salivary amylase and potassium, and tobacco aggravates this effect (Reddy *et al*., 1980). Areca-nut extracts reduce halitosis, probably by the reduction of the volatility of methyl mercaptan through arecal phenolic derivatives, and slaked lime plays an important role in this function (Wang *et al*., 2001).

(v) *Effects on endocrine systems*

Hyperglycaemic effects

The neurotransmitter GABA is functional in insulin-secreting pancreatic β cells; the GABA shunt enzyme, glutamate decarboxylase (GAD), is an autoantigen and GAD antibody formation is common in the development of the human type 1 diabetes, insulin-dependent diabetes mellitus (Martino *et al*., 1991; Sorenson *et al*., 1991). Areca alkaloids, which are inhibitors of the GABA receptor, block the inhibitory effects of GABA on the secretion of glucagon and somatotrophin, increasing their release. Glucagon release triggers the release of insulin resulting in short-term hypoglycaemia but long-term increases in glucagon cause diabetes. This provides a mechanism that could lead to diabetes in betel-quid chewers. At the same time, the inhibition of GABA reduces GAD activity and also, experimentally, the autoimmune response to GAD in rat pancreatic islets (Smismans *et al*., 1997). It is not known whether this mechanism applies to humans, in whom it could reduce the risk of type 1 diabetes and also the risk of progression to insulin requirement in patients with type 2 diabetes.

In view of the diabetogenicity of areca nut in mice (see Section 4.2.2), glycaemia and anthropometric risk factors for type 2 diabetes were investigated in relation to use of *paan* quids in ~1000 adult Bangladeshis living in East London (1992–94) (Mannan *et al*., 2000). Waist size and weight, major determinants of hyperglycaemia (Chan *et al*., 1994), increased with increasing use of betel quid (approximately 80% of men aged 30–50 and 90% of women aged 40–60 were chewers), independent of established risk factors, such as central obesity, age, smoking and parity. Reductions in body mass index (with increases in resting metabolic rate) have also been reported with increasing use of betel quid but only at higher ambient temperatures in hot climates (Strickland & Duffield, 2003). The difference in findings may reflect the more temperate weather in the United Kingdom. Alternatively, vitamin D deficiency, which is common in British Asians, is known to reduce insulin secretion and to increase glycaemia and may have obscured the features related to the use of betel quid (Boucher *et al*., 1995).

Recent work in Papua New Guinea, where vitamin D deficiency is less likely to be found, has shown that chewing betel quid without tobacco is the predominant independent risk factor for diabetes (diagnosed by fasting blood glucose ≥ 7.0 mmol/L), with age, body mass index and region of residence being the other relevant factors (odds ratio for diabetes in betel chewers, 3.4; 95% CI, 2.0–5.9) (Benjamin, 2001).

(vi) *Effects on the immune system*

Betel-quid chewers and oral submucous fibrosis patients

Antibodies

Shah *et al.* (1994) and Gupta *et al.* (1985) found that oral submucous fibrosis patients (n = 66 and 10) had higher serum levels of immunoglobulin (Ig) G, IgM and IgA than controls (n = 25 and 5), whereas Canniff *et al.* (1986) found an increase in serum IgG levels in 30 oral submucous fibrosis patients relative to normal individuals, but no difference in IgM and IgA levels; and Rajendran *et al.* (1986) observed an increase in serum IgA, IgD and IgE concentrations in 50 oral submucous fibrosis patients compared with 50 controls.

Canniff *et al.* (1986) detected the presence of serum auto-antibodies such as gastric parietal cell antibody (GPCA), thyroid microsomal antibody, anti-nuclear antibody (ANA), anti-reticulin antibody and anti-smooth muscle antibody (SMA) in 38, 23, 8, 4 and 4% of 26 oral submucous fibrosis patients, respectively. [No report of a control group is given.] Chiang *et al.* (2002a) also studied serum auto-antibodies in 109 male oral submucous fibrosis patients, all of whom were betel-quid chewers. The frequencies of serum ANA (23.9%), SMA (23.9%) and GPCA (14.7%) in oral submucous fibrosis patients were higher than those in 109 controls (9.2%, 7.3% and 5.5%, respectively). The presence of serum GPCA ($p < 0.05$) and ANA ($p = 0.066$) in oral submucous fibrosis patients was associated with daily betel-quid consumption, but the presence of serum auto-antibodies in such patients showed little correlation with maximal mouth opening.

Balaram *et al.* (1987) found an increase in circulating immune complex (CIC) in the sera of 20 healthy betel-quid chewers compared with 50 controls. Forty betel-quid chewers with oral submucous fibrosis and 85 with oral cancer had showed even higher serum levels of CIC. Remani *et al*. (1988) evaluated levels of CIC and immunoglobulins in normal controls and patients with oral leukoplakia, oral submucous fibrosis and oral cancer (n = 50 for all groups). Clearly elevated levels of CIC, and of IgG and IgM in CIC, were found in both oral submucous fibrosis and oral cancer groups.

Immune cells

An impairment of natural killer-cell activity has been detected in patients with oral leukoplakia and oral submucous fibrosis, which can be improved by treatment with interferon-α (Pillai *et al.*, 1990).

In an earlier study, total leukocyte and lymphocyte counts in peripheral blood were decreased in 25 patients with oral cancer and 30 with oral precancerous lesions and conditions (including oral leukoplakia and oral submucous fibrosis) compared with 22 healthy controls, whereas an increase in the percentage of B lymphocytes was noted in precancer patients. (Pillai *et al.*, 1987) [no oral habit data given].

Using immunohistochemical staining, Haque *et al.* (1997) reported increased numbers of CD3 cells (T lymphocytes), CD4 cells (helper/inducer T lymphocytes) and HLA-DR cells and an increase in the ratio of CD4:CD8 (suppressor and cytotoxic T lym-

phocytes) cells in the epithelium and subepithelial connective tissues of oral submucous fibrosis patients compared with normal buccal mucosa. Chiang *et al.* (2002b) also noted a marked increase in the numbers of CD3 cells, CD20 cells (B lymphocytes) and CD68 cells (macrophages) and a predominance of CD4 lymphocytes over CD8 lymphocytes in the subepithelial connective tissue of 50 oral submucous fibrosis patients compared with 10 controls.

Effects of tobacco/nicotine on the immune system

Few data are available on the effects of chewing tobacco on the immune system in humans. Nicotine, however, has been reported to affect both humoral and cell-mediated immune responses (Johnson *et al*., 1990; Geng *et al*., 1995, 1996; Kalra *et al*., 2000) and to produce an altered immune response that is characterized by a decline in inflammation, a reduction in antibody response and a decrease in T-cell receptor-mediated signalling (Sopori & Kozak, 1998). These findings suggest that nicotine is a potent immunopharmacological agent with regard to T-cell function (see IARC, 2004).

(*d*) *Other effects*

Wu *et al.* (1996) reported cases with milk-alkali syndrome caused by betel-quid chewing. The patients showed the symptom of hypercalcaemia, metabolic alkalosis, nephrocalcinosis and renal insufficiency. Lin, S.-H. *et al*. (2002) also reported one case of milk-alkali syndrome who chewed 40 pieces of betel quid per day.

Winstock *et al.* (2000) found a decrease in vitamin B_{12} level in 4 of 9 patients who had chewed betel quid for 35 years compared to historical reference range levels of supposed healthy non-chewers.

4.2.2 *Experimental systems*

(*a*) *In-vivo studies*

(i) *Chronic toxicity*

The chronic oral toxicity of *pan masala* was assessed in gavage studies in inbred male rats [strain not indicated]. The substance was ground, dispersed in water and given to the animals by gavage on 5 days per week for 6 months at 84, 420 or 840 mg/kg bw. The rats were killed 24 h after the last dose. Liver and serum enzymes (glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and alkaline phosphatase) and organ weights were measured. The results showed that chronic feeding of *pan masala* impaired liver function at the highest dose, as indicated by changes in enzyme activity, and decreased relative weights of the brain at all doses tested (22% weight loss at the highest dose) (Sarma *et al.*, 1992).

(ii) *Effect on oral soft tissues: induction of oral submucous fibrosis*

Earlier studies found that the application of arecoline to the palates of Wistar rats did not give rise to any features that were suggestive of oral submucous fibrosis (Sirsat &

Khanolkar, 1962). Application of arecaidine to hamster cheek pouch also failed to show any microscopic changes suggestive of fibrosis (MacDonald, 1987). The authors noted that the concentration chosen was too low to produce an effect *in vivo* and that the pH of the aqueous solution of aracaidine used was acid (pH 3), whereas in the human situation, the addition of lime to the betel quid would ensure an alkaline pH.

Paste made out of *pan masala* was painted onto the oral cavity (palate, cheek mucosa) of 21 Wistar albino rats on alternate days for a period of 6 months, except for a 2-week period after every biopsy. Biopsies were taken from the oral mucosa at the beginning of the study and every 2 months thereafter and were compared histopathologically with those obtained from a control group. Mild-to-moderate loss of nuclear polarity and increase in keratoses, parakeratoses, inflammatory cell infiltration and vascularity were noted compared with the controls. The increase in mitotic figures was not statistically significant and no definite changes in pigmentation or atypical cells were seen. Submucosal collagen increased sharply and steadily throughout the study. At the end of 6 months, 88% of biopsies showed thickened and condensed submucosal collagen, indicating submucous fibrosis (Khrime *et al.*, 1991).

(iii) *Neuropharmacological effects*

Arecoline, dissolved in saline, was given by subcutaneous injection to albino Swiss Nos mice at doses of 2 and 10 mg/kg bw. After 15 min, the animals were killed and the levels of acetylcholine, norepinephrine and dopamine in the cortex and subcortex (the remaining part of the brain) were studied. Arecoline at 10 mg/kg bw caused a reduction in levels of acetylcholine in the cortex and subcortex at the limit of statistical significance, and a statistically significant reduction in levels of norepinephrine. A statistically significant increase in the level of dopamine was observed only in the cortex after both doses of arecoline (Molinengo *et al*., 1986).

The dichloromethane fraction from *Areca catechu* extraction was injected intraperitoneally into rats and mice at doses of 1–13 mg/kg bw 1 h prior to observation of antidepressant effects and analysis of monoamine oxidase A. Forced swim and tail-suspension test results indicated an antidepressant effect of the dichloromethane fraction, which was associated with inhibition of monoamine oxidase-A isolated from rat brain. No such inhibition was seen with various constituents of *areca catechu* such as arecoline, arecaidine catechin, gallic acid and few aminoacids (Dar & Khatoon, 2000).

(iv) *Neurocardiovascular effects*

Intravenous injections of eugenol, safrole or an aqueous extract of *Piper betle* inflorescence (containing 6.2% eugenol and 78.9% safrole) induced hypotensive and bradycardiac effects in male Wistar rats, whereas both intra-arterial and intrathecal injections of these substances resulted in hypotensive and tachycardiac effects. Moreover, the effects of intravenous injections of the extract were reversed or inhibited by pretreatment with bilateral vagotomy, or administration of atropine (1 mg/kg intraperitoneally) or capsaicin (100 mg/kg, subcutaneously). The tachycardia resulting from intrathecal

injections of the extract was inhibited by pretreatment with propranolol (0.3 mg/kg intravenously). Eugenol and safrole induced the same pattern of changes in blood pressure and heart rate as the inflorescence extract. It was concluded that acute administration of extracts of betel inflorescence by different routes may activate C-fibre-evoked parasympathetic and sympathetic cardiovascular reflexes in rats (Chen *et al.*, 1995).

Using normal and hypertensive rats as an experimental model, areca-nut components decreased blood pressure and inhibited angiotensin I- and angiotensin II-induced increases in blood pressure, but showed little effect on heart rate (Inokuchi *et al.*, 1986). Oral intake of areca tannin (100–200 mg/kg) by hypertensive rats (6–8 per group) induced a long-lasting decrease in systolic blood pressure. Direct intravenous injection of areca tannin (10–15 mg/kg) led to hypotension (Inokuchi *et al.*, 1986). A single intraperitoneal injection of arecoline (1 mg/kg) to Fischer 344 rats (4–5 per group) induced the incorporation of arachidonate into brain microvascular endothelial cells (Williams *et al.*, 1998).

(v) *Effects on the gastrointestinal tract*

The effects of two Indian varieties of betel leaf (*Piper betle* L.), the pungent Mysore and the non-pungent Ambadi, on digestive enzymes of the pancreas and the intestinal mucosa, and on bile secretion were studied in rats. Betel leaves were despined, ground to a paste and given to female Wistar rats (eight per group) by gavage at doses of 40 and 200 mg/kg bw, comparable to one and five times the human consumption level. After 3 h, the pancreas and the small intestine were isolated and processed for assays of various enzymes. The betel leaves did not influence bile secretion or composition, but there was a significant stimulatory effect on pancreatic lipase activity. In addition, the Ambadi variety of betel leaf had a positive stimulatory influence on intestinal digestive enzymes, especially lipase, amylase and disaccharidases. A slight decrease in the activity of these intestinal enzymes was seen with the Mysore variety of the betel leaf, which also had a negative effect on pancreatic amylase. Both varieties caused a decrease in pancreatic trypsin and chymotrypsin activities (Prabhu *et al.*, 1995).

Feeding male Wistar rats (10 per group) a diet containing betel quid (30%) or its components (30%) including areca nut, *gambir* and lime for 30 or 90 days increased gastric mucosal acid back-diffusion and reduced mucous secretion. These changes were seen with experimental haemorrhagic peptic ulceration and probably contributed to increased ulceration. Alkalinization with either antacids or therapeutic blockade of acid secretion by $NaHCO_3$ and cimetidine corrected these effects (Hung & Cheng, 1994). The acetylcholine-like effect of areca nut on the bowel was due mainly to arecoline and was mediated through neuroreceptors of the autonomic nerve plexus in the gut wall.

Daily oral administration of alcoholic and aqueous areca-nut extracts to male albino rats (100 mg/kg bw extract for 1 and 4 weeks; five rats per group) significantly decreased alkaline phosphatase, Ca^{2+}-Mg^{2+}-ATPase and sucrase activities in brush border. However, in-situ exposure of the intestine to aqueous areca-nut extract for 30 min activated brush border membrane enzyme, alkaline phosphatase, Ca^{2+}-Mg^{2+}-ATPase and sucrase activities, whereas alcoholic areca-nut extract did not produce any significant changes in the

enzyme activities. Significant declines in brush border membrane constituents (total hexose, sialic acid and cholesterol) were also evident following continuous exposure to areca-nut extracts. These findings suggest that prolonged chewing of areca nut may cause significant functional alterations in the intestinal epithelial cell lining and could lead to malabsorption of nutrients (Kumar *et al*., 2000).

Feeding albino rats a diet containing 60–100% areca nut led to diarrhoea and even death of rats within 1–3 weeks. Histologically, splenomegaly, fatty liver changes, stunted skeleton and necrosis of buccal and intestinal mucosa were observed in dead rats. Diets containing 15% areca nut or more induced haemorrhagic and catarrhal enteritis (Saikia & Vaidehi, 1983).

(vi) *Lipid metabolism*

Feeding Sprague-Dawley rats with areca nut for 6 days markedly decreased plasma cholesterol and triglyceride concentrations, and also the intestinal pancreatic esterase activity involved in the cholesterol absorption process (Jeon *et al.*, 2000). Areca extracts reduce the absorption of triglycerides and cholesterol and subsequent blood levels of both through the inhibition of pancreatic cholesterol esterase, pancreatic lipase and intestinal acyl-co-enzyme A:cholesterol acyltransferase activities (Byun *et al*., 2001; Park *et al*., 2002).

(vii) *Diabetogenic effects*

Young adult CD1 mice with a low spontaneous incidence of diabetes were fed areca nut in standard feed for 2–6 days. Single time-point (90 min) intraperitoneal glucose tolerance tests were used to follow glucose tolerance up to 6 months of age. Glucose intolerance was defined as more than 3 standard deviations (SD) above mean control values. Glucose intolerance was found in 3/51 male and 4/33 female adult mice fed the areca-nut diet ($p < 0.01$). The progeny of these mice were then studied. In Group 1, which comprised matings between parents fed areca nut, glucose intolerance was found in 4/25 male and 1/22 female F_1 offspring, with significant hyperglycaemia in F_1 males born to hyperglycaemic but not to normoglycaemic mothers ($p < 0.01$). In the F_2 generation, 4/23 males and 1/16 females and, in the F_3 generation, 1/16 males and 0/20 females were glucose intolerant. In Group 2, parents fed areca nut were mated with normal controls. Glucose intolerance was found in 10/35 male and 10/33 female F_1 progeny ($p < 0.005$). The glucose-intolerant animals fed areca nut or their offspring did not develop insulin dependence. These findings suggest that consumption of areca nut may be diabetogenic and induce a heritable abnormality (Boucher *et al.*, 1994).

