Chapter 3

Arecoline Levels in Saliva
3.1. Introduction – Arecoline levels in saliva

3.1.1. The cellular response to arecoline

Chemical analysis of areca nut extracts reveal a range of compounds including: arecoline, guvacoline, methyl and ethyl esters of nicotinic acid, arecaidine, arecolidine and guracine (Lord et al., 2002). Amongst these, the alkaloid arecoline is particularly implicated by its genotoxic, mutagenic and carcinogenic effects in the pathogenesis of oral mucosal disease (Chang et al., 1998; Jeng et al., 2001; Lee et al., 2006).

The specific effects of arecoline on cells, *in vitro*, differ dependent on the cell type involved, the concentration of arecoline and the exposure time. For example, arecoline can be stimulatory (Chang et al., 2004) and cytotoxic (Chang et al., 2004; Jeng et al., 1999b; Jeng et al., 2003; Jeng JH, 1999) for keratinocytes, depending on the concentration, while fibroblasts and endothelial cells respond in a similar manner although generally at a lower concentration (Chang et al., 1998; Chang et al., 2001b; Chang et al., 2001c; Chang et al., 2001d; Harvey et al., 1986; Jeng et al., 1994; Jeng et al., 1999c; Meghji and Harris, 1995; Scutt et al., 1987; Tsai et al., 1997; Williams et al., 1998). Arecoline is also associated with increased apoptosis (Chang et al., 2004; Jeng et al., 2003).

Exposure to arecoline can induce cell cycle arrest at the G2 / M boundary in both fibroblasts and keratinocytes (Chang et al., 2001a), but did not appear to affect apoptosis in this particular study. It also depresses DNA synthesis in both keratinocytes and fibroblasts (Brandwein-Gensler and Hille, 2003; Chang et al.,
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2001a; Chang et al., 1998; Chen et al., 1995; Chung et al., 1994; Jeng et al., 1999a; Jeng et al., 1994; Jeng et al., 1999b; Nair et al., 1992; Sharan and Wary, 1992; Spalding et al., 1995; Sundqvist et al., 1989; Sundqvist et al., 1991; Wary and Sharan, 1988; Wary and Sharan, 1991; Yang et al., 2004) The depressed DNA synthesis results in reduced cell survival and lower rates of protein and DNA synthesis in a dose-dependent manner commencing at a concentration of 15μg/ml arecoline (0.1 mM) in keratinocytes (Jeng et al., 1999b).

Separately, one group reported arecoline as stimulating collagen production by fibroblasts (Harvey et al., 1986; Meghji and Harris, 1995; Scutt et al., 1987), while others have observed the reverse effect (Brandwein-Gensler and Hille, 2003; Haque et al., 2001; Shieh et al., 2004), with reports of complete inhibition of collagen synthesis (Brandwein-Gensler and Hille, 2003; Chang et al., 1999; Haque et al., 2001; Shieh et al., 2004; van Wyk et al., 1995).

Similarly, cytokine production has been reported as either reduced (Brandwein-Gensler and Hille, 2003; Haque et al., 2001) or increased in different studies (Brandwein-Gensler and Hille, 2003; Haque et al., 2001; Hsu et al., 2001). A generalized increase in a range of inflammatory cytokines has been reported in both the epithelium and lamina propria in response to arecoline, with these mediators including: interleukin (IL)-1 alpha, IL- beta, IL-6, transforming growth factor (TGF)-beta, platelet-derived growth factor, and basic fibroblast growth factor, while a reduction in interferon-gamma is seen (Haque et al., 1998). In other work, IL-8 secretion by keratinocytes has been shown to increase in response to arecoline (Cheng et al., 2000). Also IL-6 expression is significantly upregulated by arecoline and areca
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quid products (Tsai et al., 2004). Hue (2001) noted a reduction in IL-2, TNF-alpha, and TGF-beta in mononuclear circulating cells of healthy people who did not report a history of areca nut use and who were stimulated with arecoline (Hsu et al., 2001). Considering the response of fibroblasts independently, as described across multiple reports, there is a narrow range of arecoline concentrations at which cell proliferation and collagen synthesis are stimulated, this range being from 0.1 to 10 µg/ml (Canniff et al., 1986; Harvey et al., 1986; Meghji and Harris, 1995; Tsai et al., 1997). At higher concentrations, arecoline appears to be cytotoxic for fibroblasts (Chang et al., 1998; Chang et al., 2001b; Jeng et al., 1994; Jeng et al., 1999c; Tsai et al., 1997).

Similar results are reported for the effect of arecoline on keratinocytes, although these cells appear more resistant to the cytotoxic effects of arecoline. Cytotoxicity is again observed, although at higher concentrations commencing above 31-µg/ml, and causing a reduction in cell number in the order of 38% (Jeng et al., 1999c). Associated with this cytotoxicity, is an observed increase in prostaglandin E2 (PGE2) production and reduced IL-6 release by the keratinocytes (Jeng et al., 1999c).

3.1.2. Oral arecoline concentrations

The response of cultured cells to arecoline, as outlined above (3.1.1.), is highly dependent on concentration and suggests that a knowledge of arecoline concentrations in the oral cavity achieved during chewing is important to understand oral disease in nut chewers. At the time that work described in this thesis was performed, there was only one publication describing oral arecoline levels associated with areca nut chewing (Nair et al., 1985). Work described in this Chapter expands significantly upon this earlier report.
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Nair *et al.* (1985) used gas chromatography to quantitate oral arecoline levels in the saliva of six subjects chewing areca nut with and without tobacco. The concentrations of arecoline found in saliva varied greatly amongst subjects. It ranged from 2.4 to 142.9 µg/ml, with a mean value of 51.9 µg/ml in subjects who included tobacco with the 'chew'. However, if tobacco was excluded, then the levels of arecoline in saliva varied from levels below the sensitivity of the detection system up to 89.9 µg/ml, with a mean value of 29.7 µg/ml. Six subjects were included in each of the two groups. Unfortunately, it was unclear as to whether the lower arecoline levels associated with the use of tobacco reflected some particular effect of the tobacco, or of chewing habits associated with the presence of tobacco, or some other factor.

The high arecoline concentrations reported by Nair *et al.* (1985) in some individuals, would appear to be sufficient to stimulate collagen synthesis and also to reduce fibroblast proliferation, while there may also be keratinocyte cytotoxicity (Jeng *et al.*, 1999b; Jeng *et al.*, 2001; Wary and Sharan, 1991). Interestingly, however, the subjects in Nair's study were not reported as having clinical signs of oral submucous fibrosis or any other habit-associated lesions. This is consistent with the notion that the period of exposure is important, as well as perhaps individual, and potentially idiosyncratic, responses to areca nut. It is further possible that although substantial cellular injury occurs in many individuals using areca nut, that there is often no apparent detectable clinical change, with the consequence that epidemiological surveys for detectable oral disease may not fully assess the impact of areca nut use. This idea is consistent with the reported increased incidence of oral squamous cell carcinoma (OSCC) amongst areca nut users, despite relatively modest detectable pre-malignant changes (Merchant *et al.*, 2000). With regard to this, it is important to note
that increased keratinocyte ‘turn-over’ would be expected in response to arecoline cytotoxicity, and that such accelerated cellular turn-over is consistent with an increased incidence of malignant change.

3.1.2.i. Likely penetration of arecoline across the mucosal barrier

While acknowledging the concentration dependent effects of arecoline upon fibroblasts and keratinocytes outlined above in 3.1.2, it is important to recognize that this can only have biological meaning if arecoline is able to penetrate the mucosal barriers to enter the tissues. The potential of arecoline to enter the tissues and hence the circulation via the oral epithelium is important to properly interpret data presented in the chapter, and for this reason it is necessary to first discuss literature relevant to this issue.

By way of observation, the streets and thoroughfares of communities that indulge in areca nut chewing frequently have coloured markings on the ground where chewers have expectorated, discarding the areca nut along the path. This habit seems extremely common amongst users of the areca nut, and most users do not consciously swallow either the saliva or the nut. Thus while it is impossible to say that the effects of chewing the areca nut are obtained without swallowing it definitely seems to be the custom that the majority of users expectorate most or all of the saliva containing the areca nut and arecoline. Therefore, assuming that arecoline is a significant psychoactive ingredient, then appreciable levels of circulating arecoline are likely achieved by means of absorption across the oral mucosa prior to expectoration for most users.
3.1.2.ii. Demonstrated permeability of the oral mucosa

One of the primary functions of the oral mucosa is to provide a barrier, protecting the underlying tissue from substances in the oral cavity, as well as preventing the loss of body fluids into the oral cavity. This barrier function is not complete and many substances have been shown to pass across the epithelial surface (Siegel, 1984). This fact is utilized for administration of a number of medications including glycerol trinitrate, neostigmine, hyoscyamine, buprenorphine and buprenorphine/naloxone, all of which require sublingual administration (Marsh and Marsh, 2000; Moffat, 1971). These drugs not only penetrate the mucosa, but clearly achieve circulating concentrations sufficient for systemic therapeutic effects. In view of the psychotropic effects observed with areca nut chewing, as well as the known identical effect of arecoline (Asthana et al., 1996), it seems likely that similar pharmacokinetic effects are at work with arecoline during areca nut chewing.

The thickness and structure of mucosal surfaces greatly affects permeability. Mucosae with a recognized role in absorption such as those of the gastro-intestinal tract, tend to have a single layered epithelium. The oral mucosa, however, is covered by stratified squamous epithelium, while three different types of this epithelium are seen, each reflecting the highly localized functional demands of different parts of the oral cavity. The ‘masticatory’ mucosa covers the gingiva, hard palate and other areas subjected to significant mechanical forces of mastication. It has a keratinised epithelium and represents approximately 25% of the surface area in the oral cavity (Collins and Dawes, 1987; Squier, 1991). Approximately 60% of the oral cavity is covered by a non-keratinized ‘lining’ mucosa, which provides an elastic, deformable surface capable of stretching with movement during mastication and speech (Collins and
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Dawes, 1987; Squier, 1991). Being both non-keratinized and relatively thin, this area is the most permeable to exogenous agents. Finally, the ‘specialized’ mucosa covers the dorsum of the tongue which represents approximately 15% of the oral mucosal surface, and has properties in some ways intermediate to both the lining and masticatory mucosae, with both keratinised and non-keratinised epithelium (Collins and Dawes, 1987; Squier, 1991).

The main sites for absorption of drugs across the oral mucosa are the buccal and sublingual areas, in which the lining mucosa is very thin and richly vascularized. The oral mucosa is approximately ten times more permeable to water than skin. While there are differences in permeability to water within the mouth, these are relatively minor. For example, there is only a two-fold difference between the least permeable mucosal surface, being the palate, and the most permeable surface, which is the floor of the mouth. The buccal mucosa and lateral border of the tongue have a permeability value intermediate between that of the palate and floor of the mouth (Lesch et al., 1989).

3.1.2.iii. Mechanisms of transport across the oral mucosa

Substances can cross various epithelial membranes by means of simple diffusion, carrier-mediated diffusion, active transport or pinocytosis. However it appears that within the oral cavity the main mechanism is simple diffusion, and to a lesser degree carrier-mediated transport (Siegel, 1984).
3.1.2.iv. Transport by simple diffusion

Simple diffusion across an epithelial barrier can be mathematically modelled and has been expressed in the following equation (Siegel, 1984):

$$K_p = \frac{Q}{A \cdot t \cdot (C_0 - C_1)}$$

Where:

- Q is the quantity of compound traversing the tissue in time ‘t’ (minutes)
- C₀ and C₁ are the concentration on the outer (epithelial) and inner (lamina propria) sides of the specimen
- A is the area of exposed tissue, measured in square centimetres - cm²
- Kp is the permeability constant, and is expressed in units of centimetres per minute.

3.1.2.v. Solute properties influencing permeability

3.1.2.v.1. The oil/water distribution ratio (R)

Comparison has been made of the oil : water ratios (R values) of several alkaloid drugs with the ratios of sublingual to subcutaneous doses needed to produce similar pharmacological effects (Walton, 1935). Drugs with a high values for R require much lower doses than drugs with lower R values in order to produce pharmacological effects similar to those obtained by subcutaneous injection (Siegel, 1984). The effect of this is that the more lipophilic an agent is, the more effectively it will penetrate the epithelial barrier.
3.1.2.v.2. The effect of concentration gradient on diffusion rate

Consistent with the equation shown above, is a linear relationship between solute concentration gradient and the amount transferred across a diffusion barrier. For example, a 10-fold increase in the concentrations of either of the local anaesthetic agents lignocaine or prilocaine, results in an approximate 10-fold increase in the rate of transfer across canine lingual mucosa (Bergman et al., 1969), while a similar relationship is reported for ascorbic acid (Alvares and Siegel, 1981). Linearity of absorption has also been demonstrated over a 32-fold range of concentrations for two ‘adrenergic’ blocking drugs, propranolol and antenolol. Although these observations support the importance of simple diffusion in the oral absorption of drugs, a possible additional contribution by facilitated diffusion can be neither excluded nor readily confirmed from such limited kinetic data (Siegel, 1984).

3.1.2.vi. The effect of pH and other environmental factors on mucosal absorption

Observations suggesting that acidic and basic drugs penetrate the oral mucosa by passive and not facilitated diffusion, include identical absorption from mixtures as from purified preparations, and an identical percentage absorption regardless of concentration. Important for the current study, is that absorption of agents which are either weak acids or bases, depends critically upon the relative concentrations of non-ionised material, rather than the total concentration of substance present. It is also important to note that pH has a profound effect on the charge state of drugs, and thus has the potential to significantly affect passage across the oral mucosa.
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Most drugs are weak organic electrolytes and the extent of ionisation is important in determining rates of membrane penetration (Moffat, 1971). Increasing the pH of the oral environment will facilitate the absorption of basic drugs, since the concentrations of lipid soluble non-ionised forms increases, while a similar effect is seen for acidic drugs when the pH is reduced. While the pH of saliva lies between 6.0 and 7.4, changes in salivary pH affect the proportions of weak organic acid or base in non-ionised forms, and thus the rates at which such compounds cross the oral mucosa.