The possibility that betel chewing might be diabetogenic has been explored in CD1 mice (Boucher *et al*., 1994). Ground areca nut, bought in the United Kingdom and fed to young adults at 20% in a low nitrosamine feed (RM1 chow) for 5 days led to permanent diabetes in 8.3% of the animals (diagnosed by a 90-min plasma glucose concentration > 3 SDs above the mean for comparable pair-fed controls (with an incidence of spontaneous diabetes of 0.5%) at serial intraperitoneal glucose tolerance testing), in association with

obvious central obesity and pancreatic islet enlargement. Islet histology was typical of human type 2 diabetes with enlargement and vacuolation of islet cells. The F_1 offspring of the animals fed areca nut, especially males, developed diabetes in 10.6–30% in the various litters (test parents mated with animals fed control diet or animals fed areca nut when they had not developed hyperglycaemia). Affected offspring were obese and had the same islet changes; further generations (F_2–F_4) showed the same phenomena. Diabetes appeared at a higher rate in the offspring of F_0 fathers fed areca nut than in those of F_0 mothers fed areca nut. The mechanism for the inheritance of this diabetes is unknown. Damage to sperm heads, apparent by light microscopy 30 days after completion of betel feeding, may be relevant (Muhkerjee *et al*., 1991). However, subcutaneous injection of 0.2 and 0.25 mg/kg bw arecoline into alloxan-induced diabetic male rabbits (four per group) resulted in a decrease in blood sugar of 52.1 and 49.7%, respectively, which lasted for 4–6 h (Chempakam, 1993).

Tap roots of *Potentilla fulgens*, often chewed with areca nut, are also a traditional remedy for diabetes and cause dose-dependent reductions in blood glucose in normal and alloxan-induced diabetic mice (Syiem *et al.,* 2002).

(viii) *Effects on the immune system*

Arecoline

Shahabuddin *et al.* (1980) demonstrated the immunomodulatory influence of arecoline in mice, which, when injected subcutaneously with 0.5 mg arecoline twice daily for 34 days, showed suppression of both humoral and cell-mediated immunity.

Selvan *et al.* (1989) administered subtoxic doses (5, 10 and 20 mg/kg bw) of arecoline to male mice for 1, 2 and 3 weeks and found that there was a reduction not only in the weight of the thymus, spleen and mesenteric lymph nodes but also in cellularity. A marked reduction in cell numbers in the thymus and moderate effects on cellularity of the spleen and mesenteric lymph nodes were observed at a dose of 20 mg/kg bw arecoline. White and red blood cell counts decreased in a dose-dependent manner.

Selvan *et al.* (1991) explored the modulatory influence of arecoline on cell-mediated immune response. In-vivo effects of subtoxic concentrations of 5, 10 and 20 mg/kg bw arecoline given subcutaneously for 1, 2 or 3 weeks were evaluated. Delayed-type hypersensitivity (DTH) reaction to sheep red blood cells was not appreciable at the 5-mg/kg bw level, whereas at a dose of 10 mg/kg bw there was moderate reduction in DTH response. At 20 mg/kg bw given for 1, 2 or 3 weeks plus a 1-week no-exposure period, arecoline significantly suppressed the DTH reaction.

Selvan and Rao (1993) evaluated the modulatory action of arecoline on B cell-mediated immune response in male mice by administering subtoxic concentrations (5, 10 and 20 mg/kg bw) subcutaneously. After 1 week, control and experimental mice were immunized intraperitoneally with sheep red blood cells. The number of primary antibody-forming cells and haemagglutinating and haemolysis antibody titres to sheep red blood cells were assessed 4 days after immunization. Following exposure to arecoline for 1 week, there was a dose-dependent decrease in primary antibody-forming cells to sheep

red blood cells, with maximum reductions at 20 mg/kg bw, moderate reductions at 10 mg/kg bw and no effect at 5 mg/kg bw; haemagglutinating and haemolysis antibody titres were also decreased. Exposure to arecoline (10 and 20 mg/kg bw daily for 4 days) following sheep red blood cell immunization exerted dose-dependent suppression of primary antibody response. Recovery experiments in mice revealed that arecoline-mediated suppression of antibody responses is reversible.

Tobacco/nicotine

The effects of nicotine on the immune system in experimental animals have been studied extensively. Rats treated with nicotine showed a dose-dependent increase in interleukin-2 production and a suppressed splenic and peripheral blood lymphocyte response to mitogen (Caggiula *et al*., 1992; Petro *et al*., 1992). The latter effect has been attributed to the induction of a stage of anergy in these cells (McAllister-Sistilli *et al.*, 1998; IARC, 2004).

(ix) *Other biochemical effects*

Glutathione status

Studies in male Sprague-Dawley rats fed low doses of betel quid (0.53 g dry aqueous extract/kg diet) showed significantly increased hepatic activities of GSH peroxidase and cytoplasmic GST. Feeding high doses of betel quid (26.5 g dry aqueous extract/kg diet) lowered the concentrations of GSH and total glutathione (GSH and two oxidized GSH (GSSG)) (Wang *et al.*, 1999).

Na^+ and Cl^- excretion

Subcutaneous administration of arecoline (1.25–3 mg/kg) to rats increases the amount of urine and urinary excretion of Na^+ and Cl^- ions, which are associated with muscarinic receptor activation (Williams & Carter, 1965; Mujumdar *et al.*, 1979).

Thyroxine and triiodothyronine

Panda and Kar (1998) studied the effects of betel-leaf extract on thyroid hormone concentrations in male Swiss albino mice treated with 0.1, 0.4, 0.8 and 2 g/kg bw per day for 15 days. At higher doses (0.8 and 2 g/kg plant extract), betel-leaf extract increased the serum concentration of thyroxine (T4), although at lower doses (0.1 and 0.4 g/kg plant extract), the concentration of T4 was decreased. Contrasting observations were made for triiodothyronine (T3) concentrations. [Such changes could lead to thyroid dysfunction in humans but have not been recognized as being clinically significant to date.]

DNA and RNA synthesis

Male Swiss mice were given intraperitoneal injections of aqueous areca-nut extract (0.1 mL containing 1.5 mg alkaloids + 1.9 mg polyphenyl), a polyphenol fraction, tannic acid (1.9 mg per animal) or arecoline (0.06 mg/g bw). After 23 h, the mice received [^{3}H]-uridine or [^{3}H]thymidine by intraperitoneal injection and were killed 1 h later. Liver, lung,

kidney and muscle tissues were isolated and analysed for RNAse and DNAse activity, and for incorporation of tritiated nucleosides in RNA or DNA. There was no effect of different constituents of areca nut on DNAse activity but they increased RNAse activity in different tissues. The polyphenol fraction and tannic acid did not affect the RNA content of any of the tissues studied. Areca-nut extract increased the incorporation of the radiolabel [^{3}H]uridine into RNA in liver and muscle tissue, but decreased it in kidney; DNA synthesis was higher in liver and muscle, with no effect in lung and kidney. Arecoline increased [^{3}H]thymidine incorporation in DNA only in liver and lung. It decreased the deoxynucleotide pool in liver and lung, but increased it in kidney and muscle. Areca-nut extract had no effect on the deoxynucleotide pool (Shivapurkar & Bhide, 1979).

An aqueous extract of dried areca nut was injected intraperitoneally into Swiss mice. The injected amount (0.1 mL) of the extract contained 1.5 mg arecoline and 1.9 mg polyphenols. Arecoline was injected for comparison. The animals were killed after 24 h and lung, liver, kidney and muscle tissues were isolated. The areca-nut extract increased the RNA content in liver and muscle, while arecoline decreased it in lung, kidney and muscle tissue. Areca-nut extract increased the DNA content in muscle and liver, whereas arecoline decreased it in kidney and muscle and increased it in liver and lung (Shivapurkar *et al.*, 1978).

(*b*) *In-vitro studies*

(i) *Effects on oral hard and soft tissues*

Oral hard tissues

There is evidence *in vitro* suggesting that the tannin content of areca nut may have antimicrobial properties and this may contribute to the cariostatic role of areca nut (de Miranda *et al*., 1996).

Biofilms

Areca tannin (0.1–1 mg/mL) suppressed glucosyltransferase activity and consequently formation of dental plaque (Hada *et al.*, 1989).

Exposure of human peripheral blood neutrophils to aqueous extracts of ripe areca nut without husk and fresh, tender areca nut with husk abolished their bactericidal activity against oral pathogens (*Actinobacillus* and *Streptococcus* species) and inhibited the production of bactericidal superoxide anion, as measured by ferricytochrome c reduction (Hung *et al*., 2000).

Oral soft tissues

Periodontal tissues

In-vitro studies have demonstrated that areca extracts containing arecoline inhibit growth and attachment of, and suppress protein synthesis in human cultured periodontal fibroblasts (Chang, M.-C. *et al*., 1998; Jeng *et al*., 1999b). Others have confirmed that areca-nut extracts also cause growth inhibition (van Wyk *et al*., 1996) and are toxic to

human fibroblasts at a concentration of 300–500 μg/mL, leading to cell death (van Wyk *et al*., 1994).

Arecoline (0–200 μg/mL) depleted the intracellular thiols of human periodontal ligament fibroblasts *in vitro* in a dose-dependent manner. At concentrations of 25 and 100 μg/mL, depletion was approximately 18% and 56% ($p < 0.05$), respectively (Chang, Y.-C. *et al*., 2001b).

Components of areca nut stimulate the release of inflammatory mediators such as PGE_2, interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α)) from primary cultured human oral keratinocytes. Co-incubation with aspirin, specific IL-6- or TNF-α-neutralizing antibodies does not protect the cells against areca-nut cytotoxicity, cell cycle arrest or apoptosis (Jeng *et al.*, 2000, 2003).

Buccal mucosa

Early in-vitro studies showed that areca-nut alkaloids such as arecoline and arecaidine may stimulate proliferation and collagen synthesis in human cultured fibroblasts (Canniff & Harvey, 1981; Harvey *et al.*, 1986), whereas in subsequent studies, arecoline did not have similar effects on cultured oral fibroblasts (Meghji *et al.*, 1987; Jeng *et al.*, 1994a; van Wyk *et al.*, 1995). Furthermore, recent studies have shown that arecoline inhibits collagen synthesis and fibroblast proliferation *in vitro*, suggesting that it may have cytotoxic properties (Jeng *et al.*, 1994a, 1996; van Wyk *et al*., 1996), and others have confirmed that areca-nut extracts also cause growth inhibition (Jeng *et al.*, 1994a; van Wyk *et al*., 1996), and are toxic to human fibroblasts at a concentration of 300–500 μg/mL, leading to cell death (van Wyk *et al*., 1994). [The Working Group noted the inconsistent results between experiments.]

Flavanoids within the areca nut have been shown to increase the stabilization of collagen by enhancing its cross-linking, thereby increasing resistance to degradation by collagenase (Scutt *et al.*, 1987). In addition, arecoline produces dose-dependent increases in TIMP-1 expression directly in human buccal fibroblasts together with reduced secretion of both MMP-2 and MMP-9 (Chang, Y.-C. *et al*., 2002a), a phenomenon that contributes to risk for submucosal fibrosis. Tsai *et al.* (1999) further found that fibroblasts cultured from human oral submucous fibrosis tissues showed a lower capacity to phagocytose collagen-coated beads than fibroblasts from healthy buccal mucosa, and that pretreatment with arecoline and arecaidine (10 and 50 μg/mL) markedly inhibited this phagocytosis.

Exposure of human buccal fibroblasts to arecoline stimulates the expression of vimentin (25–400 μg/mL) (Chang, Y.-C. *et al*., 2002b) and inhibits GST activity (50–400 μg/mL) but shows little effect on lipid peroxidation (25–200 μg/mL) (Chang, Y.-C. *et al.*, 2001a).

Lysyl oxidase activity and basal collagen synthesis of fibroblasts cultured from oral submucous fibrosis patients are greater than those of fibroblasts from healthy buccal mucosa (Meghji *et al.*, 1987; Ma *et al.*, 1995). Production of type I collagen trimer (digested with difficulty by collagenase) by oral submucous fibrosis fibroblasts but not normal fibroblasts has also been reported (Kuo *et al*., 1995a).

(ii) *Effects on cultured human buccal epithelial cells*

Cell survival and DNA repair capacity

Exposure of buccal cell cultures to various organic or water-based extracts of products related to the use of tobacco and betel quid (bidi-smoke condensate, betel leaf, snuff, areca nut) decreased both cell survival (measured by reduction of tetrazolium dye) and activity of O^6-methylguanine–DNA methyltransferase (MGMT), enzyme that catalyses the repair of the premutagenic O^6-methylguanine lesion in DNA. Organic extracts of bidi-smoke condensate or betel leaf showed higher potency than those of tobacco or snuff. An aqueous snuff extract also decreased both parameters, whereas an aqueous areca-nut extract had no effect. While significant MGMT activity was demonstrated in buccal tissue specimens and in the major buccal mucosal cell types *in vitro*, inhibition of MGMT activity was observed in the buccal mucosa of tobacco and areca-nut chewers after exposure to complex mixtures present in the saliva (Liu *et al.*, 1997).

Cell survival, membrane integrity and DNA breakage

The pathobiological effects of an aqueous areca-nut extract, four areca-nut alkaloids and four areca-specific nitrosamines were investigated in cultured human buccal epithelial cells. Areca-nut extract decreased cell survival, vital dye accumulation and membrane integrity in a dose-dependent manner. Depletion of cellular free low-molecular-weight thiols also occurred, but at fairly toxic concentrations. Comparison of the areca nut-related *N*-nitroso compounds and their precursor alkaloids, at concentrations up to 5 mM, indicated that, on a molar basis, MNPA is the most potent at decreasing both cell survival and thiol content. Arecoline, guvacoline and NGL also decreased cell survival and thiol content, whereas arecaidine, guvacine, NGC and MNPN had only minor effects on these variables (Sundqvist *et al.*, 1989).

Cell growth, differentiation and morphology

The effects of an aqueous extract of areca nut on growth, differentiation, morphology and DNA damage were studied in cultured human buccal epithelial cells. Acute exposure (3 h) of the cells to the extract altered their morphology and induced ridges in the plasma membrane, with indications of internalization of extract particles. It also caused formation of DNA single-strand breaks, which accumulated during post-treatment culture, indicating continuous exposure to residual particles and/or the possibility of inhibited DNA repair. The extract accelerated terminal differentiation of the cells, measured as involucrin expression, at relatively non-toxic levels. The extract caused similar loss of colony-forming efficiency in normal cells and in a buccal carcinoma cell line (SqCC/YI), which was defective in its ability to undergo differentiation, indicating that extract toxicity could occur independently of this response. These findings *in vitro* suggest that betel-quid carcinogenesis in the human oral cavity may involve cytopathic alterations of normal cell morphology, growth and differentiation, by areca-nut-related agents extracted or formed in saliva (Sundqvist & Grafström, 1992).

(iii) *Effects on the cardiovascular system*

Exposure of isolated rat aorta to areca-nut extract, areca tannin or arecoline induces vasodilatation (Goto *et al.*, 1997). Activation of muscarinic receptors by arecaidine propargyl ester induced 6-keto-$PGF_{1\alpha}$ and cGMP production in bovine aortic endothelial cells, but not in rabbit vascular aortic smooth muscle cells (Jaiswal *et al.*, 1991).

Areca-nut extract stimulated rabbit platelet aggregation and thromboxane B_2 synthesis, which was inhibited by 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, an intracellular calcium chelator, and neomycin, a phospholipase C inhibitor. Areca nut-induced platelet thromboxane production can be also inhibited by catalase, dimethylthiourea, two specific scavengers of reactive oxygen species and genistein (a tyrosine kinase inhibitor), indicating the participation of H_2O_2, hydroxyl radicals and tyrosine kinase activation. On the contrary, aqueous extracts of betel leaf and *Piper betle* inflorescence inhibited the aggregation and thromboxane synthesis of rabbit platelets. In addition, betel-leaf extract inhibited thromboxane B_2 production in platelet aggregates induced by collagen, arachidonic acid, thrombin or platelet activator factor (Jeng *et al*., 2002; Lei *et al*., 2003), but had no effect on areca nut-induced platelet aggregation (Jeng *et al*., 2002).

(iv) *Effects on the gastrointestinal system*

The aqueous fraction of betel-leaf extract (1–10 mg/mL) induced a spasmogenic effect in isolated guinea pig ileum, which was inhibited by atropine, a muscarinic receptor antagonist, and inhibited the spontaneous contraction of isolated rabbit jejunum at concentrations ranging from 0.03 to 3 mg/mL, indicating that it contains a potent spasmolytic component (Gilani *et al*., 2000).

Extracts of areca nut [10–1000 mg/mL] stimulated the contractile frequency of isolated colonic smooth muscle strips in rats dose dependently. This effect was partially inhibited by atropine (Xie *et al.*, 2002).

(v) *Effects on the immune system*

Betel quid

Yang *et al*. (1979) studied the effect of areca-nut extract on the proliferation of phytohaemagglutinin-stimulated cultured human lymphocytes. At concentrations up to 2.5%, the extracts inhibited [^{3}H]thymidine incorporation by 10–100%, in a dose-dependent manner, demonstrating the adverse effect of areca-nut extract on cell proliferation.