Under physiological conditions, the absorption of weak electrolytes with pKa values close to physiological pH are significantly affected by slight changes in oral pH (Siegel, 1984).

When considering arecoline, which has a pKa of 6.84, there is a preponderance of non-ionised, and thus lipophilic molecular forms in saliva. If an alkaline environment is created in the oral cavity, by including slaked lime with the betel quid, there is ready hydrolysis of uncharged arecoline to the negatively charged arecaidine (Von Nieschulz, 1968). Arecaidine penetrates the mucosa at a slower rate than arecoline, which is its methyl ester, while conversely, neutral or acidic conditions appear to promote transmucosal diffusion of arecoline (van der Bijl et al., 2001).

Separately, mucosal permeability can also be altered independent of pH by exogenous substances, including surface-active agents widely used in toothpaste and mouthwashes (Siegel, 1984). Some enzymes and mucolytic substances are also reported to enhance penetration of the oral mucosa, presumably by removing or altering surface coverings. The presence of areca nut extract itself, leads to a
3.1.3. Oral mucosal permeability can be changed by systemic factors

3.1.3.i. The effect of nutritional deficiencies on mucosal absorption

Systemic factors can alter the permeability of the oral mucosal membrane. Measurements of permeability, in vitro, demonstrate that vitamin C deficiency leads to an increase in mucosal permeability (Alfano et al., 1975; Alvares and Siegel, 1981; Siegel, 1984). In addition, the permeability of the sulcular epithelium is decreased in patients given supplements of vitamin C (Siegel, 1984). From this it is perhaps not surprising that malnutrition, also associated with alcohol abuse, is linked to discernable changes in mucosal integrity (Enwonwu, 1994; Enwonwu and Meeks, 1995; Strickland, 1998).

3.1.3.ii. The effect of age and oral mucosal disease upon absorption

Clinically mucosal atrophy is observed with age; however, there seems no clear effect of this apparent thinning on mucosal permeability (Alfano, 1978; Squier, 1991).

Separate to ageing, is oral mucosal atrophy and necrosis associated with pathological conditions such as lichen planus, viral infections, pemphigus, allergic reactions and
radiation or chemotherapy-associated mucositis. In such situations, atrophy or loss of
the epithelium, with or without altered mucin production, may disrupt the major
permeability barrier (Squier, 1991).

3.1.4. A need for further investigation of oral
arecoline concentrations and mucosal absorption

Although a wide range of intraoral arecoline concentrations are reported consequent
to chewing the areca nut, with or without tobacco, a need for further evaluation is
apparent (Nair et al., 1985). Unfortunately, the published study does not provide
information on the kinetics of oral arecoline levels during chewing, or any potential
daily variability in concentration.

In addition, the basis for the wide range of concentrations observed remains unclear
from the single available published study (Nair et al., 1985). Salivary concentrations
are reported to range from 2.4 to 142.9 μg/ml when tobacco is included in the ‘chew’,
and from 0.0 to 89.9 μg/ml when tobacco is not included, with corresponding
differences in mean arecoline level between the two groups. Assuming that Nair et al.
(1985) provided a similar amount of areca nut to each participant, the highly variable
salivary concentrations observed suggest potentially interesting kinetic or other
idiosyncratic variability amongst users of the nut, as well as possible effects of
tobacco on the release, uptake and distribution of arecoline between saliva and
tissues.
As noted in Chapter 2, oral mucosal changes are present in a sub-group of the population using the areca nut. The frequency of such presentations is reported to reach up to 40% of the areca nut chewing population (Pearson et al., 2001), and although this is substantially higher than observed in the study described in Chapter 2, the wide range in the incidence of oral lesions seen could reflect underlying differences amongst users in the concentrations of salivary arecoline achieved during chewing.

3.1.5. A need for development of a new protocol for the measurement of salivary arecoline using high pressure liquid chromatography

Most of the burden of disease related to the use of the areca nut is within the developing countries of Asia, so that research into areca nut use is facilitated by development of relevant laboratory techniques that may be more readily available in these countries. Previously reported studies used gas chromatography to determine arecoline concentration or to distinguish arecoline from other alkaloids in saliva (Nair et al., 1985; Self et al., 1999). One objective of the study described in this Chapter was to develop an alternative approach to arecoline measurement which was of comparable accuracy to gas chromatography but more accessible. As described in this Chapter, high performance liquid chromatography (HPLC) was found to be an effective alternative technology to gas chromatography, while at the same time being more widely available in the developing world.
3.2. Materials and Methods

3.2.1. Materials

3.2.1.i. High pressure liquid chromatography and mass spectrometry equipment

A Shimadzu HPLC-MS system was used for the detection of arecoline (Shimadzu Pty Ltd Kyoto, Japan). The HPLC system included a LC 10AD model dual piston pump, DGU-3A degasser, SIL-10A automated injector, CBM-10A controller, and a CTO-10A oven. All experiments used a Luna 5 μm particle size, 100 Å pore size, 250 mm x 4.6 mm internal diameter, stainless steel, C18 reversed-phase column (Waters Pty. Ltd., Sydney, Australia). Electron spray mass spectrometry (MS) was used to detect arecoline (Shimadzu Pty Ltd, Kyoto, Japan), with an atmospheric pressure chemical ionisation electro-spray interface (ESI), controlled by Shimadzu HPLC (Version 1.0) software on a 486 PC computer.

3.2.1.ii. Reagents

Arecoline (methyl-1,2,5,6-tetrahydro-1-methylnicotinate), arecaidine (1,2,5,6-tetrahydro-1-methylnicotinic acid), triethylamine, and caffeine were from Sigma Pty. Ltd. (St Louis, USA). HPLC analytical grade acetonitrile was from Unichrom Co (Kuang-Fu Rd, Taipei 100, Taiwan ROC). Lignocaine was from Astra Zenica (North Ryde, Sydney, Australia). Areca nut was obtained from local asian ‘nut and spice’ shops in Sydney.
The areca nut chips were obtained supplied in 50 gm packages from Roshan Naidu’s (Importer and Distributor, Parramatta, NSW), while the inert rubber-based impression material was 3M Imprint II (vinyl polysiloxane, Light body) from 3M Dental Products, (St Paul MN).

3.2.1.iii. Plastic ware

Ten mL polyethylene tubes with screw tops as well as Eppendorf disposable plastic tubes were purchased from Crown Scientific Pty. Ltd (Sydney Australia). Disposable C_{18} extraction cartridges were from Alltech (Deerfield, IL., USA).

3.2.1.iv. Calibration solutions

Calibration solutions of arecoline from 50 to 500 ng/ml, and lignocaine from 0.001 ug/ml to 50 ug/ml were prepared in Milli-Q water via serial dilutions.

3.2.2. Methods

3.2.2.i. Questionnaire completed by volunteers

To obtain relevant information about participants, a survey essentially similar to that used for the earlier Australian survey (Appendix) was used. Important refinements included questions relating to the different localities of habitation, the type of product used, the frequency of use, the amount chewed, the duration of chewing and the last occasion when the habit was practised.

The modified questionnaire was reviewed from the perspective of clarity of meaning and flow of questions by two staff members at the Westmead Centre for Oral Health
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as well as by four students, three of whom spoke a language other than English at home. Minor modifications were made in response to this review. An important change was inclusion of a word list in multiple languages for the areca nut, which recognized that while some participants may be unfamiliar with the term ‘areca nut’, they would be familiar with the traditional name of the nut.

3.2.2.ii. Collection of mixed and parotid saliva

Thirty-two subjects who had an areca nut chewing habit were recruited into the study. Mixed saliva was collected from 32 subjects who were asked to spit into 10 ml disposable centrifuge tubes before being given 0.5 gm of areca nut to chew, and then collecting all saliva generated by spitting into further centrifuge tubes, with fresh tubes provided at 1, 3, 5, 10, 15, 20 and 25 minutes. Participants were then asked to remove all remaining nut particles from the mouth, and then saliva samples were collected for up to a further 20 minutes, changing tubes at five minute intervals.

While thirty-two subjects provided saliva during areca nut chewing, six of these subjects repeated the procedure over three consecutive days. In addition, parotid saliva was also collected from these six subjects using a Lashley cup, normally placed over the punctum of the right parotid duct.

Saliva samples were centrifuged at 402 x g (times gravity or 3,000 rpm), decanted into fresh tubes and snap frozen in liquid nitrogen before storage at -20°C. Controls consisted of subjects with no reported areca nut chewing habit, who instead of being given areca nut to chew, were asked to chew inert rubber-base impression material cut to approximate a size similar to the 0.5gm of areca nut.
3.2.2.iii. Determination of salivary flow rates

Salivary flow rates were determined by weighing centrifuge tubes and assuming a specific gravity for saliva of 1g/ml. Parotid salivary flow rates were determined both before and after chewing areca nut. In brief, saliva was collected using Lashley cups placed over the opening of Stenson’s duct. Stimulation of salivary flow was achieved by giving the subject 5.0 gm of areca nut to chew. The volume of saliva was measured in tubes by weight assuming the specific density of saliva to be 1g/ml. The flow rate for each subject was expressed in ml/min. All samples were collected in the morning between 9 am and 11.30 am. Mixed saliva was also collected from these individuals by asking subjects to spit into 10 ml plastic cups. The measurements of both the mixed and parotid saliva were recorded over the three consecutive days.

3.2.2.iv. Conditions for detection of arecoline by mass spectrometry

MS instrument parameters were optimised to obtain protonated molecules together with some characteristic fragments. The following ESI-MS conditions were found to be effective for detection of arecoline: nitrogen for drying was heated to 350°C and applied at a flow rate of 10.0L/min; nitrogen as a nebulizer was applied at a pressure of 40 psi; capillary voltage was set at 1500V; fragmentor voltage applied at the exit end of the capillary was at 110V; the dwell time was 139 ms; and the mass peak width was 0.7th (FWHM). Qualifying ions were identified with mass : charge (m/z) of 156, and 197 for arecoline, and m/z 235 for lignocaine. The acceptance criterion for ion intensity ratios was a deviation of approximately 20% of the average of the ion intensity ratios of all the calibrators. The ions at m/z 156 and m/z 197, for arecoline,
were used for quantification. These optimum MS parameters for arecoline were
determined following direct infusion of a solution in acetonitrile into the mass
spectrometer. The mass spectrum obtained by negative ion electron-spray contained
an intense [M-H]- molecular anion isotope group (Fig 3.1). Two of these ions, at m/z
156 and m/z 197 produced similar product-ion mass spectra when subjected to
collision-induced dissociation using argon. Lignocaine with a m/z of 235 was used as
the internal standard. Factors other than concentration contribute to the height and
width of peaks measured in MS spectra. To correct for this variability, arecoline
levels in individual samples were expressed in terms of a ratio between peak heights
for the arecoline peak as compared with that of the internal lignocaine standard.

3.2.3.v.  The preparation of samples for HPLC/MS

3.2.3.v.1.  The preparation of saliva samples for HPLC

All saliva samples were centrifuged to remove areca nut and any solid material prior
to freezing. Fifty-micro-litre volumes of thawed saliva were mixed with 50μl volumes
of stock Lignocaine and added to 200 μl of acetonitrile. In most cases, the
concentration of stock lignocaine was 3 μg/ml, however, where arecoline levels were
very low and required re-evaluation by HPLC, the stock solution of lignocaine was at
a concentration of 1.5 μg/ml. Solutions were vortexed for 1 minute and centrifuged
for 5 minutes prior to HPLC.
Figure 3.1

A typical mass spectrograph for arecoline dissolved in water. Major peaks for arecoline was at m/z 156, (and 197 for an adduct of arecoline) and lignocaine was at 235, and these were used for quantitation. Minor peaks were also observed.
3.2.3.v.2. **The preparation of standards for quantitation of arecoline in saliva**

To prepare standards for quantitation of arecoline in saliva, fresh saliva samples from the author, were spiked with arecoline at increasing concentrations to achieve final concentrations in saliva ranging from 0.01 μg/ml to 50 μg/ml. In addition, lignocaine (3μg/ml) was used as an internal standard. The ratio of the relative proportion of arecoline to lignocaine, as determined by the height of the MS spectral peaks was used to establish standard curves ranging in arecoline concentration from 0.01 to 50 μg/ml. To further evaluate the accuracy and reproducibility of this measurement approach, fresh saliva samples were spiked with 50 ng/ml of arecoline and repeatedly measured. Recovery was estimated by using four different concentrations of arecoline in duplicate spiked saliva: 100, 150, 200, 250, and 300 ng/ml.

3.2.3.v.3. **The effect of pH on the recovery of arecoline from HPLC/MS**

To determine the effect of pH on arecoline recovery, aliquots of Milli-Q water were adjusted with a solution of 0.1 M sodium phosphate and a solution of 0.1M calcium carbonate to achieve pH values of 4, 6, 7, 10.5 and 11, with the pH level confirmed using a TPS digital pH meter (Model 1852, TPS Pty Ltd Brisbane). The solutions were then spiked with arecoline, 1 μg/ml, prior to the evaluation of arecoline concentration by MS.
3.2.3. vi. High pressure liquid chromatography

Samples of neat saliva were analysed by reverse-phase chromatography using a Luna C_{18} column at 30°C and a mobile phase composed of 50% acetonitrile and 50% 0.01 M sodium hydrogen-phosphate (pH 7.8) with 0.01% triethylamine at isocratic flow rates ranging from 0.3 to 1.2 ml/min. Dependent on the levels of arecoline found in samples, accurate quantitation with reference to standards required application of either 3, 6 or 10 ul volumes to the column. Where the concentration of arecoline was less than 1 μg/ml, samples were diluted at a ratio of 1:2 with Milli-Q water in acetonitrile.

3.3. Results

3.3.1. Salivary arecoline measurements by mass spectrometry were reliable and reproducible

As indicated in Figure 3.1, arecoline appeared as a discrete spectral peak with MM of 155 Da, and a column retention time of 1.6 min, while the internal standard found most appropriate for MS detection was lignocaine, with a MM of 234 Da and a column retention time of 3.7 min. It was possible to quantitate arecoline concentration using MS spectra, by measurement of the peak height specific for arecoline, and comparison with a standard constructed from a range of samples of known concentration (Figure 3.2). Standards were assessed each time that MS was performed, so that new standard curves were prepared for each experimental session. The lower limit of detection was 50 pg of arecoline.
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When further characterizing the reproducibility of arecoline measurement in this way using saliva spiked with arecoline (50 ng/ml) and repeating measurements 5 times on one occasion, there was less than 1.2% variability in the recorded measurements.