Hsu *et al.* (2001) cultured peripheral blood mononuclear cells (PBMCs) from normal persons (n = 10) and betel-quid chewers with either oral submucous fibrosis (n = 10) or oral cancer (n = 10). The levels of IL-2, transforming growth factor β (TGF-β)) and interferon-γ (IFN-γ) production by peripheral blood mononuclear cells from betel-quid chewers with oral submucous fibrosis or oral cancer were lower than those from healthy individuals without these habits. Only peripheral blood mononuclear cells isolated from betel-quid chewers with oral cancer, and which were stimulated by arecoline, produced more IL-2.

Arecoline

Both dose-dependent and time-dependent cytotoxic effects were observed in spleen cells incubated with varying concentrations of arecoline. Arecoline (10^{-6}–10^{-4} M) concomitantly with concanavalin A markedly inhibited both [^{3}H]-thymidine incorporation and IL-2 production in spleen cells (Selvan *et al*., 1991).

Concomitant treatment of IL-2-dependent murine cytolytic lymphocytes with arecoline (10^{-4} M) and IL-2 decreased proliferative response up to 43% (Selvan *et al*., 1991). Arecoline at concentrations of 10^{-5} and 10^{-4} M added to spleen cells *in vitro* concomitantly with poke-weed mitogen inhibited the induced proliferative response by 17 and 21%, respectively (Selvan & Rao, 1993).

Exposure of oral KB cells to 1 and 100 μM of arecoline for 72 h stimulated IL-1β and IL-1α production and also intercellular adhesion molecule-1 expression (Cheng & Tsai, 1999). A 72-h exposure of KB cells to arecoline (1 and 100 μM) also stimulated IL-8 production, which was decreased by the addition of IL-1α and IL-1β antibodies, suggesting that IL-8 secretion by KB cells may be partially mediated by IL-1 (Cheng *et al.*, 2000).

(vi) *Other effects*

Betel-quid components increased basal adrenal medullary catecholamine secretion in bovine chromaffin cells isolated from the adrenal glands but inhibited that induced by carbachol or potassium *in vitro* (Wang & Hwang, 1997).

4.3 Reproductive and developmental effects

4.3.1 *Humans*

The mean weight of newborn babies of 70 Indian tobacco chewers (the tobacco was either chewed or ingested alone or mixed with betel leaf or with lime) was 14% less than that of the babies of 70 matched controls (Verma *et al*., 1983).

de Costa and Griew (1982) carried out a study to determine whether betel chewing had any adverse effect on pregnancy outcome. The antenatal records of 400 Papua New Guinean women who had chewed betel quid throughout pregnancy were examined together with a control group of 400 pregnant women who did not smoke, drink or take any drugs and who had never chewed betel quid. At birth, body weight of the babies, stillbirths, neonatal deaths and abnormalities, if present, were recorded. Babies born to betel-chewing mothers had a mean weight of 2998.5 ± 492.5 g (SD), and non-chewing mothers gave birth to babies with a mean weight of 3079.5 g ± 464.1 g (SD); the difference was statistically significant ($0.02 > p < 0.01$). There were no outstanding differences between the two groups with regard to the occurrence of congenital abnormalities. Perinatal mortality rates were 25/1000 and 27.5/1000, respectively, in chewing and non-chewing groups. [The Working Group questioned the statistics applied.]

Betel-quid chewing is very common among the aboriginal tribes in southern Taiwan, China, and many women consume betel quid throughout pregnancy. Yang *et al.* (1999)

conducted a study to estimate the prevalence of substance use among aborigines during pregnancy, and to assess the extent of the adverse effects of betel-quid chewing on pregnancy outcomes. Betel quid consisted of areca nut, slaked lime and a piece of unripe fruit of Piper betle that contains safrole. Women of the Bunum tribe of aborigines, aged 15–50 years, were asked to participate in the study, and a group of 186 women was recruited consisting of 62 subjects who had experienced adverse pregnancy outcomes and 124 age-matched women who had had normal pregnancy outcomes (control group). The participants were interviewed using a questionnaire. Prevalence of the use of various substances in aborigines with adverse pregnancy outcomes was estimated as follows: alcohol, 43.6%; smoking, 14.5%; betel-quid chewing, 43.6%; and drug use, 4.8%. In the control group, the prevalence was: alcohol, 38.7%; smoking, 8.1%; betel-quid chewing, 28.2%; and drug use, 0%. Univariate analysis revealed that adverse pregnancy outcomes were associated with maternal betel-quid chewing, maternal illness during pregnancy and number of pregnancies (gravidity) experienced. After adjusting for maternal illness and number of previous pregnancies as covariates, the prevalence of adverse pregnancy outcome was computed to be 2.8-fold higher among women chewing betel quid compared with non-chewers (adjusted odds ratio, 2.8; 95% CI, 1.2–6.8). In addition to reductions in birth weight, the adverse pregnancy outcomes included spontaneous abortion, premature delivery, stillbirth and fetal malformation.

Yang, M.S. *et al.* (2001) studied adverse birth outcomes among pregnant aboriginal women who chewed betel quid. The study population comprised 32 cases and 197 controls. The betel quid consisted of three ingredients: areca nut, slaked lime and a piece of unripe fruit from *Piper betle*. A statistically significant association was found between low birth weight, pre-term birth and maternal betel-quid chewing. The mean birth weights for the neonates of betel-quid chewers and non-chewers were 3030 and 3200 g, respectively, the difference for which was statistically significant. The estimated odds ratio of adverse birth outcome was statistically significantly higher in women who were betel-quid chewers during their pregnancy (adjusted odds ratio, 5.0; 95% CI, 1.1–23.0). [The Working Group noted the limited size of the study.]

4.3.2 *Experimental systems*

(*a*) Pan masala

Mukherjee *et al.* (1991) studied the effect of one popular brand of *pan masala* on the germ cells of male mice that received 84, 420 and 840 mg/kg bw per day by intragastric intubation for 5 consecutive days. Sperm recovered from the tail of the epididymes showed significant ($p < 0.05$) increases in morphological abnormality.

Sarma *et al.* (1992) studied the effects of *pan masala* in different organs including testes in male rats. *Pan masala* was fed at dose levels of 84, 420 or 840 mg/kg bw per day for 6 months (5 days per week). Statistical evaluation of data on organ weight showed no significant difference from the control group (untreated rats) except for a decrease in absolute weight of testis ($p < 0.05$) at all dose levels.

(*b*) *Areca nut*

Sinha and Rao (1985a) investigated the effect of areca nut on the intrauterine development of mice. Extracts of processed and unprocessed varieties of areca nut (1, 3 or 5 mg) were given daily to pregnant mice from day 6 through day 15 of gestation. Animals were killed on day 17 of gestation, and fetal anomalies were assessed. Pregnant dams exposed to either variety of areca nut showed an increase in the percentage of resorption as well as dead, macerated fetuses, clearly indicating embryotoxicity of areca-nut extract. The percentage of dead fetuses per litter in all test groups was dose-dependent. Another very significant ($p < 0.01$) effect of the administration of areca-nut extract was a dose-related decrease in the average total body weight per litter, especially in the group receiving unprocessed areca nut. There was also a decrease in the ossification of coccygeal vertebrae, which was dose-dependent and was more pronounced after exposure to unprocessed areca nut.

Paul *et al.* (1996) studied the teratogenicity of processed areca-nut extract in chick embryos. Different doses (0.001, 0.1, 0.25 and 0.5 mg) were injected into the yolk sac of 4-day-old embryos and the latter were killed after 14 days of incubation. Higher doses caused dose-dependent mortality, with a significant effect at doses of 0.1, 0.25 and 0.5 mg per egg. Fetuses had reduced body size. In another experiment, Paul *et al.* (1997) injected 5-day-old chick embryos with different (0.015–0.375 mg per egg) doses of areca-nut extract. The embryos were killed after 8 days of incubation. Reduced body weight, abnormal hindlimb digits and everted viscera were prominent. Mortality rates were dose related. Paul *et al.* (1999) studied the teratogenicity of arecoline hydrobromide (0.25, 0.50, 0.75 and 1.0 mg per egg) given at 2, 3 or 4 days of incubation. On day 14 of incubation, the experiment was terminated and fetal anomalies were recorded. The rate of anomalies was greatest for embryos injected on day 2 of incubation. Developmental defects included reduced body size, sparse feathering, everted viscera, shortened lower beak and arthrogryposis. Unossified vertebrae, missing or unossified ribs, shortening of long bones and unossified phalanges also occurred.

(*c*) *Arecoline*

Anisímov (1978) studied the influence of arecoline on compensatory ovarian hypertrophy in rats. After unilateral ovariectomy, rats were given different doses (0.15, 0.5, 5.0 mg/kg bw) of arecoline for 7 days, and it was found that higher doses suppressed compensatory hypertrophy.

Sinha and Rao (1985b) investigated the influence of arecoline on the morphology of sperm in mice. Intraperitoneal injections of arecoline at doses of 20, 40 and 80 mg/kg bw was given for 5 consecutive days and mice were killed 35 days after the first injection. Sperm was recovered from the tail of the epididymes. A linear increase in the percentage of abnormal sperm was observed following exposure to arecoline, and treatment with arecoline increased unscheduled DNA synthesis response in the germ cells of mice.

Intraperitoneal administration of arecoline at three dose levels (20, 40 and 80 mg/kg bw) to Swiss albino mice on day 17 of gestation resulted in a significant increase in the frequency of micronucleated polychromatic erythrocytes in fetal mouse blood compared with that of control animals, which was found to be dose-dependent (Sinha & Rao, 1985c).

(*d*) *Stems of* Piper betle

Adhikary *et al.* (1990a,b) studied the effect of oral administration of extracts of *Piper betle* stems on the reproductive function of female and male rats. In female rats, the treatment caused disturbance in estrus cycle, inhibition of fertility in a dose-dependent manner, reduction in implantation at higher doses, reduction in ovarian and uterine weights at higher doses and a fall in Δ^5-3β-hydroxysteroid dehydrogenase activity (Adhikary *et al*., 1990a). In male rats, significant reduction in fertility and in the number and motility of sperm were observed at the higher doses, as well as a reduction in the relative weight of the testis and accessory sex organs of treated animals.

Another study by Adhikary *et al.* (1989) revealed the antigonadal property of extracts of *Piper betle* stems in rats. Following subcutaneous injection for 21 days with either extract or vehicle, males and females were allowed to mate; *Piper betle*-stem extract caused a 100% reduction in male fertility and a 63% reduction in female fertility when mated with fertile partners. A reduction in the weight of gonads and other reproductive organs was also observed. Sarkar *et al.* (2000) studied the antifertility effect of an alcoholic extract of air-dried leaf stem of *Piper betle* in male mice. The weights of the reproductive organs (testes, epididymes, seminal vesicles and prostate) of the treated animals decreased, and a decrease in the sperm count and sperm motility was also observed. Recovery experiments revealed that these effects were reversible.

[The Working Group noted that the stem is usually removed from the betel leaf before preparation of the betel quid in the Indian subcontinent.]

(*e*) *Nicotine*

There is a vast literature on the effects of nicotine on reproductive and developmental systems in experimental animals of different species and during different stages of gestation. Examples are the delay in ovum cleavage and implantation (Yoshinaga *et al.*, 1979), the interference with fetal brain differentiation and development (Slotkin *et al*., 1986; Slotkin, 1992; Slotkin *et al*., 1993) and with fetal testosterone levels (Lichtensteiger & Schlumpf, 1985), adverse effects on the adrenal glands (Monheit *et al.*, 1983), an increase in the number of stillbirths (Arbeille *et al.*, 1992) and impairment of cardiorespiratory defence to hypoxia (Hafström *et al.*, 2002). The reader is referred to the monograph on tobacco smoking (IARC, 2004) and references therein.

4.4 Genetic and related effects

4.4.1 *Humans*

(*a*) *Genotoxicity and mutagenicity*

(i) *DNA adduct*

DNA extracted from exfoliated oral mucosal cells collected from Canadian non-smoking controls (n = 19), Indian areca-nut chewers (n = 22), Filipino inverted smokers (who smoke with the burning end of the cigar in their mouth; n = 15) and Indian *Khaini* tobacco chewers (n = 22) was used for analysis of aromatic DNA adducts by the ^{32}P-post-labelling technique. Differential amounts of five aromatic DNA adducts were found within these four groups, but there were no differences among the groups (tobacco chewing, betel-quid chewing or smoking) (Dunn & Stich, 1986).

In Taiwan, China, Chen *et al*. (1999) identified several safrole-like DNA adducts from 77% (23/30) of tissues from oral squamous-cell carcinomas obtained from betel-quid chewers and 97% (29/30) of tissues from adjacent non-cancer areas, whereas no such adducts were identified in squamous-cell carcinomas obtained from non-chewers. Six of seven (86%) oral submucous fibrosis tissues obtained from betel-quid chewers also exhibited the same safrole-like DNA adducts.

(ii) *Micronuclei, chromosomal aberrations and sister chromatid exchange*

In a multicountry study, Stich *et al*. (1986) scored scraped or brushed [erroneously referred to in the literature as exfoliated] micronucleated epithelial cells from oral mucosa. The number of betel quids consumed was an average of 44 quids each day in Taiwan, China, compared with an average of 20 quids per day in India (Stich *et al*., 1982). Betel-quid chewers (n = 36) in Orissa (India) who regularly chewed dried areca nut, slaked lime, betel leaf, tobacco and catechu had the highest frequencies of micronucleated cells (6.1%), followed by those in Khasis (4.7%) (India, who chewed fresh areca nut [husk removed], slaked lime and betel leaf), the Philippines (3.9%) (fresh areca nut [husk removed], betel leaf, slaked lime and tobacco), Guam (1.8%) (fresh green areca nut with husk, slaked lime and betel leaf) and Hualien (1.7%) (Taiwan, China, fresh areca nut with husk, slaked lime and betel leaf).

Stich *et al*. (1984b,c, 1988, 1989, 1991) explored the possibility of reversal of the formation of micronuclei in the oral cavity (shown in exfoliated cells) using vitamin A and/or β-carotene. The treatment decreased the frequency of micronuclei in users of betel quid with tobacco. However, the reappearance of micronucleated cells was noted after termination of the treatment (Stich *et al.*, 1991).

Dave *et al*. (1991) compared 30 healthy controls and 15 *pan-masala* consumers with respect to cytogenetic effects in peripheral blood lymphocytes and exfoliated cells from buccal mucosa. Sister chromatid exchange and chromosomal aberrations of peripheral blood lymphocytes were more frequent in cells of *pan-masala* chewers' than in those iso-

lated from control subjects. The percentage of micronucleated cells in exfoliated buccal mucosal cells was also higher in *pan-masala* consumers than in healthy controls [population size seems too small: $n = 5$ for *pan masala* + tobacco, $n = 10$ for *pan masala*, $n = 15$ for controls]. Consistently, the frequencies of micronuclei in exfoliated mucosal cells were shown to be higher in chewers (with or without oral submucous fibrosis) of tobacco plus slaked lime, *mava* (areca nut, slaked lime and tobacco), *tamol* (raw fermented areca nut, betel leaf and slaked lime) and areca nut in different regions of India compared with healthy individuals with no habit ($n = 10$–36 for each group) (Kayal *et al*., 1993).

Nair *et al*. (1991) reported that the frequency of micronuclei in exfoliated human oral mucosa cells was 4.83 ± 0.7 per 1000 cells in chewers of betel quid with tobacco ($n = 35$; tobacco, areca nut, betel leaf, slaked lime and catechu) and 5.2 ± 0.66 per 1000 cells in chewers of tobacco and slaked lime ($n = 35$), whereas the frequency in the control group ($n = 27$) was 2.59 ± 0.37 per 1000 cells. No correlation between the frequencies of micronucleated cells and the duration or frequency of the chewing habit was noted.

Adhvaryu *et al*. (1986) compared the frequency of sister chromatid exchange in cultured lymphocytes isolated from controls ($n = 15$), tobacco chewers ($n = 10$) and oral submucous fibrosis patients ($n = 10$) who chewed a combination of tobacco, areca nut and slaked lime. Marked elevation of sister chromatid exchange frequency in lymphocytes cultured from tobacco chewers and oral submucous fibrosis patients was noted. Moreover, Adhvaryu *et al*. (1991) further analysed sister chromatid exchange and chromosomal aberrations in peripheral lymphocytes and micronuclei in exfoliated mucosal cells in healthy *mava* (tobacco, areca nut and lime) chewers, *mava* chewers with oral submucous fibrosis and *mava* chewers with oral cancer ($n = 15$ for all groups). Chromosomal aberrations and sister chromatid exchange were significantly higher for all three groups of chewers than for controls. The frequencies of micronuclei were higher in healthy chewers and oral submucous fibrosis patients. The micronucleus assay was not conducted in oral cancer patients.

Desai *et al*. (1996) further analysed the frequencies of micronuclei in exfoliated oral mucosa cells and sister chromatid exchange in lymphocytes isolated from healthy volunteers, and patients with oral submucous fibrosis, oral leukoplakia and oral lichen planus. These patients chewed *pan masala*, areca nut, areca nut plus tobacco, betel quid plus tobacco or had mixed habits. The number of micronuclei in exfoliated cells was elevated from 1.9/1000 cells (normal) to 11.6, 10.8 and 11.7/1000 cells in patients with oral submucous fibrosis, oral leukoplakia and oral lichen planus, respectively. A large number of micronucleated cells was also seen in circulating lymphocytes obtained from these three groups.

Dave *et al*. (1992a) evaluated cytogenetic effects in controls ($n = 15$), healthy areca-nut chewers ($n = 10$), areca-nut chewers with oral submucous fibrosis ($n = 10$) and areca-nut chewers with oral cancer ($n = 8$). All three groups of areca-nut chewers showed significantly large numbers of chromosomal aberrations and sister chromatid exchange in peripheral blood mononuclear cells and the frequencies of micronucleated exfoliated buccal cells increased by approximately 3.8-fold.

(*b*) *Genomic instability*

Analysis of cytogenetic changes in betel quid- and tobacco-associated oral squamous-cell carcinomas showed most common gains in chromosomes 8q, 9q, 11q, 17q and 20q and most frequent losses in chromosome arms 3p (genes *FHIT*, *RARβ* and *VHL*), 4q, 5q, 9p21–23 and 18q, a high frequency of breakage and exchanges at the 1cen-1q12 region and allelic imbalance in short tandem repeat markers (Rupa & Eastmond, 1997; Mahale & Saranath, 2000; Lin, S.-C. *et al.*, 2002; Pai *et al.*, 2002). Loss of 3p was significantly associated with poor survival of patients (Lin, S.-C. *et al.*, 2002). Lee, H.-C. *et al.* (2001) showed mitochondrial DNA (mtDNA) deletions (4977-bp deletion) in oral squamous-cell carcinomas of betel-quid chewers. This study revealed that, irrespective of the history of betel-quid chewing, the mtDNA deletions detected in oral tumours were less abundant than those in the surrounding non-tumorous tissues. Moreover, betel-quid chewing significantly enhanced the accumulation of mtDNA deletions in non-tumorous oral tissues. Collectively, these observations are consistent with the mutagenic effects of betel quid and tobacco.

(*c*) *Oncogenes and tumour-suppressor genes*

Analyses of *TP53* mutations in oral carcinomas associated only with betel-quid chewing are limited. These studies demonstrate infrequent *TP53* mutations in oral cancers in South-East Asia including Sri Lanka, Taiwan, China, and India, as well as in Papua New Guinea (summarized in Table 71) (Chiba *et al*., 1998; Hsieh *et al*., 2001).

No *TP53* mutations were observed in oral leukoplakia or squamous-cell carcinomas of chewers of betel quid without tobacco ($n = 6$) in Indian populations (Heinzel *et al.*, 1996; Ralhan *et al.*, 2001), or in 48 cases of oral tumour from eastern India in chewers of betel quid with tobacco (Patnaik *et al.*, 1999). However, in Sri Lankan populations, Chiba *et al.* (1998) reported *TP53* mutations in 10/23 (43%) oral squamous-cell carcinomas in betel-quid chewers. [The Working Group noted that 7/10 cases harbouring *TP53* mutations had been betel-quid chewers and smokers for 10–30 years.]

In oral squamous-cell carcinomas associated with betel-quid chewing and tobacco smoking, *TP53* mutations were clustered in exons 5, 7 and 8 comprising A:T→T:A transversions or G:C transitions, G:C→A:T transitions and G:C→T:A transversions (Table 71) (Thomas *et al.*, 1994; Heinzel *et al*., 1996; Chiba *et al.*, 1998; Wong *et al*., 1998; Hsieh *et al.*, 2001; Ralhan *et al.*, 2001). In addition to point mutations in G + C-rich regions, small deletions or insertions were also observed (Chiba *et al.*, 1998). The G:C→C:G transversions were observed in betel-quid chewers or smokers, and codons 135 and 136 were frequently mutated (G→T; A→G) in oral squamous-cell carcinomas associated with betel-quid and tobacco consumption (Chiba *et al.*, 1998; Hsieh *et al.*, 2001; Ralhan *et al.*, 2001).

TP53 mutations were reported in 8/36 (22%) Taiwanese betel quid/tobacco smoking-associated oral squamous-cell carcinomas (Wong *et al.*, 1998), and in oral preneoplastic lesions (leukoplakia) in betel-quid and tobacco consumers (Ralhan *et al.*, 2001).

Table 71. Analysis of *TP53* alterations in human oral premalignant and malignant lesions

Total no. of cases	Exons analysed	*TP53* mutations	Population	Betel quid without tobacco		Betel quid + tobacco smoking/chewing		Betel quid + tobacco smoking/chewing + alcohol		*TP53* mutational analysis	Reference
				Total cases	*TP53* mutation cases	Total cases	*TP53* mutation cases	Total cases	*TP53* mutation cases		
5 SCCs	5–9	0	Sri Lankan	–	–	5	0	–	–	ND	Ranasinghe *et al.* (1993a)
30 SCCs	5–9	3	Papua-New Guinean	–	–	5[a]	1	17[a]	2	Exon 6 codon 193 (C→T) (2 cases) Exon 7 codon 248 (G→A)	Thomas *et al.* (1994)
23 SCCs	5, 7	4[b]	Indian	6	0	2	1	–	–	Exon 5 codon 153 (C→T) (non-user) codon 158 (G→A) smoker codon 176 (T→C) (unknown) Exon 7 codon 239 (A→G) (betel-quid chewer + smoker)	Heinzel *et al.* (1996)
23 SCCs 7 leukoplakia 2 OSF	5–8	10 0 0	Sri Lankan	9 2	3 ND	14 4 2	7 ND ND	– – –	– – –	Exon 5 codon 130 C deletion codon 135 (G→T) codons 144–148 deletion (2 cases) codon 164 (G→T) codons 172–187 deletion codon 176 (C→G) Exon 7 codon 245 (G→A) codon 248 (G→A) codon 250 C insertion	Chiba *et al.* (1998)

Table 71 (contd)

Total no. of cases	Exons ana-lysed	*TP53* mutations	Population	Betel quid without tobacco		Betel quid + tobacco smoking/chewing		Betel quid + tobacco smoking/chewing + alcohol		*TP53* mutational analysis	Reference
				Total cases	*TP53* mutation cases	Total cases	*TP53* mutation cases	Total cases	*TP53* mutation cases		
50 SCCs	5–9	12	Taiwanese	–	–	36	8	–	–	Exon 5 codon 161 (C→A) Exon 5 codon 175 (G→A) Exon 6 codon 222 (C→G) Exon 7 codon 255 (A→T) Exon 8 codon 266 (G→T) Exon 8 codon 273 (C→T), 273 (G→T) Exon 8 codon 277 (G→A) (2 cases), 277 (T→C)	Wong *et al.* (1998)
						1				Exon 8 codon 282 (C→T) (2 cases)	
				–	–	14	3	–	–	Allelic deletion of *TP53*	
37 SCCs	5–8	2	Taiwanese	3	–	23	2 (1 smoker, + 1 non-user)	–	–	Exon 5 codon 177 (C→T) Exon 8 codon 266 (G→A)	Kuo *et al.* (1999a)
48 oral tumours	5–8	0	Indian	–	–	–	–	48[c]	0	Rearrangement	Patnaik *et al.* (1999)
187 SCCs	5–9	91	Taiwanese	7	4	51	15	75	43	Predominantly GC→ AT in betel-quid chewers alone Exon 5 codon 135 (G→T) Exon 6 codon 195 deletion Exon 8 codon 267 (G→C) codon 273 (C→T)	Hsieh *et al.* (2001)

Table 71 (contd)

Total no. of cases	Exons ana-lysed	*TP53* mutations	Population	Betel quid without tobacco		Betel quid + tobacco smoking/chewing		Betel quid + tobacco smoking/chewing + alcohol		*TP53* mutational analysis	Reference
				Total cases	*TP53* mutation cases	Total cases	*TP53* mutation cases	Total cases	*TP53* mutation cases		
30 SCCs	5–9	7	Indian	6	ND	12	8	–	–	Predominantly missense (5) Exon 5 codons 126 (C→G), 136 (A→G), 174 (G→T) Exon 7 codons 233 (CA→TC), 234 (TAC→AAA) Exon 8 codon 267 (G→C), 270 (T→A)	Ralhan *et al.* (2001)
30 leukoplakia		5		6	ND	13	6	–	–	Predominantly missense (3 missense, 2 nonsense) Exon 5 codons 126 (C→G), 136 (A→G) (2 cases), 174 (G→T) Exon 6 codon 196 (C→T)	

SCC, squamous-cell carcinoma; ND, not detected; OSF, oral submucous fibrosis

[a] Includes four former alcohol consumers

[b] Patients with *TP53* mutations: one was a non-user and data on betel/tobacco habit were not available for the other case.

[c] Patients were chewers of betel quid with tobacco and tobacco paste; no information about alcohol consumption was available.

Hsieh *et al*. (2003) reported that polymorphism in the DNA repair gene *XRCCI,399 Gln/Gln* phenotype showed an independent association with the frequency of *TP53* mutations (after adjustment for smoking, areca-quid chewing and alcohol drinking) in oral cancer patients in Taiwan, China. [The Working Group noted that, in absence of specific information on betel quid and tobacco consumption habits of oral squamous-cell carcinoma patients, the data from the reports of Chang *et al.* (1992), Munirajan *et al.* (1996), Ravi *et al.* (1996, 1999), Saranath *et al.* (1999), Tandle *et al.* (2001), Nagpal *et al.* (2002a) and Pande *et al*. (2002) were not included.]

Several studies reported a high incidence of p53 protein expression in oral premalignant lesions and squamous-cell carcinomas from betel quid and/or tobacco consumers (summarized in Table 72).

Comparison of p53 protein expression in 22 baseline biopsies of oral precancerous lesions that transformed to cancer 4–25 years later with that in 68 similar lesions that did not transform over the same period did not show a significant relationship between p53 protein expression and malignant transformation (Murti *et al.*, 1998). All cancers were detected among betel-quid chewers who included tobacco in their quid and/or smoked. However, the acquisition of p53 protein expression in 9/10 biopsies that did not show p53 expression at baseline occurred once they had undergone progression to squamous-cell carcinoma.

Cox and Walker (1996) reported p53 protein accumulation in 14/20 (70%) and proliferative cell nuclear antigen (PCNA) in 31.8% of Nepalese oral submucous fibrosis patients. Trivedy *et al*. (1998) reported p53 protein expression in 15/20 (75%) oral submucous fibrosis cases, 3/6 (50%) squamous-cell carcinomas arising from oral submucous fibrosis and 15/21 (67%) squamous-cell carcinomas not arising from this disease. Alterations in *TP53* were reported in 13/21 cases of oral submucous fibrosis and 15/27 cases of squamous-cell carcinoma, showing a concordance between results from immunocytochemistry and single-strand conformation polymorphism results in a majority (33/48) of cases. Chiang *et al*. (2000a,b) reported a significant increase in PCNA and p53 expression in oral submucous fibrosis, epithelial hyperplasia and epithelial dysplasia compared with normal oral mucosa in a population in Taiwan, China, population. However, no association was observed between PCNA and p53 expression in oral submucous fibrosis lesions associated with areca chewing and tobacco smoking. Srinivasan and Jewell (2001a) showed higher expression of the proliferation markers, epidermal growth factor receptor, TGF-α, PCNA and one genomic marker, *c-myc*, in oral submucous fibrosis lesions of betel quid and tobacco consumers compared with normal oral mucosa (Srinivasan & Jewell, 2001b). [The Working Group noted the absence of detailed information on betel quid and tobacco consumption habits of patients in these studies.]

Thongsuksai and Boonyaphiphat (2001) showed no independent association of betel-quid chewing with p53 expression. Moreover, the lack of correspondence between p53 protein expression and *TP53* mutations (Ranasinghe *et al.*, 1993a,b) suggests other pathways of p53 inactivation. The pathways identified include interaction of p53 with other cellular proteins: murine double minute 2 (MDM2), 70-kDa heat shock protein (HSP70)

Table 72. Analysis of p53 protein expression in human oral premalignant and malignant lesions

Total cases	Total p53-positive cases	Population	Betel quid without tobacco		Betel quid + tobacco smoking/chewing		Reference
			Total cases	p53-Positive cases	Total cases	p53-Positive cases	
34 SCCs	25	Indian	5	3	14	13	Kaur *et al.* (1994)
27 leukoplakia	15		3	1	9	9	
30 SCCs		Papua New Guinean	–	–	21[a]	5	Thomas *et al.* (1994)
23 SCCs	13	Indian	–	–	20	13	Kuttan *et al.* (1995)
60 SCCs	27	Taiwanese	44[b]	17	–	–	Yan *et al.* (1996)
145 SCCs	102	Indian	12	5	59	51	Kaur *et al.* (1998a)
75 leukoplakia	39		8	2	33	22	
48 SCCs	22	Indian	–	–	48 (TC)[c]	22	Baral *et al.* (1998)
81 SCCs	47	Taiwanese	1	1	56	29	Chiang *et al.* (1999)
38 SCCs	4	Sri Lankan	–	–	38	4	Ranasinghe *et al.* (1993b)
50 oral submucous fibrosis	30	Taiwanese	–	–	50	30	Chiang *et al.* (2000a)
10 hyperplasia	4		–	–	10	4	
10 dysplasia	4		–	–	10	7	
10 normal mucosa	0		–	–	3[d]	0	
106 SCCs	74	Thai	64	42	–	–	Kerdpon *et al.* (2001)

Table 72 (contd)

Total cases	Total p53-positive cases	Population	Betel quid without tobacco		Betel quid + tobacco smoking/chewing		Reference
			Total cases	p53-Positive cases	Total cases	p53-Positive cases	
156 SCCs	58[e]	Thai	29	11	36	9	Thongsuksai & Boonyaphiphat (2001)
232 OELs		Taiwanese					Chang, K.-C. *et al.* (2002)
25 verrucous hyperplasia[f]	0		23	0	–	–	
13 dysplasia	3		10	3	–	–	
6 verrucous carcinoma[g]	0		5	0	–	–	
7 epithelial hyperplasia	1		7	1	–	–	
10 keratosis	1		9	1	–	–	
10 squamous papillomas	0		10	0	–	–	
5 verruca vulgaris	0		5	0	–	–	
104 SCCs	37[h]		87	33	–	–	

SCC, squamous-cell carcinoma; ND, not detected; OEL, oral epithelial lesion

[a] Include 17 alcohol consumers

[b] Some of the patients were smokers, data not available

[c] TC, tobacco chewers

[d] Smokers only

[e] Data on other habits not included here

[f] Betel quid/tobacco habit data unknown for two cases

[g] Betel quid/tobacco habit data unknown for one case

[h] Four p53-positive cases were SCC unrelated to betel quid.

or E6 protein of human papilloma virus (HPV) (Kaur & Ralhan, 1995; Agarwal *et al.*, 1999; Nagpal *et al.*, 2002b).

[The Working Group noted the lack of information on specific *TP53* mutations associated with the habit of chewing betel quid without tobacco.]

High incidences of H-*ras* mutations (codons 12, 13 or 61) and loss of allelic heterozygosity were reported in oral squamous-cell carcinomas in Indian populations in comparison with populations in the West (Saranath *et al.*, 1991a,b; Munirajan *et al*., 1998). [The Working Group noted that betel-quid chewing with or without tobacco is a common habit in this population. However, detailed data on chewing habits were not given in these studies.] *Ki-ras* codon 12 mutations or p21 ras protein accumulation have been observed in areca-quid chewing- and tobacco smoking-related oral squamous-cell carcinomas in a population in Taiwan, China (Kuo *et al.*, 1994, 1995b).

Mutations and/or alterations in the expression of cancer-related genes (*MTSI*/p16, pRb, *FHIT*, *APC*, H-*ras*, Ki-*ras*, cyclin D1, MDM2, c-myc, $p21^{WAF1}$, Stat-3, $p27^{kip1}$, Bcl-2, Ets-1, RARβ, RARα and HSP70) associated with betel-quid chewing with or without tobacco have been observed in oral squamous-cell carcinomas (summarized in Table 73 and Figure 8).

A high prevalence of HPV-16 infection in 13/17 (76.4%) oral squamous-cell carcinomas was reported in betel-quid chewers and smokers in a Taiwanese population (Chang *et al*., 1989). A high prevalence of HPV-18 in addition to HPV-16 was also reported in 67/91 (74%) oral squamous-cell carcinomas in betel-quid chewers and smokers in an Indian population (Balaram *et al*., 1995). A lower prevalence of HPV infection (37/110; 33.6%) was observed in oral squamous-cell carcinomas associated with reduced frequency of Pro/Pro allele frequency at codon 72 of *TP53* in an eastern Indian population consuming betel quid and chewing tobacco (Nagpal *et al*., 2002a).

(*d*) *Polymorphism in carcinogen-metabolizing enzymes*

Several isozymes of cytochrome P450 (CYP) are involved in the metabolic activation of polycyclic aromatic hydrocarbons and nitrosamines, while phase II enzymes such as GST are predominantly involved in detoxification. Numerous alleles that cause defective, qualitatively altered, diminished or enhanced rates of drug metabolism have been identified for many of the phase I and phase II enzymes and can result in marked interindividual differences in carcinogen metabolism. The underlying molecular mechanisms of a number of these genetic polymorphisms have been elucidated (reviewed in Vineis *et al*., 1999; Nair & Bartsch, 2001).