Providing further confidence in the reproducibility of arecoline measurements by HPLC/MS, when single samples were assessed on 5 separate occasions, there was at most only 2.5% variability between measurements. Importantly, when individual samples were evaluated for arecoline concentration up to 2 months apart, there was no significant or consistent variation in quantity detected, indicating sufficient stability of arecoline in saliva for meaningful quantitation of samples over time.
Figure 3.2

Arecoline standard curves for concentrations ranging from 0.01 – 1 µg/ml (A) and 0.01 – 50 µg/ml (B) relating the ratio of the peak height of arecoline to lignocaine. A linear relationship was consistently seen between the concentration of arecoline and the ratio of arecoline and the internal standard, Lignocaine. The standard working solutions for arecoline were as follows: 50µl of arecoline (1M) in water plus 50µl of 3µg/ml of Lignocaine in water plus 200µl of Acetonitrile. The mobile phase was 30%ACN, 10mM NH₄F, 0.0025%FA, pH 5-6, 0.2ml/min.
A

\[ y = 0.128162x \]
\[ R^2 = 0.999511 \]

B

\[ y = 0.127074x \]
\[ R^2 = 0.999601 \]
3.3.2. Ionization of arecoline increased with rising pH

The concentration of arecoline in Milli-Q water, as detected by HPLC/MS, varied with the alteration of the pH of the solution. Figure 3.3 demonstrates an apparent variation in the concentration of arecoline (relative to a neutral pH of 7) in acidic and alkaline solutions. This possibly reflects increasing ionization of arecoline with increasingly alkaline conditions. It was found that arecoline measurements increased substantially with rising pH, such that at pH 10 there was approximately three times more arecoline detected while at pH 10.5 this rose to over four times the level detected at pH 7. There was a minimal arecoline detected in acidic conditions.
Figure 3.3

The concentration of arecoline as detected by HPCL/MS in aqueous solutions of varying pH. The detected concentration of arecoline altered according to the pH of the solution. High levels were detected in alkaline solutions with the highest level at pH 10.5, being over four times that at a neutral pH.

Arecoline solution of 1μg of arecoline in 20 μL of Milli-Q water (equivalent to 500ng on the column)
3.3.3. Participants were primarily from the Indian Subcontinent and used areca nut on a regular basis

Table 3.1 summarizes the data obtained from the questionnaire for participants in this study. The majority of the 32 participants who used the areca nut were from the Indian sub-continent, with 17 coming from India, 6 from Sri-Lanka, and 1 from Bangladesh. Other participants using the nut were from Malaysia (1), Taiwan (1) and Australia (6). Four of the 6 participants who did not use the areca nut but who participated as control subjects were from India, while 2 were from Sri Lanka.

Approximately one half of participants were male (53%), while the age of participants varied from 17 to 49 years, with a mean age for males of $28.8 \pm 10.5$ years and $34.2 \pm 10.1$ years for females.

The frequency of areca nut use was important for this study, since it was considered possible that arecoline levels might be constitutively high in users of the nut. The distribution of 'frequency of use' for the areca nut by participants according to sex is shown in Figure 3.3, and it is apparent that most participants used areca nut on a regular/semi-regular basis with 78% using it at least once a month.

The age at which areca nut use commenced is also displayed in Figure 3.4. Most participants commenced use of the areca nut during early childhood or adolescence with the earliest age of use being 5 years, although a few participants first used the areca nut in early adult life,
### Table 3.1 Summary of data obtained by questionnaire from participants

<table>
<thead>
<tr>
<th>Participant</th>
<th>Age</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Age when areca nut use commenced</th>
<th>Frequencies of areca nut use</th>
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<td>1 / Month</td>
</tr>
<tr>
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<td>F</td>
<td>Goan</td>
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<td>1 / Month</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>39</td>
<td>M</td>
<td>Indian</td>
<td>14</td>
<td>1-2 / Month</td>
</tr>
<tr>
<td>9</td>
<td>34</td>
<td>F</td>
<td>Indian</td>
<td>19</td>
<td>2-3 / Month</td>
</tr>
<tr>
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<tr>
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<td>M</td>
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<td>25</td>
<td>F</td>
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**Controls**

<table>
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<th></th>
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</tr>
</thead>
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<td>F</td>
<td>Indian</td>
</tr>
</tbody>
</table>

Thirty two subjects indicated they had an areca nut chewing habit, while 6 subjects did not report any areca nut use and comprised the control group. There was no significant difference between males and females with regard to age, while the mean age was 31.3 ± 10.5 years, with the mean ages of males (17) and females (15) being 28.8 ± 10.5 and 34.2 ± 10.1 years respectively. (The mean age for the control was 36.7 ± 9.0 years)
Figure 3.4

Histogram demonstrating the frequency of areca nut use amongst a sample group of Australian females (closed bars), and males (open bars). Approximately 16% of the subjects used areca nut more than four times in a month, 80% of the females and 76% of males chew areca nut on a monthly basis, while only 22% considered themselves as occasional users of the areca nut.
Frequency of Oral Habit

- **Daily/Weekly**
- **4+ per Month**
- **3-4 per Month**
- **1-2 per Month**
- **Occasional**

**Number of Individuals**

- **Female**
- **Male**
Figure 3.5

Histogram showing the age at which participants first started to use the areca nut.

Twenty-eight percent of participants commenced using the nut before the age of 10, with 66% commencing between the ages of 10 and 20.
3.3.4. Arecoline levels in whole mixed saliva before, during and after chewing areca nut were sufficient to stimulate cellular responses and cytotoxicity

Figure 3.6 shows the maximum and minimum values for salivary arecoline observed in individual participants in each experiment. It is clear that in all cases studied the levels of arecoline in saliva of individuals chewing areca nut exceeded either the 0.1 µg/ml or the 10 µg/ml thresholds for cell stimulation or cytotoxicity respectively.

Only seven of the 32 areca nut chewers studied had no detectable arecoline in their saliva at the commencement of the study, ‘0’ time point, so that 25/32 (78%) of subjects had residual salivary arecoline from earlier chewing episodes. Baseline levels of arecoline measured before chewing ranged from undetectable levels to 2.4 µg/ml, and in 21 of the subjects the level was above the 0.1 µg/ml, considered sufficient to stimulate cellular responses.