(i) *Oral cancer and oral lesions* (Table 74)

The association between *CYP2E1*, *GSTM1* and *GSTT1* polymorphisms and oral cancer was reported in Taiwan, China, in a small case–control study of 41 male oral cancer cases from the National Taiwan University Hospital and 123 healthy controls taken among residents living in Taipei City and Taipei County and frequency-matched for ethnicity, sex and age (Hung *et al*., 1997). Cigarette smoking, alcohol drinking and betel-quid

Table 73. Analysis of cancer-related genes/proteins in premalignant and malignant oral lesions

Gene/protein	Population	Total cases	Total altered	Betel quid without tobacco		Betel quid + tobacco smokers/chewers		Alteration	Reference
				Total cases	Cases altered	Total cases	Cases altered		
MTSI/p16	Taiwanese	110 SCCs	7	–	–	77	7	Mutations exon 2 intron 1/exon 2 splice site	Lin *et al.* (2000a)
			15			56	15	Methylation of promoter region exon 1	
MTSI/p16	Indian	23 SCCs	3	6	–	2	1	2 mutations exon 2, 1 deletion	Heinzel *et al.* (1996)
p16	Indian	23 SCCs	22	–	–	31	22	Loss of p16 expression	Pande *et al.* (1998)
		22 leukoplakia	13	–	–	19	13		
pRb	Indian	35 SCCs	23	–	–	31	23	Loss of pRb expression	Pande *et al*. (1998)
		22 leukoplakia	14	–	–	19	14		
FHIT (exon 3–10)	Taiwanese	39 SCCs	3	–	–	39	3	Deletion/mutation	Chang, K.W. *et al.* (2002)
			8			29	8	Promoter methylation	
			11			31	11	Aberrant transcription	
			26			63	26	Abnormal immunoreactivity	
APC (codon 279–1673)	Taiwanese	40 SCCs	6	6	–	21	6	5 missense mutations 1402 G→A 1367 A→G 1382 T→C 1352 T→C 1652 C→T 1 base pair deletion 1593 A<u>A</u>GC→AGC	Kok *et al.* (2002)
H-*ras*	Indian	57 SCCs	20	–	–	57	20	Mutations Codon 12 G→T, G→A Codon 13 G→A Codon 61 A→G, A→T, G→T	Saranath *et al*. (1991a)

Table 73 (contd)

Gene/protein	Population	Total cases	Total altered	Betel quid without tobacco		Betel quid + tobacco smokers/chewers		Alteration	Reference
				Total cases	Cases altered	Total cases	Cases altered		
Ki-*ras*	Taiwanese	33 SCCs	6	28	6	–	–	Codon 12 G→A, G→T	Kuo *et al.* (1994)
Ki-*ras*	Taiwanese	51 SCCs[a]	47	32	32	47	47	p21 ras expression	Kuo *et al.* (1995b)
		4 dysplasia	4	–	–	4	4		
		7 hyperplasia	7	–	–	7	7		
		6 normal	6	–	–	6	1		
Cyclin D1	Taiwanese	88 SCCs	73	1	1	62	54	Overexpression	Kuo *et al.* (1999b)
MDM2	Indian	65 SCCs	51	–	–	50	40	Overexpression	Agarwal *et al.* (1999)
		33 leukoplakia	17	–	–	27	15		
MDM2 and p53	Indian	65 SCCs	39	–	–	50	31	Overexpression	Agarwal *et al.* (1999)
		33 leukoplakia	16	–	–	27	14		
MDM2	Taiwanese	52SCCs	36	1	1	31	25	Overexpression	Huang *et al.* (2001)
MDM2	Japanese	40 SCCs	29	–	–	40	29	Overexpression	Shwe *et al.* (2001)
MDM2 and p53	Japanese	40 SCCs	25	–	–	40	25	Overexpression	Shwe *et al.* (2001)
c-myc	Indian	48 SCCs	27	–	–	48 (TC)[b]	27	Overexpression	Baral *et al.* (1998)
c-myc and p53	Indian	48 SCCs	9	–	–	48 (TC)[b]	9	Overexpression	Baral *et al.* (1998)
$p21^{WAF\ 1}$	Taiwanese	43 SCCs	31	1	1	29	20	Overexpression	Kuo *et al.* (2002a)
Stat-3	Indian	90 SCCs	74	–	–	90 (TC)[b]	74	Overexpression	Nagpal *et al.* (2002b)
$p27^{Kip\ 1}$	Taiwanese	63 SCCs	47	3	2	44	33	Loss of expression	Kuo *et al.* (2002b)
Bcl-2	Indian	87 SCCs	87	–	–	87	87	Overexpression	Ravi *et al.* (1999)
		38 leukoplakia	15	–	–	38	15		
Ets-1	Indian	100 SCCs	62	14	8	32	25	Overexpression	Pande *et al.* (2001)

Table 73 (contd)

Gene/protein	Population	Total cases	Total altered	Betel quid without tobacco		Betel quid + tobacco smokers/chewers		Alteration	Reference
				Total cases	Cases altered	Total cases	Cases altered		
RARβ	Taiwanese	38 SCCs[c]	16	25	13	34	–	Loss of expression	Kao *et al.* (2002a)
RARβ	Indian	64 SCCs	18	–	–	64	18	Overexpression	Chakravarti *et al.* (2001)
		45 leukoplakia	18	–	–	45	18		
RARα	Indian	64 SCCs	43	–	–	64	43	Overexpression	Chakravarti *et al.* (2001)
		45 leukoplakia	18	–	–	45	18		
RARα	Indian	115 SCCs	67	26	14	–	–	Overexpression	Chakravarti *et al.* (2003)
HSP70	Indian	125 SCC	92	9	4	52	48	Overexpression	Kaur *et al*. (1998b)
		64 leukoplakia	38	6	2	28	21		

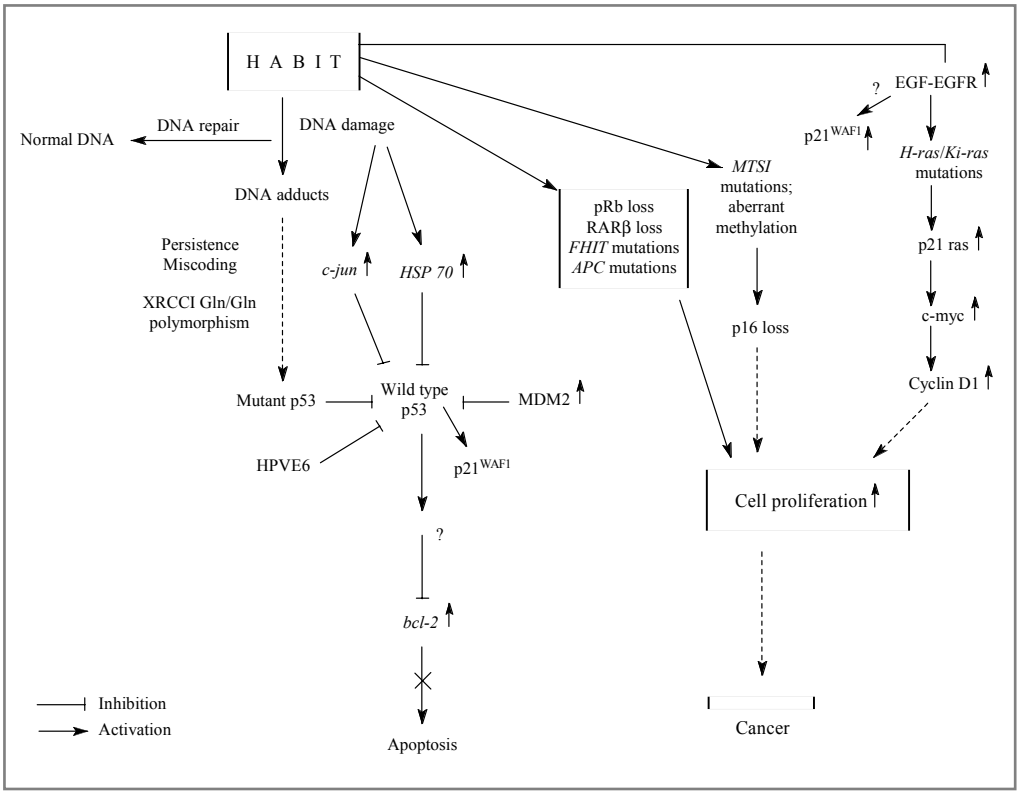
SCC, squamous-cell carcinoma

[a] Some chewers of betel quid were also tobacco smokers; detailed habit data not available; four SCC patients with negative p21 ras staining were nonsmokers and non-chewers of betel quid

[b] TC, tobacco chewers

[c] Data on betel quid users only were included.

Figure 8. Molecular targets affected by chewing betel quid with tobacco, often in combination with smoking



[The Working Group noted that in almost all cases, betel-quid chewing was combined with smoking.]

chewing were significantly associated with the risk for oral cancer and a dose–response relationship was observed. All betel-quid chewers also smoked cigarettes (30 cases and 15 controls). In the multiple logistic regression analysis, individuals with null genotypes of *GSTM1* and/or *GSTT1* had an increased risk for oral cancer compared with those who had non-null genotypes of both *GSTM1* and *GSTT1*, showing a multivariate-adjusted odds ratio of 4.6 (95% CI, 0.9–23.7). The *CYP2E1 c1/c2* and *c2/c2* genotypes were associated with a significantly increased risk for oral cancer compared with the *c1/c1* genotype among those who did not chew betel quid (odds ratio, 4.7; 95% CI, 1.1–20.2). This association was not observed among betel-quid chewers. [However, no adjustment was made in this study for cigarette smoking as a potential confounder as all betel-quid chewers were also smokers. Therefore, the relative importance of betel-quid chewing and smoking as risks for oral cancer could not be determined in this study.]

The effect of genetic variants of *GSTM1* and *GSTT1* on modifying the risk for oral leukoplakia was ascertained in genomic DNA from biopsies taken from 98 oral leuko-

Table 74. Genetic polymorphism and oral cancer

Gene	Mutation/allele	Country/ethnicity	No. of cases/controls	Significance: odds ratio (95% CI)	Comments	Site	Reference
CYP2E1	RsaI	Taiwan Chinese: 71% Fukienese 7% Hakka 22% mainland	41/123	NS: *c1/c2+c2/c2* vs *c1/c1* Among non-chewers only, S: 4.7 (1.1–20.2) *n* = 7/40	Cases all men, age and ethinicity adjusted	Oral cancer	Hung *et al*. (1997)
GSTM1 *GSTT1*	Gene deletion			NS: *GSTM1* and/or *GSTT1*	NS: combination with *GSTM1* and /or *GSTT1*	Oral cancer	Hung *et al*. (1997)
GSTM1 *GSTT1*	Gene deletion	India	98/82	S*: GSTM1* null, 22 (10–47) S: *GSTT1* null, 11 (5–21)	Association significantly higher for non-homogenous than homogenous leukoplakia	Oral leukoplakia	Nair *et al*. (1999)
GSTM1 *GSTT1*	Gene deletion	Thailand	53/53	S: *GSTM*1 null, 2.6 (1.04–6.5) NS: *GSTT1* null		Oral cancer	Kietthubthew *et al*. (2001)
CYP1A1	*MspI* RFLP , *2A Exon 7 *Ile/Val*, *2C	Taipei (Taiwan)	166/146 (106 SCC, 60 OPL)	S: *ile/val* vs *ile/ile* OSCC, 5.1 (2.6–9.76); OPL, 2.67 (1.32–5.4) *val/val* vs *ile/ile* SCC, 18.86 (3.61–98.52); OPL, 15.23 (2.8–83.98) NS: *MspI* RLFP genotype A, B or C	Predominantly men No statistically significant difference in genotypes A (*m1/m1*), B (*m1/m2*) or C (*m2/m2*) between cases and controls	Oral cancer and oral premalignant lesions	Kao *et al*. (2002b)
CYP2A6	Wild-type *1A Conversion type *1B Gene deletion *4C	Sri Lanka	286/135 (15 SCC, 62 OSF, 209 leukoplakia)	S: *1B/ *4C vs *1A/ *1A, 0.2 (0.05–0.88) *4C/ *4C vs *1A/ *1A, 0.14 (0.03–0.72)	Hospital controls Only 5% SCC cases	Oral lesions	Topcu *et al*. (2002)
GSTM1	Gene deletion			NS: *GSTM1* null	S: Combination of *CYP2A6* deletion allele and *GSTM1* null		Topcu *et al*. (2002)

CI, confidence interval; S, significant; NS, not significant; SCC, oral squamous-cell carcinoma; OPL, oral precancerous lesion; OSF, oral submucous fibrosis

plakia patients and exfoliated cells from 82 healthy controls from India (Nair *et al*., 1999). Most leukoplakia cases were heavy chewers (betel quid with or without tobacco; 15–20 quids per day), whereas the chewers among controls were regular but not heavy chewers (betel quid with or without tobacco; 1–2 quids per day). *GSTM1*-null genotype was found in 81.6% of cases compared with 17% of controls and was significantly associated with risk for oral leukoplakia [odds ratio, 22; 95% CI, 10–47]. Similarly, *GSTT1*-null genotype was found in 75.5% of cases and 22% of controls and was also significantly associated with risk for leukoplakia (odds ratio, 11; 95% CI, 5–22). Combined null genotypes of *GSTM1* and *GSTT1* prevailed in 60.2% of the cases with none detected in controls. Although the low prevalence of null genotypes in the control population may have contributed to the high relative risks observed, this study suggested an extremely strong association between null genotypes of *GSTM1* and *GSTT1* singly or in combination with high risk for oral leukoplakia.

Another population-based case–control study of 53 matched pairs conducted in Thailand (Kietthubthew *et al*., 2001) assessed the risk for oral cancer in relation to genetic polymorphism of the GST genes in cigarette smokers, alcohol drinkers and betel-quid chewers. The *GSTM1*-null genotype was found in 56.6% of cases compared with 30.2% of controls and was significantly associated with risk for oral cancer for each habit (odds ratio, 2.6; 95% CI, 1.04–6.5). Among the null *GSTM1* individuals, smoking (odds ratio, 4.0; 95% CI, 1.2–13.7), alcohol consumption (odds ratio, 7.2; 95% CI, 1.5–33.8) and/or betel-quid chewing (odds ratio, 4.4; 95% CI, 1.1–17.8) resulted in a significantly increased risk for oral cancer. Interactions between any two of the lifestyle habits for oral cancer risk, however, were not found. The frequency of the *GSTT1*-null genotype was 34.0% among cases and 47.2% among controls. No association was observed between the *GSTT1*-null allele and risk for oral cancer. This study indicates that *GSTM1*-null genotype predisposes towards oral cancer in individuals exposed to cigarette smoke, alcohol and betel quid.

The impact of *GSTM1*-null genotype on oral cancer risk was also analysed in separate groups of individuals from India with different tobacco habits (297 cancer patients and 450 healthy controls). The odds ratios associated with *GSTM1*-null genotype was 3.7 (95% CI, 2.0–7.1) in chewers of tobacco with lime or with betel quid, 3.7 (95% CI, 1.3–7.9) in bidi smokers and 5.7 (95% CI, 2.0–16.3) in cigarette smokers. Furthermore, increased lifetime exposure to tobacco chewing appeared to be associated with a twofold increase in oral cancer risk in *GSTM1*-null individuals (Buch *et al.*, 2002). [Gene–environment interaction was not estimated for chewing betel quid with or without tobacco.]

The effect of *CYP2A6* and *GSTM1* genes on susceptibility to oral lesions among habitual betel-quid chewers was investigated in a Sri Lankan population in a hospital-based case–control study (Topcu *et al*., 2002). Of a total of 286 cases, 15 had oral squamous-cell carcinoma, 209 had leukoplakia and 62 had oral submucous fibrosis. The control group of 135 subjects was composed of patients who visited the hospital for clinical reasons other than oral cancer and had no oral lesions. The frequency of homozygotes for *CYP2A6*4C*, a gene deletion polymorphism, was significantly lower in the cases compared with controls and a reduced risk was observed in the group with this deletion geno-

type (odds ratio, 0.1; 95% CI, 0.03–0.7). While all subjects were betel-quid chewers, 68% of cases and 45% of controls were also smokers. The significant association was stronger in nonsmoking betel-quid chewers than in those with both habits. Further, the *GSTM1*-null genotype had no effect on risk, but was more frequently found in cases with the susceptible genotype of *CYP2A6* than among controls.

The effect of *CYP1A1* polymorphisms on susceptibility to oral lesions associated with betel-quid use was investigated in an Indian population in a hospital-based case–control study (Kao *et al.*, 2002). The percentage of betel-quid users was 62.3% in cases of oral squamous-cell carcinoma, 75% in cases of oral precancerous lesions and 15% among controls. No significant difference was observed for CYP1A1 Msp1 (*2A) polymorphism between cases and controls. However, the frequence of CYP1A1 *Ile/Val* and *Val/Val* genotypes in exon 7 of cases with squamous-cell carcinoma (79.2 and 7.6%) and oral precancerous lesions (68.3 and 10%) was significantly higher than that in controls (53.2 and 1.4%). The study of the genetic polymorphism of CYP1A1 and risk for cancer associated with the duration of betel-quid use has shown no statistically significant difference for squamous-cell carcinoma. Although the *Val/Val* genotype in betel-quid chewers of the oral precancerous lesions group had a history of betel use much shorter than that of other genotypes, no statistically significant difference could be found due to the small number of *Val/Val* genotype (n = 2) betel chewers. [The Working Group noted that all betel users were also smokers.]

(ii) *Oesophageal cancer* (Table 75)

Alcohol dehydrogenase-2 (ADH2) converts ethanol to acetaldehyde and aldehyde dehydrogenase-2 (ALDH2) converts acetaldehyde to acetate. The association of lifestyle habits and polymorphism of *ADH2* and *ALDH2* genes with the risk for oesophageal cancer was investigated in a Thai population (Boonyaphiphat *et al*., 2002) in a hospital-based case–control study of 202 cases and 261 controls. The results of multivariate logistic analysis showed that alcohol consumption (> 60 g per day), smoking (> 10 cigarettes per day) and chewing betel (≥ 10 quids per day) significantly increased the risk (odds ratios, 5.8; 95% CI, 3.2–10.9; 4.7 (2.0–10.8) and 4.7 (2.1–10.7), respectively). *ADH2*1/*1* also increased the risk significantly (odds ratio, 1.6; 95% CI, 1.01–2.4); *ALDH2* resulted in a similar but statistically non-significant odds ratio (odds ratio for *ALDH2*1/*2*, 1.6; 95% CI, 0.9–2.8). The combined genotypes, *ADH2*1/*1* and *ALDH2*1/*2*, increased the risk fourfold. In addition, significant gene–environment interaction was found for heavy drinkers (> 60 g per day) with *ADH2*1/*1* or *ALDH2*1/*2* genotype who had an 11-fold increased risk. [Gene–environment interaction was not estimated for chewing betel quid with tobacco.]

To assess the risk of *CYP1A1* polymorphisms on oesophageal cancer, a study was conducted using 146 cases of oesophageal squamous-cell carcinoma and 324 malignancy-free controls from three hospitals in Taiwan, China (Wu *et al*., 2002). Habitual use of cigarettes, alcohol and areca nut provided the most significant predictors of oesophageal cancer risk. The proportion of areca-nut chewers was 21.2% in cases and 9.3% among controls

Table 75. Genetic polymorphism and oesophageal cancer

Gene	Mutation/allele	Country/ ethnicity	No. of cases/ controls	Significance: odds ratio (95% CI)	Comments	Reference
ADH2 *ALDH2*	*ADH2*2* *ALDH2*2*	Thailand	202/261	S: *ADH2*1/*1*, 1.6 (1.01–2.4) NS: *ALDH2*1/*2* S: Combined *ADH2*1/*1* + *ALDH2*1/*2*, 4.4 (1.8–10.8)	Mixed ethnicity Significant gene interaction with heavy drinking; gene interaction with betel-quid chewing not estimated	Boonyaphiphat *et al*. (2002)
CYP1A1	MspI RFLP, *2A exon 7 *ile*/*val*, *2C	Taiwan	146/324	S: *val*/*val* vs *ile*/*ile*, 2.5 (1.2–5.3)	91% of cases and 92% of controls were men. Adjusted for age, education, ethnicity, smoking, alcohol and areca consumption	Wu *et al*. (2002)
SULT1A1	*arg*/*his*, G→A, codon 213	Taiwan	187/308	*arg/his* vs *arg/arg*, 3.5 (2.1–5.9) Positive association stronger in no-habit group, adjusted odds ratios, 4.0 for non-chewers, 4.5 for non-drinkers and 4.8 for nonsmokers	Men only Adjusted for age, education, ethnicity, smoking, alcohol and areca consumption	Wu *et al*. (2003)

CI, confidence interval; S, significant; NS, not significant

[data on mixed habits not shown]. No significant difference was observed for the *CYP1A1* MspI polymorphism between carcinoma cases and controls. However, the frequency of wild-type *Ile/Ile*, *Ile/Val* and mutant *Val/Val* in exon 7 was 46.6% (68/146), 42.5% 62/146) and 11.0% (16/146) in carcinoma cases and 55.3% (179/324), 39.2% (127/324) and 5.6% (18/324) in controls, respectively. Patients with *Val/Val* showed a 2.5-fold (95% CI, 1.2–5.3) greater risk for developing oesophageal cancer than those with *Ile/Ile* after adjustment for age, ethnicity, education, and cigarette, alcohol and areca habits. A slightly (not significantly) greater risk was identified in subjects with *Ile/Val* (odds ratio, 1.3; 95% CI, 0.9–2.1). These findings suggest that an exon 7 polymorphism, not a MspI polymorphism, in *CYP1A1* may be pivotal in the development of oesophageal cancer.

The association between the sulfotransferase *SULT1A1 arg/his* genotype and oesophageal cancer in men was studied in 187 cases of oesophageal squamous-cell carcinoma and 308 controls enrolled from three medical centers in Taiwan, China (Wu *et al*., 2003). [The population appears to overlap with that of Wu *et al*. (2002)]. The frequencies of *SULT1A1 arg/his* heterozygote cases (27.8%) and controls (11.0%) were significantly different ($p < 0.0001$). No subjects carried the *his/his* genotype. After adjusting for age, ethnicity, education and cigarette, alcohol and areca habits, individuals with the *arg/his* genotype had a 3.5-fold (95% Cl, 2.1–5.9) higher risk of developing oesophageal cancer than those with *arg/arg* genotype. Remarkably, this positive association was found to be even stronger among non-smokers, non-drinkers and non-chewers. Among the 163 non-substance users, 35.4% (17/48) of the cases and 8.7% (10/115) of the controls carried the *arg/his* genotype. Adjusted for age, education and ethnicity, the odds ratio was 5.7 (95% CI, 2.3–13.9). [The Working Group noted that data are not given regarding mixed-habit groups.]

(iii) *Other susceptibilities and polymorphisms*

As oral submucous fibrosis is a collagen-related disorder induced by cumulative exposure to betel-quid chewing, Chiu *et al*. (2002) investigated polymorphisms of six collagen-related genes, collagen 1A1 and 1A2 (*COL1A1* and *COL1A2*), collagenase-1 (*COLase*), transforming growth factor β1 (*TGF-β1*), lysyl oxidase (*LYOXase*) and cystatin C (*CST3*), among patients with low and high exposure to betel quid [tobacco content not specified]. The subjects comprised 166 patients with oral submucous fibrosis from a medical center in Taiwan, China, and 284 betel-quid chewers free of the disease and oral cancer from the same hospital and five townships. Polymerase chain reaction-restriction fragment length polymorphism assays were used to genotype the genes situated on different chromosomes. Genotypes associated with the highest risk for oral submucous fibrosis for *COL1A1*, *COL1A2*, *COLase*, *TGF-β1*, *LYOXase* and *CST3* were CC, AA, TT, CC, AA and AA, respectively, for the low-exposure group, and TT, BB, AA, CC, GG and AA, respectively, for the high-exposure group. A trend was noted for an increased risk for oral submucous fibrosis with increasing number of high-risk alleles for those with both high and low exposures to betel quid. The results imply that susceptibility to oral

submucous fibrosis could involve multigenic mechanisms modified by the dose of exposure to betel quid.

The secretion of TIMP-1 in response to increases in circulatory MMP-9 in humans varies with the *Taq 1* polymorphism of the vitamin D receptor (VDR) gene (*Taq 1 VDR* genotypes) and also increases in relation to areca-nut consumption (Timms *et al.*, 2002a). No study of this polymorphism as a possible risk factor for submucosal fibrosis and/or cancer has been carried out.

A study on an ethnic Chinese population in Taiwan, China, was conducted to investigate the effect of DNA-repair *XRCCI* genes on susceptibility to oesophageal cancer (Lee, J.-M. *et al*., 2001). *XRCCI* polymorphisms at codons 194, 280 and 399 were genotyped in 105 patients with oesophageal squamous-cell carcinoma and 264 healthy controls matched for age (± 3 years), sex and ethnicity. The distribution of the three genotypes was not significantly different among patients and controls. However, among alcohol drinkers, the *XRCCI399 Arg/Arg* genotype was frequently found in patients with oesophageal cancer. After adjustment for other potential environmental confounders, the odds ratio for the genotype of *XRCCI399 Arg/Arg* was 2.8 (95% CI, 1.2–6.7) compared with the *XRCCI399 Arg/Gln* and *XRCCI399 Gln/Gln* genotypes in alcohol drinkers. Similar trends, not reaching significance, were observed among cigarette smokers (adjusted odds ratio for *XRCCI399 Arg/Arg*, 1.7; 95% CI, 0.7–3.9) and areca chewers (adjusted odds ratio for *XRCCI399 Arg/Arg*, 2.8; 95% CI, 0.5–16.7) .

4.4.2 *Experimental systems*

(*a*) *Genotoxicity and mutagenicity* (see Table 76 for details and references)

(i) Pan masala

Aqueous, aqueous/ethanolic and hot chloroform extracts of *pan masala* were not mutagenic to *Salmonella typhimurium* TA98 or TA100 in the presence or absence of an exogenous metabolic activation system in one study, but the aqueous extract induced mutation in these two strains both in the absence or presence of exogenous metabolic activation in another study. Only the ethanolic extract showed mild direct mutagenicity toward *S. typhimurium* TA98. These extracts were still not mutagenic to TA98 or TA100 even after addition of an exogenous metabolic system or after nitrosation at acidic pH (pH 3) with nitrite (Bagwe *et al.*, 1990). In another study, aqueous extract of *pan masala* was positive in *S. typhimurium* TA98 with or without bioactivation.

Aqueous extracts of both *pan masala* and *pan masala* with tobacco induced chromosomal aberrations, sister chromatid exchange and micronucleated cells in Chinese hamster ovary (CHO) cells in the presence and absence of an exogenous metabolic system, although metabolic activation markedly inhibited the chromosomal damaging effect, implicating the presence of direct-acting mutagens and clastogens (Jaju *et al.*, 1992). The clastogenic effect of *pan masala* towards CHO cells was evaluated in the presence of ethanol, which markedly decreased the mitotic index of CHO cells from 4.3

Table 76. Genetic and related effects of components of pan masala, betel quid and areca nut

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Pan masala				
Salmonella typhimurium TA100, TA98, reverse mutation, aqueous extract	–	–	154 mg/plate	Bagwe *et al.* (1990)
Salmonella typhimurium TA100, TA98, reverse mutation, aqueous/ethanol extract	–	–	37.5 mg/plate	Bagwe *et al.* (1990)
Salmonella typhimurium TA100,TA98, reverse mutation, hot chloroform extract	–	–	1.4 mg/plate	Bagwe *et al.* (1990)
Salmonella typhimurium TA100, reverse mutation, aqueous extract	(+)	+	100 µg/plate	Polasa *et al.* (1993)
Salmonella typhimurium TA98, reverse mutation, ethanol extract	(+)	–	25 mg/plate	Bagwe *et al.* (1990)
Salmonella typhimurium TA98, reverse mutation, aqueous extract	+	+	100 µg/plate	Polasa *et al.* (1993)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*, aqueous extract with or without tobacco	+	(+)	5 µL/mL	Jaju *et al.* (1992)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro,* DMSO extract with or without tobacco	+	(+)	5 µL/mL	Patel *et al.* (1994a)
Sister chromatid exchange, Swiss albino mouse bone-marrow cells *in vivo*, aqueous suspension	+		25 mg/kg ip	Mukherjee & Giri (1991)
Micronucleus formation, Chinese hamster ovary cells *in vitro*, aqueous extract with or without tobacco	+	(+)	5 µL/mL	Jaju *et al.* (1992)
Micronucleus formation, Chinese hamster ovary cells *in vitro,* DMSO extract with or without tobacco	+	(+)	5 µL/mL	Patel *et al.* (1994a)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*, aqueous extract with or without tobacco	+	(+)	5 µL/mL	Jaju *et al.* (1992)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro,* DMSO extract with or without tobacco	+	(+)	5 µL/mL	Patel *et al.* (1994a)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*, aqueous extract with or without tobacco plus ethanol	+	NT	5 µL/mL + 1 or 2% ethanol	Patel *et al.* (1994b)

Table 76 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Betel quid				
Salmonella typhimurium TA100, TA1535, reverse mutation, aqueous BQ extract	+	+	10 µg/plate	Shirname *et al.* (1983)
Salmonella typhimurium TA100, TA1535, reverse mutation, aqueous BQ + tobacco extract	+	+	5 µg/plate	Shirname *et al.* (1983)
Salmonella typhimurium TA100, TA1535, reverse mutation, aqueous BQ + tobacco extract	–	+	1 µg/plate	Shirname *et al.* (1983)
Salmonella typhimurium TA100, TA98, reverse mutation, aqueous BQ extract	–	–	100 µg/plate	Wang *et al.* (1999)
Gene mutation, Chinese hamster V79 cells *in vitro* , aqueous BQ extract	–	–	10 µg/mL	Shirname *et al.* (1984)
Gene mutation, Chinese hamster V79 cells *in vitro*, aqueous BQ + tobacco extract	+	+	5 µg/mL	Shirname *et al.* (1984)
Sister chromatid exchange, Chinese hamster ovary-K1 cells *in vitro*, aqueous BQ extract	(+)	–	100 µg/mL	Wang *et al.* (1999)
Micronucleus formation, Swiss mouse polychromatic erythrocytes *in vivo*, aqueous BQ extract	–		2 mg, ip × 2	Shirname *et al.* (1984)
Micronucleus formation, Swiss mouse polychromatic erythrocytes *in vivo,* aqueous BQ + tobacco extract	+		0.3 mg ip × 2	Shirname *et al.* (1984)
Chromosomal aberrations, Chinese hamster ovary-K1 cells *in vitro*, aqueous BQ extract	–	–	1000 µg/mL	Wang *et al.* (1999)
Areca nut				
Salmonella typhimurium TA100, TA1535, reverse mutation, aqueous areca-nut extract	+	+	10 µg/plate	Shirname *et al.* (1983)

Table 76 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA98, reverse mutation, aqueous extract of *Supari*	(+)	+	200 µg/plate	Polasa *et al.* (1993)
Saccharomyces cerevisiae, mitotic conversion, aqueous phenolic extract and tannin fraction	+	NT	3 mg/mL (pH > 10)	Rosin (1984)
DNA strand breaks, mouse kidney cells *in vitro*, aqueous areca-nut extract	+	NT	100 µg/mL	Wary & Sharan (1988)
Gene mutation, Chinese hamster V79 cells *in vitro*, aqueous areca-nut extract	+	+	5 µg/mL	Shirname *et al.* (1984)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*, aqueous areca-nut extract	+	NT	2.5 µL/mL	Dave *et al.* (1992b)
Sister chromatid exchange, Swiss albino mouse bone-marrow cells *in vivo*, aqueous ripe sun-dried areca-nut extract	+		12.5 mg/kg × 5, 10 or 15 d	Panigrahi & Rao (1986)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*, aqueous areca-nut extract	+	NT	8 mg/mL	Stich & Dunn (1986)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*, aqueous areca-nut extract	+	NT	5 mg/mL	Stich & Tsang (1989)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*, aqueous areca-nut extract	+	NT	5 µL/mL	Dave *et al.* (1992b)
Cell transformation, mouse C3H10T1/2 cells *in vitro*, aqueous extract	+	NT	0.6 mg/mL	Stich & Tsang (1989)
DNA strand breaks, DNA–protein cross-links, human primary buccal epithelial cells *in vitro*, aqueous areca-nut extract	+	NT	300 µg/mL	Sundqvist *et al.* (1989)
DNA strand breaks, human oral mucosal fibroblasts *in vitro*, aqueous areca-nut extract	+	NT	3 mg/mL	Jeng *et al*. (1994a)
Unscheduled DNA synthesis, human Hep 2 laryngeal carcinoma cells *in vitro*, aqueous, acetic acid, hydrochloric acid and ethanol areca-nut extracts	+	NT	10–25 µg/mL	Sharan & Wary (1992)