The maximum concentrations of arecoline achieved during areca nut chewing ranged from 5.66 to 97.39 µg/ml. No clear difference was seen between male and female subjects with regard to the peak concentrations of arecoline achieved (Figure 3.7).
Figure 3.6

The range of concentrations of arecoline detected in saliva of individual participants chewing areca nut, with participants coded as numbered in Table 3.1. In all subjects studied, arecoline levels reached concentrations above the 0.1 µg/ml reported to stimulate cell activity, while in 24 out of 32 subjects, arecoline levels were above the 10 µg/ml concentration threshold (marked with a horizontal dashed line) reported to cause cell death.
Figure 3.7

Maximum concentrations of arecoline achieved during areca nut chewing according to sex. In all cases, arecoline levels were sufficient to stimulate cellular responses (>0.1 μg/ml), or cytotoxicity (>10 μg/ml) (marked with a horizontal line), while there was no clear difference between the sexes with regard to maximum levels of arecoline achieved.
Peak levels of salivary arecoline were always reached during the time of chewing (p < 0.0001), although the precise time that this occurred varied during the 25 minutes that participants chewed areca nut (Figure 3.8), with high peak levels usually reached by 3 minutes of chewing (p < 0.03). In 14 participants, more than one peak in salivary arecoline concentration was observed (Figure 3.9), and this occurred before the highest peak level was reached in six cases. In one participant, there was a surprising third and lower peak in salivary arecoline levels collected between 40 and 45 minutes, being 20 minutes after the nut had been removed from the mouth. No significant difference was noted between males and females or with regard to the response of people with different countries of origin. Peak concentrations for arecoline in saliva was rarely the same between the subjects despite the same quantity of areca nut being chewed.

Although arecoline levels were substantially lower after removal of the nut from the mouth at the 25-minute time point (p < 0.001), levels still exceeded those required to stimulate cells in culture in most participants (p < 0.001) (Figure 3.8). Note that the concentration of arecoline in saliva was generally above 0.1 µg/ml.
Figure 3.8

Arecoline concentrations in saliva samples of all subjects chewing areca nut over a 25 minute period (arrow) as well as for 15 minutes after removal of areca nut from the mouth. The concentration of arecoline was above 0.1 µg/ml in all subjects for almost all of the time (dotted line), while the 1.0 µg/ml (light intermittent line) threshold was reached most of the time in most subjects and the cytotoxic concentration of 10 µg/ml (dashed line) was achieved in all but five cases.
Figure 3.9

Times at which the highest, second highest and third highest peak salivary concentrations were reached in participants. In most participants, the highest levels of arecoline were achieved within 3 minutes of chewing, while in one subject, this did not occur until shortly before removal of nut from the mouth at 25 minutes (arrow). A second and lower peak concentration was observed in many participants, while in one case, a third and still lower peak was found in the final salivary sample collected.
3.3.5. Secretion of arecoline by the parotid glands

To investigate the possible secretion of arecoline by the salivary glands, parotid and whole mixed saliva was collected simultaneously from six subjects. Figure 3.10 summarises the observations of experiments with all six participants. Parotid salivary arecoline levels were typically an order of magnitude lower than those found in mixed saliva (p < 0.01). Levels of arecoline in parotid saliva paralleled those in whole saliva, although a delay of up to 30 minutes was seen in peak parotid salivary levels for 4 out of 6 subjects. Similar results were obtained for individuals on each of the three days that they were studied, particularly with regard to the highest concentrations of salivary arecoline achieved, while the major differences between individuals were seen across all three experiments performed on consecutive days.
**Figure 3.10**

The concentration of arecoline (μg/ml) in parotid saliva over three consecutive days (●, +, ×), as well as in whole mixed saliva during parotid saliva collection (■, ◆, ▲) for each of the six subjects participating in the study. Higher levels of arecoline were always detected in whole mixed saliva as compared with parotid saliva. There was a high level of consistency for readings over consecutive days for individual subjects. Two of the subjects (E,F) had arecoline levels in the parotid saliva consistently below 0.1μg/ml whereas four subjects (A,B,C,D) had concentrations that rose up to 1 ug/ml, and two of these (C,D) rose above this level, though not consistently. The presence of appreciable levels of arecoline in parotid saliva suggest secretion of arecoline in the saliva after initial absorption, and this appeared to occur within 10 minutes of initiating chewing.
3.3.6. Areca nut chewing substantially increased salivary flow rates

Figure 3.11 shows differences in salivary flow rate between individuals during and after areca nut chewing. The mean salivary flow while chewing the areca nut was 1.6 ml/min, (SD 0.9), while chewing, and 1.0ml/minute (SD 0.5) after the nut was removed from the mouth (p<0.000). The flow of parotid saliva via Stensen’s duct was 0.2 ml/min while chewing the nut, and 0.1 ml/min after the nut was removed from the mouth. There was no significant difference in the flow rates of the parotid gland.
**Figure 3.11**

Scatter diagrams demonstrating the salivary flow rate with or without areca nut chewing in whole mouth saliva (A) and parotid gland saliva (B).

**A.** Individuals chewing the areca nut had a mean whole saliva flow of 1.6 ml/min ranging from 0.06 to 4.5 ml/min and then reduced to 1.0 ml/min ranging from 0.02 to 2.3 ml/min after chewing ceased. The difference in flow rate between chewing with and without areca nut was significant ($p<0.007$).

**B.** Similar observations were made for parotid flow where areca nut chewing induced a mean flow rate of 0.2 ml/min, ranging from 0.01 to 0.9 ml/min and dropping to 0.1 ml/min, range 0.002 to 0.4 ml/min, after chewing. The difference in flow rate in parotid secretions between chewing with and without areca nut was again significant ($p<0.02$).

(The flow rates were measured over varying time periods (1, 2, 5 and 10 minute intervals).
3.4. Discussion

This is the first time that HPLC has been used to determine the concentration of arecoline in saliva. The use of HPLC allows for a relatively rapid and inexpensive method for determining the concentration of arecoline in saliva, thus making this technique available to many developing countries, which have a high prevalence of oral habits involving the areca nut. Hence this is a means of enabling basic research in countries that experience a heavy disease burden that is linked to this habit.

It was anticipated that the results obtained using reverse-phase liquid chromatography with the HPLC-MS system would be comparable to those obtained with the gas chromatography method for determining the concentration of arecoline in saliva (Nair et al., 1985), although HPLC had not been previously used in this regard. A high level of reproducibility and precision was achieved as demonstrated in experiments spiking saliva with known quantities of arecoline. The results of the present study demonstrated arecoline at a level slightly lower than that found in the study by Nair et al (1985). Nair demonstrated levels of arecoline in the saliva up to 140 µg/ml with a mean value of 52 µg/ml in subjects who both smoked cigarettes and chewed areca nut, although levels were lower (mean of 30 µg/ml) in subjects who had the single habit of chewing areca nut. The results of the present study gave a maximum arecoline concentration of 97.4 µg/ml with a mean of 37.2 µg/ml during the 'chewing' phase of the study. It would therefore seem that the results of the current study confirm those of Nair, although the methodology in the Nair study is perhaps less clear since saliva samples were obtained once or twice during chewing and then snap frozen and stored at -20°C. The present study found that the level of arecoline was always higher if the residue of the nut had not been removed from samples prior to
freezing. Indeed each cycle of ‘freezing-thawing’ lead to an increase in the level of arecoline concentration if remnants of the nut were not first removed from the saliva. (Nair et al., 1985).

The presence of arecoline in the saliva of some of the subjects prior to the study prompted a re-examination of the data related to the breakdown of arecoline in the body. The presence of a second peak in many subjects also raises the question as to whether arecoline is re-secreted in the saliva of subjects who are chewing the nut.