Table 76 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Unscheduled DNA synthesis, human primary gingival keratinocytes *in vitro*, aqueous areca-nut extract	+	NT	200 µg/mL	Jeng *et al.,* (1999b)
Micronucleus formation, fibroblasts from healthy subjects *in vitro*, aqueous areca-nut extract	+	NT	2.6 mg/mL	Yi *et al.* (1990)
Micronucleus formation, fibroblasts from ataxia telangiectasia patients *in vitro*, aqueous areca-nut extract	+	NT	1.3 mg/mL	Yi *et al.* (1990)
Micronucleus formation, Swiss mouse bone-marrow cells *in vivo*, areca-nut extract	+		20 mg ip × 2	Shirname *et al.* (1984)
Micronucleus formation, hamster cheek pouch buccal epithelial cells *in vivo,* areca-nut extract with catechu and lime	+		1 mg + 1 mg catechu + 2 mg lime, cpp × 5	Nair *et al.* (1992)
Micronucleus formation, hamster cheek pouch buccal epithelial cells *in vivo,* areca-nut extract with lime and without catechu	+		1 mg + 2 mg lime, cpp × 5	Nair *et al.* (1992)
Areca alkaloids				
Escherichia coli PQ37, SOS induction (chromotest), arecoline	–	–	156.8 µg/assay	Kevekordes *et al.* (1999)
Salmonella typhimurium TA100, TA1535, reverse mutation, arecoline	+	+	10 µg/plate	Shirname *et al.* (1983)
Salmonella typhimurium TA100, TA1535, reverse mutation, arecaidine	+	+	100 µg/plate	Shirname *et al.* (1983)
Salmonella typhimurium TA100, reverse mutation, crude alkaloid extracts	–	(+)	4 mg/plate	Wang & Peng (1996)

Table 76 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, reverse mutation, oil arecoline	+	+	6.5 µmol [1 mg]/plate	Wang & Peng (1996)
Salmonella typhimurium TA98, reverse mutation, crude alkaloid extracts	–	–	4 mg/plate	Wang & Peng (1996)
Salmonella typhimurium TA98, reverse mutation, oil arecoline	–	–	26 µmol [4 mg]/plate[c]	Wang & Peng (1996)
DNA strand breaks, mouse kidney cells *in vitro*, arecoline	+	NT	10 µg/mL	Wary & Sharan (1988)
Gene mutation, Chinese hamster V79 cells *in vitro*, arecoline	+	+	5 µg/mL	Shirname *et al.* (1984)
Gene mutation, Chinese hamster V79 cells *in vitro*, arecaidine,	–	+	10 µg/mL	Shirname *et al.* (1984)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro,* arecoline	+	NT	12.5 µg/mL	Dave *et al*. (1992b)
Sister chromatid exchange, Chinese hamster ovary cells *in vitro*, arecoline	+	NT	12.5 µg/mL	Trivedi *et al.* (1993)
Micronucleus formation, Chinese hamster ovary cells *in vitro,* arecoline	+	NT	0.2 µM	Lee *et al.* (1996)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro,* arecoline	+	NT	50 µg/mL	Dave *et al*. (1992b)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*, arecoline	–	NT	12.5 µg/mL	Trivedi *et al.* (1993)
DNA strand breaks, human primary buccal epithelial cells *in vitro*, four areca alkaloids	–	NT	5 mM	Sundqvist *et al.* (1989)
DNA strand breaks, human buccal mucosal fibroblasts *in vitro,* arecoline	–	NT	400 µg/mL	Jeng *et al.* (1994a); Chang, Y.-C. *et al.* (1998)
Unscheduled DNA synthesis, human Hep 2 laryngeal carcinoma cells *in vitro*, arecoline	+	NT	5 µg/mL	Sharan & Wary (1992)

Table 76 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Unscheduled DNA synthesis, primary human gingival keratinocytes *in vitro*, arecoline	–	NT	1.6 mM	Jeng *et al.* (1999a)
Micronucleus formation, human lymphocytes *in vitro,* arecoline	+	+	12.5 µM [3 µg/mL] for 20 h	Kevekordes *et al.* (2001)
Micronucleus formation, human hepatoma cell line Hep-G2 *in vitro,* arecoline	+	+	25 µM [6 µg/mL] for 24 h	Kevekordes *et al.* (2001)
Unscheduled DNA synthesis, Swiss albino mouse early spermatids *in vivo*, arecoline	+		20 mg/kg ip	Sinha & Rao (1985b)
Sister chromatid exchange, Swiss albino mouse bone-marrow cells *in vivo*, arecaidine	+		100 mg/kg ip × 5 or 10	Panigrahi & Rao (1984)
Sister chromatid exchange, Swiss albino mouse bone-marrow cells *in vivo*, arecoline	+		20 mg/kg ip	Deb & Chatterjee (1998)
Sister chromatid exchange, Swiss albino mouse bone-marrow cells *in vivo*, arecoline	+		40 mg/kg ip × 1, 5 or 15 d	Chatterjee & Deb (1999)
Sister chromatid exchange, Swiss albino mouse bone-marrow cells *in vivo*, arecoline	+		170 µg/mL po × 1, 5 or 15 d	Chatterjee & Deb (1999)
Micronucleus formation, Swiss mouse bone-marrow polychromatic erythrocytes *in vivo,* arecoline	+		2 mg/animal/d ip × 2	Shirname *et al.* (1984)
Micronucleus formation, Swiss mouse bone-marrow polychromatic erythrocytes *in vivo,* arecaidine	(+)		14 mg/animal/d ip × 2	Shirname *et al.* (1984)
Micronucleus formation, pregnant Swiss albino mouse fetal polychromatic erythrocytes *in vivo*, arecoline	+		20 mg/kg ip	Sinha & Rao (1985b)
Chromosomal aberrations, Swiss albino mouse bone-marrow cells *in vivo*, arecoline	+		20 mg/kg ip	Deb & Chatterjee (1998)

Table 76 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Chromosomal aberrations, Swiss albino mouse bone-marrow cells *in vivo*, arecoline	+[d]		40 mg/kg ip × 1, 5 or 15 d	Chatterjee & Deb (1999)
Chromosomal aberrations, Swiss albino mouse bone-marrow cells *in vivo*, arecoline	+		170 µg/mL po × 1, 5 or 15 d	Chatterjee & Deb (1999)
Inflorescence *Piper betle*				
DNA strand breaks, human oral mucosal fibroblasts *in vitro*, aqueous extract	+	NT	3 mg/mL	Jeng *et al.* (1994a)
Unscheduled DNA synthesis, primary human gingival keratinocytes *in vitro*, aqueous extract	–	NT	1600 µg/mL	Jeng *et al*. (1999b)
***Piper betle* leaf**				
Salmonella typhimurium TA100, TA1535, reverse mutation, aqueous extract	–	–	200 µg/plate	Shirname *et al.* (1983)
Salmonella typhimurium TA100, TA1535, TA1537, TA98, reverse mutation, aqueous and acetone extract	–	–	200 µg/plate	Nagabhushan *et al.* (1987)
Micronucleus formation, human lymphocytes *in vitro*, acetone/ether extract	–	NT	200 µg/mL	Ghaisas & Bhide (1994)
Micronucleus formation, Swiss albino mouse bone-marrow cells *in vivo*, extract	–		250 mg/kg × 2	Amonkar *et al*. (1989)
Eugenol and hydroxychavicol				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation, eugenol	–	–	600 µg/plate	Amonkar *et al.* (1986)
Salmonella typhimurium TA100, TA1535, TA1538, TA98, reverse mutation, hydroxychavicol	–	–	300 µg/mL	Amonkar *et al.* (1986)

Table 76 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA98, TA97, reverse mutation, hydroxychavicol	–	–	100 µg/plate	Lee-Chen *et al.* (1996)
Salmonella typhimurium TA102, reverse mutation, hydroxychavicol	+	–	0.1 µg/plate	Lee-Chen *et al.* (1996)
Micronucleus formation, Chinese hamster ovary-K1 cells *in vitro,* hydroxychavicol	+	NT	20 µM	Lee-Chen *et al.* (1996)
Chromosomal aberrations, Chinese hamster ovary-K1 cells *in vitro,* hydroxychavicol	+	NT	20 µM	Lee-Chen *et al.* (1996)
DNA strand breaks, human oral mucosal fibroblasts *in vitro*, eugenol	–	NT	3 mM	Jeng *et al.* (1994b)
Catechu				
Salmonella typhimurium TA100, TA1535, TA1538, TA98, reverse mutation, ethyl/alcohol extract and catechin	–	–	250 µg/plate	Nagabhushan *et al.* (1988)
Micronucleus formation, human lymphocytes *in vitro,* acetone/ether extract	–	NT	200 µg/mL	Ghaisas & Bhide (1994)
Sister chromatid exchange, Swiss albino mouse bone-marrow cells *in vivo*, extract	+		3 mg/kg ip × 1	Giri *et al.* (1987)

[a] +, positive; (+), weakly positive; –, negative; NT, not tested; BQ, betel quid

[b] LED, lowest effective dose; HID, highest ineffective dose unless otherwise stated; in-vitro tests, µg/mL; in-vivo tests, mg/kg bw/day; ip, intraperitoneal; cpp, cheek pouch buccal painting; po, oral

[c] The highest ineffective dose with an exogenous metabolic system is 39 µmol/plate (6 mg/plate), but this dose was not tested without an exogenous metabolic system.

[d] Chromosomal aberrations induced after 5 or 15 days of intraperitoneal treatment with arecoline were not statistically significant.

to 2.1 (Patel *et al*., 1994b). Exposure of CHO cells to extract of *pan masala*/*pan masala* with tobacco plus ethanol also increased the frequency of chromosomal aberrations. A dimethyl sulfoxide (DMSO) extract of *pan masala* with and without tobacco induced chromosomal aberrations and sister chromatid exchange and raised the frequency of micronuclei in CHO cells without metabolic activation. In the absence of an exogenous metabolic activation system, the genotoxic effect of the two extracts was markedly increased, indicating the content of direct-acting mutagens. The decrease in genotoxic response in the presence of an exogenous metabolic system is probbly due to detoxification of mutagens by microsomal enzymes (Patel *et al.*, 1994a) [cytotoxicity data and the concentration of the extract of *pan masala* and *pan masala* with tobacco were not given].

Intraperitoneal injection of *pan masala* suspensions into Swiss albino mice *in vivo* induced sister chromatid exchange in bone-marrow cells. Higher doses resulted in delay in cell cycle progression of bone-marrow cells as revealed by a decrease in the proliferation rate index (Mukherjee & Giri, 1991).

(ii) *Betel quid*

Betel-quid extract was mutagenic to *S. typhimurium* TA100 and TA1535 in the presence or absence of metabolic activation in one study, but not to TA100 or TA98 in another. Betel quid plus tobacco induced stronger mutagenicity towards TA100 and TA1535.

Betel-quid extract did not induce 8-azaguanine gene mutation in Chinese hamster V79 cells in the presence or absence of an exogenous metabolic activation system, whereas betel quid plus tobacco did. Exposure of Chinese hamster ovary (CHO)-K1 cells to betel-quid extract resulted in a weak induction of sister chromatid exchange in the absence of an exogenous metabolic system and no change in chromosomal aberrations compared with unexposed cells.

Intraperitoneal injection of betel-quid extract into Swiss mice induced only a slight non-significant increase in micronucleated cells of polychromatic erythrocytes in the bone marrow, whereas injection of an extract of betel quid plus tobacco induced a greater increase ($p < 0.05$).

(iii) *Areca nut*

Areca-nut extract was mutagenic to *S. typhimurium* TA100 and TA1535 in the presence and absence of an exogenous metabolic activation system. Aqueous extract of *Supari* (processed areca nut with or without flavourings) was weakly positive in *S. typhimurium* TA100 and TA98 in the absence of bioactivation but clearly induced mutation in the presence of an exogenous metabolic activation system. Exposure to aqueous areca-nut extract and tannin induced mitotic gene conversion in *Saccharomyces cerevisiae* at pH values higher than 10.

Exposure of mouse kidney cells in culture to aqueous extract of ripe areca nut (after removal of coated fibres) induced a low but significant level of DNA strand breaks, but

reduced cell cycle time [single dose tested only] (Wary & Sharan, 1988). Areca-nut extract induced 8-azaguanine gene mutation in Chinese hamster V79 cells, which was enhanced by the presence of exogenous metabolic activation [exposure time not given]. Continuous or pulse treatment of Chinese hamster ovary (CHO) cells with aqueous extract of ripe and sun-dried areca nut increased the frequency of sister chromatid exchange and chromosomal aberrations in a dose-dependent fashion. Chromatid-type aberrations were more frequent than the chromosomal type (Dave *et al*., 1992b). Although clinically β-carotene treatment may decrease the frequency of micronucleated buccal epithelial cells in betel-quid/tobacco chewers (Stich *et al*., 1984b,c), a 48-h pretreatment of CHO cells with β-carotene showed little protective effect against gallic acid (12 μg/mL)-, tannic acid (60 μg/mL)-, aqueous areca-nut extract (8 mg/mL)- and H_2O_2 (5–25 μM)-induced chromosomal aberrations and micronucleus formation. The last end-point was not analysed for aqueous areca-nut extract (Stich & Dunn, 1986) [only a single dose of β-carotene was used in this study; no cytotoxicity data on CHO cells were given]. In another study, aqueous areca-nut extract (5 mg/mL) induced a significant number of chromosomal aberrations in CHO cells.

Transfection of mouse C3H/10T1/2 cells with a plasmid containing bovine papillomavirus DNA (*pdBPV-1*) induced transformed foci. When transfection of C3H/10T1/2 cells by the plasmid *pdBPV-1* was followed by exposure to areca-nut extract (0.6–2.5 mg/mL) for 7 days, an observable elevation of transformed foci was noted (Stich & Tsang, 1989).

Areca-nut extract induced DNA strand breaks in human oral mucosal fibroblasts *in vitro*. Aqueous areca-nut extract induced DNA strand breaks and DNA–protein cross-links in human primary buccal epithelial cells *in vitro*. Exposure of Hep 2 human laryngeal carcinoma cells to aqueous, acetic acid, hydrochloric acid or ethanol extracts of areca nut induced unscheduled DNA synthesis [the assay seems unable to discriminate between total DNA synthesis and unscheduled DNA synthesis; no negative control data were given in the unscheduled DNA synthesis assay. This assay cannot distinguish between synthesis by induction of S-phase arrest by arecoline and areca nut and that due to DNA damage and subsequent DNA repair.] An aqueous areca-nut extract induced unscheduled DNA synthesis in human primary gingival keratinocytes *in vitro*. Arecoline (0.042–0.339 mM) [6.5–52.5 μg/mL) and sodium nitrite (0.145 mM) [10 μg/mL] showed additive cytotoxicity to cultured Hep 2 cells in the presence of an exogenous metabolic system at pH 4.2, with an exposure time of 30 min (Wary & Sharan, 1991). Four strains of fibroblasts cultured from ataxia telangiectasia patients were exposed to H_2O_2 or aqueous areca-nut extract and showed a stronger response in the cytokinesis-block micronucleated cell assay than control fibroblasts. This effect could be partially prevented by addition of catalase (100 U/mL) to the fibroblasts, indicating the involvement of H_2O_2 (Yi *et al.*, 1990).

Chang, M.C. *et al*. (2002b) reported that areca-nut extract (1200 μg/mL)-induced unscheduled DNA synthesis in human gingival keratinocytes can be prevented by *N*-acetyl-L-cysteine (NAC, 1 and 3 mM), GSH (1 and 3 mM) and vitamin C (200 μg/mL), but not by specific reactive oxygen species scavengers such as DMSO (2%), SOD (50, 200 U/mL) or catalase (200, 400 U/mL).

Intraperitoneal administration of aqueous extracts of ripe and sun-dried areca nut to Swiss albino mice induced sister chromatid exchange in bone-marrow cells. However, areca-nut tannin induced only a mild elevation of sister chromatid exchange (Panigrahi & Rao, 1986). Intraperitoneal administration of areca-nut extract to Swiss mice increased the frequencies of micronuclei in bone-marrow polychromatic erythrocytes from 0.27 to 0.73% (Shirname *et al*., 1984). Painting of hamster cheek pouch with lime (50 μL, 4%, w/v) [2 mg] and areca-nut extract [50 μL of a 20 mg/mL solution; 1 mg] plus catechu extract [50 μL of a 20 mg/mL solution; 1 mg] increased the frequency of micronucleated cells of the cheek pouch. Painting of hamster cheek pouch with ripe and tender areca-quid extract (areca nut plus lime, 125 μL [concentration not given]) for 14 days induced a significantly higher amount of 8-OHdG formation in the buccal pouch than was seen in untreated control mucosa (Chen *et al*., 2002).

(iv) *Areca alkaloids*

Arecoline did not elicit SOS induction in *Escherichia coli* PQ37. Arecoline and arecaidine induced reverse mutation in *S. typhimurium* TA100 and TA1535 in the presence and absence of an exogenous metabolic activation system. Crude areca alkaloid extracts were not mutagenic to *S. typhimurium* TA98 but were weakly mutagenic to *S. typhimurium* TA100 in the presence but not in the absence of metabolic activation. Oily arecoline isolated from areca nut was not mutagenic to *S. typhimurium* TA98 but was mutagenic to TA100.

Arecoline induced DNA strand breaks in mouse kidney cells *in vitro* and inhibited the proliferation of these cells (Wary & Sharan, 1988). It induced gene mutation in Chinese hamster V79 cells, which was greater in the presence of an exogenous metabolic activation system (Shirname *et al*., 1984). Arecaidine was also mutagenic to V79 cells but only in the presence of metabolic activation. Arecoline induced sister chromatid exchange and the formation of micronuclei in Chinese hamster ovary (CHO) cells. It also impaired the division of CHO cells at concentrations equal to or higher than 2 μM (Lee *et al*., 1996). Arecoline induced chromosomal aberrations in Chinese hamster ovary cells in one study but not in another. Addition of nicotine plus arecoline showed synergistic effects in inducing chromosomal aberrations and sister chromatid exchange in CHO cells (Trivedi *et al*., 1993).

Arecoline did not induce DNA strand breaks in human buccal mucosal fibroblasts. It induced unscheduled DNA synthesis in human Hep 2 laryngeal carcinoma cells but not in primary human gingival keratinocytes. [Arecoline is cytotoxic to Hep 2 cells at concentrations greater than 10 μg/mL. No negative control data are given in the unscheduled DNA synthesis assay in human Hep2 laryngeal carcinoma cells.] Exposure of cultured human peripheral blood lymphocytes and the human hepatoma cell line Hep-G2 to arecoline induced micronucleus formation.

After intraperitoneal injection, arecoline induced unscheduled DNA synthesis in early spermatid cells, and sister chromatid exchange, micronucleus formation and chromosomal aberrations in the bone-marrow cells of Swiss mice *in vivo*. Intraperitoneal injec-

tion of arecaidine induced sister chromatid exchange and micronucleus formation in Swiss albino mouse bone-marrow cells *in vivo*.

(v) Piper betle *inflorescence*

Aqueous extract of *Piper betle* inflorescence (> 2 mg/mL) induced DNA breaks in human oral mucosal fibroblasts but did not stimulate unscheduled DNA synthesis in primary human gingival keratinocytes *in vitro* (< 1.6 mg/mL).

(vi) Piper betle *leaf*

Extract of *Piper betle* was not mutagenic to *S. typhimurium* TA100, TA1535, TA1537 or TA98 in the presence or absence of an exogenous metabolic activation system.

An acetone/ether extract of *Piper betle* leaf did not induce micronucleated cells in cultured human peripheral blood lymphocytes *in vitro*.

Administration of betel-leaf extract to Swiss albino mice twice failed to induce micronucleated bone-marrow cells.

(vii) *Eugenol and hydroxychavicol*

In the absence but not in the presence of an exogenous metabolic activation system, hydroxychavicol was mutagenic to *S. typhimurium* TA102, but not to TA100, TA98 or TA97. Eugenol and hydroxychavicol were not mutagenic to *S. typhimurium* TA100, TA1535, TA1537, TA1538 or TA98, even after metabolic activation. Catalase and SOD showed a protective effect towards hydroxychavicol-induced mutation in *S. typhimurium* TA102, indicating the participation of H_2O_2 and superoxide radicals in mediating these events (Lee-Chen *et al*., 1996).

Hydroxychavicol induced DNA strand breaks in the presence of transition metal. Reaction of hydroxychavicol with supercoiled pRSVcat plasmid DNA in the presence of copper (100 μM) for 30 min induced DNA breaks. At concentrations ranging from 6.25 to 100 μM [1–15 μg/mL], hydroxychavicol dose-dependently induced oxidative damage with the addition of metal ions, as shown by the formation of 8-OHdG in CHO-K1 cells (Lee-Chen *et al*., 1996).

Eugenol did not induce DNA strand breaks in human oral mucosal fibroblasts. Chen *et al*. (2000) found that hydroxychavicol was cytotoxic to human hepatoblastoma HepG2 cells at concentrations higher than 50 μM [7.5 μg/mL]. Pretreatment for 24 h with buthionine sulfoximine (BSO; 25 μM [49 μg/mL]), an inhibitor of γ-glutamyl cysteine synthetase, enhanced the cytotoxicity of hydroxychavicol. Hydroxychavicol weakly induced 8-OHdG formation, apoptosis and DNA fragmentation in HepG2 cells, which were enhanced by BSO. Catalase at a high dose (1000 U/mL) suppressed BSO/hydroxychavicol-mediated apoptosis (Chen *et al*., 2000).

Exposure of Chinese hamster ovary (CHO)-K1 cells to hydroxychavicol induced chromosomal aberrations and increased micronucleus frequencies.

(viii) *Catechu* (Acacia catechu)

Catechu extract (< 250 μg/plate) and catechin were not mutagenic to *S. typhimurium* TA98, TA100, TA1535 or TA1538.

Exposure of human lymphocytes to catechu (200 μg/mL) for 6 days did not induce a statistically significant increase in the number of micronuclei. However, sister chromatid exchange in bone-marrow cells was significantly elevated in a dose-dependent manner following intraperitoneal administration of catechu extract (3 mg/kg bw) to male Swiss albino mice.

(ix) *Urine from chewers*

Urine isolated from controls and chewers of *masheri* (n = 23) and betel quid with tobacco in addition to *masheri* (n = 34) induced little or weak mutation in *S. typhimurium* TA98 and TA100. In the presence of metabolic activation and nitrite, the mutagenicity of urine samples collected from controls and chewers showed greater mutagenicity in *S. typhimurium* TA98 but not in TA100 compared with urine samples from control subjects (Govekar & Bhisey, 1993).

Cultured CHO cells were exposed to urine concentrates (10 μL/mL) collected from 20 tobacco and areca-nut chewers (without smoking or alcohol drinking) for 3 h. Marked elevation of chromosomal aberrations and sister chromatid exchange was noted compared with exposure to urine concentrates from control subjects, even without metabolic activation (Trivedi *et al*., 1995).

(*b*) *Oncogenes and tumour-suppressor genes*

Treatment of oral mucosal fibroblasts with areca-nut extract (200 μg/mL) or arecoline (10 μg/mL) for 1 h induced an approximately threefold increase in c-*jun* mRNA levels. This increase was transient and c-*jun* mRNA returned rapidly to control levels thereafter (Ho *et al.*, 2000). However, areca-nut extract and arecoline did not induce c-*fos* mRNA expression. Furthermore, preincubation of cells with either *N*-acetyl-cysteine, a GSH precursor, or BSO, a specific inhibitor of GSH biosynthesis, had a minimal effect on arecoline-induced c-*jun* expression, suggesting that this effect is independent of GSH status (Ho *et al*., 2000).

Lin *et al.* (2000b) established a cell line, HCDB-1, from tumours induced by the application of 7,12-dimethylbenz[*a*]anthracene (DMBA)/betel-quid extract from Taiwan, China, to hamster buccal pouch. Mutational analysis of *TP53* revealed a C→T transition at codon 141 (Ala→Val) in these cells. The HCDB-1 cells were tumorigenic in nude mice.

The *APC* gene of cultured cells from oral submucous fibrosis patients (8/8) had a CGA→GGA missense mutation at codon 498 (Arg→Gly) and 7/8 cell cultures from these patients had an adenine deletion at nucleotide 1494 that created a stop codon (TGA) at codon 504, while all (8/8) normal human gingival fibroblast cultures expressed the wild-type APC protein (Liao *et al.*, 2001).

(*c*) *Polymorphisms in carcinogen metabolizing genes*

No studies on genetic polymorphisms in experimental systems were available to the Working Group.

(*d*) *Markers of tumour promotion*

The promoting effects of betel quid and lime–piper additives (*Piper betle* flower and slaked lime paste) in betel quid on epidermal hyperplasia were investigated in CD-1 mouse skin. Aqueous extracts of these materials were condensed to powder and then kept as a 200-mg/mL stock solution in water/ethanol (5:3 v/v). They were applied twice daily for 4 days to the dorsal skin of female CD-1 mice, which were killed 18 h after the last dose. At concentrations of 50, 100 and 150 mg/mL, both extracts caused a significant induction of hyperplasia. The extract of the lime–piper additives at concentrations of 25, 50 and 75 mg/mL caused an increase in the activity of epidermal ornithine decarboxylase (ODC) by 1.4, 1.6 and 1.6 compared with the control, and an increase in the production of H_2O_2 of 2.4-, 3.9- and 3.8-fold, respectively. In addition, the activity of myeloperoxidase (MPO) was increased by 1.4-, 2.7- and 2.3-fold, respectively. Application of both extracts at 50, 100 and 150 mg/mL caused induction of protein kinase C-α (PKC-α) and NF-κB, with the lime–piper additives showing the strongest effect. The results show that betel quid has potential tumour-promoting activity, that the lime–piper additives probably play a major role in enhancing the effects of betel quid-induced skin hyperplasia and inflammation, and that only lime–piper additives induced the ODC activity. These promoting effects on mouse skin were associated with the induction of the expression of PKC and NF-κB (Lee *et al.*, 2002).

(*e*) *Preventive effects*

Padma *et al*. (1989b) found that *Piper betle* leaf extract dose-dependently decreased the mutagenicity of tobacco-specific nitrosamines, NNN and NNK, to *S. typhimurium* TA100 in the presence of metabolic activation. It also decreased the mutagenicity of *N*-methyl-*N*-nitrosourea towards TA100 and TA1535 (Nagabhushan *et al*., 1989).

Piper betle leaf extract (250 mg/kg, twice) almost completely prevented the NNN- and NNK (250 mg/kg bw)-induced formation of micronuclei in the bone marrow of Swiss albino mice *in vivo* (Padma *et al*., 1989a). This could be partly attributed to the content of eugenol and hydroxychavicol in *Piper betle* leaf (Amonkar *et al*., 1989).

Administration of hydroxychavicol (100 mg/kg bw) decreased NNN- and NNK (500 mg/kg bw)-induced formation of micronucleated cells in the bone marrow of Swiss mice *in vivo* (Amonkar *et al*., 1989). Furthermore, hydroxychavicol (6–96 μg/plate) and eugenol (56–222 μg/plate) inhibited DMBA (5 μg/plate)-induced mutation in *S. typhimurium* TA98 in the presence of exogenous metabolic activation systems (Amonkar *et al*., 1986).

In addition, catechu extract (25–20 μg/plate) and catechin (12–100 μg/plate) inhibited the benzo[*a*]pyrene- and DMBA-induced mutation in *S. typhimurium* TA98. In another

assay, catechu extract (2–10 mg/mL) and catechin (0.5–6 mg/mL) inhibited *N*-methyl-*N*-nitrosourea-induced mutation in *S. typhimurium* TA1535 (Nagabhushan *et al*., 1988). Catechu (30–200 μg/plate) and catechin (15–100 μg/plate) inhibited the mutagenic effects of cigarette-smoke condensate (150 μg/plate), tobacco (100 μg/plate) and *masheri* (100 μg/plate) extract in *S. typhimurium* TA98 in the presence of an exogenous metabolic activation system (Nagabhushan & Bhide, 1988).

Acetone/ether extract of Purnark (a mixture of turmeric, betel leaf and catechu; 100 and 200 μg/mL) was shown to give 40–60% protection against benzo[*a*]pyrene (5 and 10 μg/mL)-induced formation of sister chromatid exchange and micronuclei in human lymphocytes after 3 days of exposure (Ghaisas & Bhide, 1994).

In the presence of mouse or rat liver microsomal fractions, [^{3}H]benzo[*a*]pyrene showed efficient binding to calf thymus DNA within 30 min of incubation at 37 °C (n = 10). Hydroxychavicol (25 μM) [3.7 μg/mL] prevented [^{3}H]benzo[*a*]pyrene binding to DNA by 97%, whereas eugenol (25 μM) [4 μg/mL] and catechin (25 μM) [7.3 μg/mL] inhibited this binding by 84% and 82%, respectively (Lahiri & Bhide, 1993) [only a single dose was tested]. Chang, M.C. *et al*. (2002a) reported the reactive oxygen species scavenging effect of hydroxychavicol. Effective hydroxychavicol concentrations toward H_2O_2, superoxide radicals and hydroxyl radicals are > 10 μM [1.5 μg/mL], > 0.02 μM [0.003 μg/mL] and > 1.6 μM [0.25 μg/mL], respectively.

Nair, U.J. *et al*. (1987) detected the production of superoxide radicals and H_2O_2 by catechu at pH higher than 9.5. The production of superoxide was enhanced by Fe^{2+}, Fe^{3+} and Cu^{2+}, but inhibited by Mn^{2+}. When incubated with calf thymus DNA under alkaline conditions, catechu and catechin also promoted the formation of 8-OHdG, especially in the presence of Fe^{2+} and Fe^{3+}. It was suggested that decreasing the lime content of betel quid prevented its toxicity. Aqueous catechu extract (5–60 μg/mL) inhibited the γ-radiation-induced lipid peroxidation of rat liver microsome and was an effective scavenger of 2,2′-diphenyl-1-picrylhydrazyl radicals (Naik *et al*., 2003).

4.5 Mechanistic considerations

Betel-quid and areca-nut chewing without tobacco

Evidence for possible human exposure to carcinogenic compounds following the use of areca nut and other betel-quid ingredients, such as lime, catechu, betel leaf and arecoline — the principal alkaloid of areca nut — was considered. Data on toxicity, genotoxicity, mutation in cancer-related genes, immunological effects and gene–environment interactions were evaluated. [The Working Group noted that tobacco chewing and smoking probably modulate the effects of betel-quid use because of the presence of numerous carcinogens in tobacco and tobacco smoke. Therefore, the discussion in this section is restricted to the use of betel quid and areca nut without tobacco.]

The areca-nut-derived nitrosamines MNPN, a rodent carcinogen, NGC and NGL have been detected in the saliva of betel-quid chewers. In-vitro nitrosation experiments using nitrite and arecoline yielded MNPA in addition to MNPN and NGL. In-vitro nitrosation

of betel quid with nitrite and thiocyanate (both are present in human saliva) yielded NGL. By use of a modified NPRO test, the formation of NPRO in the oral cavity has been demonstrated to occur during chewing of betel quid to which proline was added. Bacterial plaque also plays a role in the formation of nitroso compounds in the oral cavity. MNPN forms the DNA adducts 7-methylguanine and O^6-methylguanine (a pro-mutagenic DNA adduct) as well as (2-cyanoethyl)guanines in treated animals (Prokopczyk *et al*., 1987, 1988; see the monograph on some areca-nut-derived *N*-nitrosamines, Section 4.4.2 of this volume). Such DNA adducts could trigger the tumorigenic process in the oral cavity of betel-quid chewers. There is some evidence that betel-leaf extract can inhibit O^6-methylguanine–DNA methyltransferase (MGMT), an enzyme responsible for removal of O^6-alkylated guanine residues from DNA.

The formation of reactive oxygen species has been demonstrated in the oral cavity of betel-quid chewers. In-vitro studies suggest that the superoxide anion is generated because of auto-oxidation of polyphenols found in areca nut and catechu, which is enhanced by the alkaline pH of lime. Superoxide anion is converted to H_2O_2, which reacts in the presence of transition metals, such as copper and iron, to generate hydroxy radicals. Copper and iron are present in microgram per gram amounts in areca nut, *pan masala*, catechu and slaked lime. This generation of hydroxyl radicals may be a critical event in carcinogenesis. In-vitro studies have demonstrated that areca nut and catechu in the presence of lime can induce oxidation of deoxyguanosine in DNA to yield 8-OHdG, which could trigger the tumorigenic process in the oral cavity. In addition, reactive oxygen species can induce DNA strand breaks.

Depletion of GSH (a cellular anti-oxidant) and reduction of the activity of GST — a detoxifying enzyme for carcinogenic electrophiles — have been demonstrated in cultured human oral keratinocytes and fibroblasts, and in the liver of mice treated with areca-nut extract and arecoline. GSH depletion and decrease in GST activity can lead to increased cellular and DNA damage, which is known to induce several response signals implicated in carcinogenesis.

Micronuclei and other markers of DNA damage have been demonstrated in exfoliated cells obtained from chewers of betel quid and users of *pan masala*. Animal studies and in-vitro test systems support the suggestion that extracts of areca nut and *pan masala* can exert genotoxic effects.

Arecoline caused the inhibition of both humoral and cell-mediated immune responses in mice. Areca-nut extract produced adverse effects on the proliferation of phytohaemagglutinin-stimulated human lymphocytes *in vitro*, suggesting that there may be impaired immune surveillance in areca-nut chewers.

Arecoline modulates matrix metalloproteinases and their tissue inhibitors, as well as the activity of lysyl oxidase, which leads to the accumulation of collagen in oral mucosal fibroblasts. Areca-nut polyphenols inhibit collagenases and increase the cross-linkage of collagen, reducing its degradation. These events may underly the generation of oral submucous fibrosis, which could be further enhanced by the presence of copper ions.

Continuous local irritation and trauma caused by betel quid and *pan masala* can generate chronic inflammation, oxidative stress and cytokine production. In-vitro studies have demonstrated the adhesive nature of areca-nut particles to cultured oral mucosal cells, leading to morphological changes and membrane damage. Oxidative stress and the generation of reactive oxygen species can drive affected cells to proliferation, senescence or cell death. Chronic occurrence of these events can lead to hyperplastic/dysplastic lesions in the oral cavity and could drive some of these preneoplastic lesions to malignancy.

In summary, multiple features of the carcinogenic process have been observed to occur *in vitro* and *in situ* in the oral cavity of betel-quid chewers, and in experimental animals treated with the betel-quid ingredients areca nut, arecoline, catechu and slaked lime.