Arecoline can exist in both non-ionised and ionised forms in the mouth, with a pKa of 6.8, and therefore will become more ionised if the pH of the oral environment rises, which can occur if the subject uses slaked lime or smokes tobacco (Johnson and Bain, 2000). The study by Nair et al. (1985) indicated that higher levels of arecoline were found in subjects who used tobacco (Nair et al., 1985). This therefore presents an anomaly, for it seems that the higher the pH the more ionised it is and hence harder to cross the mucosal barrier. This may indicate that higher levels of arecoline were found in the mouths of those who had a more alkaline pH, due to the tobacco, and hence less of the arecoline remained in the non-ionised form available for absorption across the mucosal barrier, thus recording a higher level of arecoline in the mouth.

It would be interesting to investigate this further by examining arecoline levels in the plasma of subjects who use and who do not use tobacco and or slaked lime with the areca nut.
Arecoline Levels in Saliva

Arecoline was identified in the saliva from the parotid duct in subjects who had been instructed not to swallow any saliva during the period of the study. The resultant chromatograph demonstrated the presence of arecoline in the saliva from the parotid duct within the first minute, and concentration levels of the arecoline frequently ‘shadowed’ the levels found in the mouth but less, by a factor of approximately ten. These levels also appear with a time delay. However, there were some subjects who presented with arecoline in their saliva at time zero, indicating they had low levels of arecoline in their mixed saliva even before the study commenced. There are at least three possible explanations for this. Firstly, that the residual arecoline is possibly not secreted completely by the kidneys. The reason could possibly be due to the chemical structure of arecoline preventing movement of the drug into a high salt fluid such as urine, but instead establishing a ‘steady state’ equilibrium and allowing arecoline to move from systemic blood into saliva in the salivary glands, and from a tissue bank back into saliva. Secondly, it is possible that the non-ionized form of arecoline becomes sequestered in fat, and is slowly released into the circulation between chewing episodes. Finally, it is possible that arecoline is absorbed into dental plaque and slowly released, hence the level of arecoline in saliva might reflect the oral hygiene state of the patient.

This research demonstrates that arecoline has been detected in the saliva from the duct of the parotid gland. The concentration levels of the arecoline in the saliva is delayed compared to that found in the mouth, and the subsequent rise and fall in whole saliva is shadowed by the concentrations of arecoline observed in the duct saliva. In view of the fact that all participants in the study were advised to expectorate all saliva into the collection vials and not to swallow any saliva, then a reasonable explanation for the
Arecoline Levels in Saliva

presence of arecoline in the saliva in the duct of the parotid gland is that arecoline has been absorbed across the mucosal membrane of the mouth. Absorption of substances across the oral mucosal membrane has been described previously for other substances (section 3.1.2) and thus the absorption of arecoline across the oral mucosal barrier is probable.

This appears to be the first time that a substance absorbed across the oral mucosa has been documented to be present in the saliva. Previously entero-salivary recirculation has been observed with a number of substances including heavy metals and alkaloids (Lord et al., 2002; Richelmi et al., 1980; Wilhelm et al., 2002).

These results demonstrate that the concentration level of arecoline in the mouth varies significantly over time, if the subject spits out the juice of the areca nut. These levels are generally well above those required for stimulation of collagen synthesis, (0.1 µg/ml) as well as being cytotoxic for the cell (above 10.0 µg/ml). While the time period in the study was over a forty-five minute period it is accepted that the possibility exists for very high concentrations to be present in the mouth if the nut is used over extended periods of time, especially if the subject were to sleep with the areca in the mouth. Cytotoxicity would apply to fibroblasts as well as keratinocytes and endothelial cells.

The monitoring of the arecoline concentration levels, presented in this document, provides a dynamic picture of the fluctuation of these levels, with levels in the parotid duct saliva shadowing the levels found in the mouth, though greatly reduced. For the first time there is documentation supporting the idea that some individuals are more
Arecoline Levels in Saliva

'at risk' of developing an unusual response to the chewing of the areca nut, and that a small portion of the population that chew the areca nut can achieve a very high concentration level of arecoline. This could reflect a number of variables including pH of the saliva, rate of chewing of the areca nut, which in turn may reflect different personalities, stress levels. However, it was notable that there seemed to be a small sub-group of females who tended to have higher levels.

Unstimulated salivary flow rate is approximately 0.48 ml/min (SD 0.1-2.0 ml/min) (Fenoll-Palomares et al., 2004). This research demonstrates that the areca nut functions as a sialogogue. The mean value of the salivary flow rate for the 6 subjects in this study was 1.54 ml/min (SD 0.73) over three consecutive days. Once the nut was removed from the mouth the flow rate dropped to 1.05 ml/min (SD 0.41), but remained above the unstimulated salivary for the duration of the study.

Further research should explore these unknowns. Saliva samples should be taken from individuals who routinely use the areca quid at times when they are about to expectorate, to explore the levels of concentration normally reached by regular chewers. This research has identified individuals who appear to have a particular propensity to develop very high levels of arecoline on a regular and repeatable basis. The pH and buffering capacity of the saliva should be analysed in these individuals and to determine whether chewing patterns are a significant contributing factor. The mean concentration of arecoline in saliva is lower than expected. The highest concentration of arecoline in the mixed saliva of subjects in this study was 97.4 (SD 25.3) μg/ml, however this level was not sustained over any significant period of time.
Arecoline Levels in Saliva

The average of the highest concentrations during the active period (20 min of active chewing of the areca nut) in this study was 37.2 μg/ml of arecoline.

This may have implications for the laboratory studies that have been documented, where tissue is placed in high concentrations of arecoline for a long period of time. This situation does not reflect the general nature of the mouth, although it may reflect local areas immediately adjacent to the nut or betel quid.

In addition, this study demonstrates the reliability of the data, and for the first time demonstrates that individuals are unique in the manner in which they achieve a concentration of arecoline in the saliva. While Table 3.4 provides results that are similar to those of Nair et al (1985), Table 3.5 demonstrates that each individual achieves a level of arecoline concentration that is similar to their own previous studies but different from others (Nair et al., 1985).

Some factors that may contribute to the variation in arecoline concentration include salivary flow rate, the pH of the saliva, the rate at which the nut is masticated, the rate at which the arecoline is absorbed across the mucosal membrane. The results presented in Table 2 demonstrate the variability of the salivary flow rate amongst individuals as well as the significant variation in arecoline concentration, despite all individuals being given the standardized 0.5 gm sample of areca nut.

These results demonstrate degree of consistency within the one subject over the three day period. While the peaks in arecoline concentration may occur at slightly different times, the maximum concentration of arecoline obtained in the mouth, by any one
arecoline was similar for the three days. This was particularly if the concentration of arecoline was less than 20 μg/ml, with variation being less than 5 μg/ml. However, if the concentration was greater than 20 μg/ml then the variation was significant, and the period of time that the concentration was above 10 μg/ml was extensive.

One practical aspect of this study is that it provides a means of biochemically validating the data from the questionnaire and any intervention strategies undertaken in the future.
Chapter 4

A Clinical and Histopathological Investigation of Oral Submucous Fibrosis in Nepal
4.1. Signs and symptoms of oral submucous fibrosis

OSF is a chronic disease of insidious onset affecting the oral mucosa, with associated involvement of the pharynx and oesophagus. Both sexes are affected by OSF, with many studies showing a predominance of females (Gupta et al., 1980; Pindborg et al., 1968; Rao, 1962). The initial presentation of most patients is between 20 and 40 years of age; however, the age-range of people affected with OSF varies according to region (Gupta et al., 1980; Pindborg et al., 1980).

All clinical studies of OSF report the presence of constrictive fibrous bands, either in the buccal mucosa, the posterior part of the palate, or the labial mucosa (Ahuja and Agrawal, 1971; Akbar, 1976; Barnes and Duke, 1975; Chiu et al., 2002b; Cox, 1991; Dockrat and Shear, 1969; Dudani and Kher, 1971; Gupta et al., 1998; Hamner et al., 1974; Harvey et al., 1986; Jeng et al., 2001; Joffe, 1971; Lal, 1953; Mukherjee and Biswas, 1972; Pindborg, 1989; Seedat and van Wyk, 1988b). One of the main criteria for the clinical diagnosis of OSF is the presence of fibrous bands either in the buccal mucosa, the posterior part of the palate or the labial mucosa (Pindborg, 1989).

Reduced oral opening appears to be associated with stiffening of the oral mucosa and formation of fibrous bands (Haider et al., 2000). These fibrous bands run between the maxilla and mandible in the buccal mucosa, and are initially discrete, but gradually broaden and coalesce such that the whole of the involved mucosa becomes firm, with tissues assuming a leathery consistency. Involvement of the lips is characterized by the presence of fibrous bands running parallel but seemingly superficial to the *orbicularis oris* muscle. In the palate, the bands often radiate from the median raphe...
to the anterior faucial pillars. The faucial pillars usually become short and thick, and frequently the tonsils appear atrophic. When the soft palate is affected, its mobility is reduced, the voice becomes nasalized, and the uvula is often shrunken and bud-like. (Figure 1.2.D). Mucosal petechiae are described in about one fifth of OSF patients (Bhonsle et al., 1981).

There are regional differences in the presentation of OSF, and it has been postulated that these reflected the individual chewing habits of those who use the areca nut, including where the quid is held or the side where the nut is chewed, and whether the juice is spat out or swallowed (Bhonsle et al., 1987). In regions where the juice and quid are generally spat out, there is a higher involvement of the anterior sites of the mouth, compared with regions where the juices and quid are swallowed, in which case the posterior sites are primarily affected (Bhonsle et al., 1987). Dysaesthesia or a 'burning' sensation involving the oral mucosa is another common symptom associated with OSF, seemingly across all the regions where OSF is prevalent. This is in addition to reduced oral opening, associated with slowness and difficulty in eating, increased salivation, changed gustatory sensation, dryness of the mouth, and a nasalized voice (Chiu et al., 2002b; Chang et al., 2001; Huang et al., 1993).

4.1.1. The mechanism for induction of oral submucous fibrosis by areca nut use is not known

4.1.1.i. OSF is occasionally observed in non-chewers

The areca nut, is the fourth most common drug used worldwide, yet the incidence of OSF, allowing for geographic variations, remains relatively low at approximately
0.5% of the population using the nut (Canniff and Harvey, 1981; Winstock, 2002). Significant geographic variations are observed between countries as well as within national boundaries, with the incidence in India and China ranging from 0.05% to 1.4% and 0.9% to 4.7% respectively (Mehta et al., 1971; Zhang and Reichart, 2007).

OSF has traditionally been associated with chewing of the areca nut, however, some large population-based studies report individuals with OSF who have no apparent areca nut habit (Mehta et al., 1971; Seedat and van Wyk, 1988a). One study found for example, that only 77% of OSF patients used the areca nut in some form (Daftary, 1992). A further study involving 100,000 subjects reported that only 52% of OSF patients chewed areca nut. The same study demonstrated an incidence of OSF of 2.1% (Mehta et al., 1972). While areca nut use appears to have a strong association with the occurrence of OSF, the mechanism by which this develops is poorly understood.

A possible immune basis to the disease is suggested by a number of observations including altered lymphocyte subsets, a decrease in the total number of lymphocytes, and a reduction in cell mediated immune activity of both isolated peripheral blood mononuclear cells and lymphocytes infiltrating tumours in OSF patients (Chang et al., 2005a). It is possible that the areca nut exacerbates the immune responsiveness to some other, as yet unidentified, environmental agent responsible for OSF.

As noted in the earlier literature review (Section 3.1.1-2), although arecoline is toxic at high concentrations, it is also able to modify cell behavior at lower concentrations, and this is consistent with an indirect rather than direct role for the areca nut in OSF.
4.1.1.ii. **Progression of oral submucous fibrosis is not well documented**

Any discussion that considers the aetiological mechanisms for OSF, would have to consider whether the disease is progressive. An early histopathological description of OSF implied a progression through four stages, defined as ‘very early’, ‘early’, ‘moderately advanced’ and ‘advanced’ (Pindborg *et al*., 1964; Pindborg *et al*., 1965). Although this histopathological classification implies a predictable progression of OSF, there has been little documentation of OSF progression in individual patients. This may have clinical consequences, for although intervention therapies vary, the literature lacks any clear idea that different forms of therapy may be appropriate for different stages of OSF. In addition, the period of observation in most studies is very short and many studies appear to lack a control group (Maher *et al*., 1997; Pindborg, 1989). One objective of the work described in this chapter, was to determine if OSF affects oral tissue in a predictable order using a cross-sectional population approach, with a view to later longitudinal studies if appropriate.

4.1.1.iii. **Possible aetiological mechanisms for oral submucous fibrosis**

Chillies have been suggested as a possible aetiological agent in the development of OSF. A connective tissue response to capsaicin, the active ingredient of chillies, has been observed in Wistar rats (Sirsat and Khanolkar, 1960). Unfortunately, these observations could not be replicated by latter workers (Hamner *et al*., 1974) and no aetiological role for capsaicin was found in separate work comparing people with and
without OSF with regard to the amount and the duration of consumption of chilies (Wahi et al., 1966b).

Transforming growth factors – alpha and beta (TGF-alpha, -beta), known to stimulate proliferation of fibroblasts in vitro, have been linked to OSF (Chiu et al., 2002a; Haque et al., 1998; Hsu et al., 2001; Ma et al., 1995; Rajalalitha and Vali, 2005; Tilakaratne et al., 2005; Trivedy et al., 1999; Vilcek et al., 1986). Enzymes associated with the production and modification of collagen such as collagenases and lysyl oxidase have also been suggested as playing a possible role in the development of OSF (Chiu et al., 2002a; Ma et al., 1995; Tilakaratne et al., 2005; Trivedy et al., 1999). Collagen-related genes are potentially modified by some of the components of betel quid, while TGF-beta stimulates the transcriptional activation of pro-collagen genes in OSF (Rajalalitha and Vali, 2005). There is also correlation between susceptibility for OSF and specific genotypes for collagen genes 1A1 and 1A2, collagenase-1, TGF-beta, and lysyl oxidase (Chiu et al., 2002a). Over-deposition of ECM (extracellular matrix molecules) is suggested as the basis for tissue fibrosis in OSF (Chang et al., 2002). Complex interactions between cytokines have also been suggested as possibly contributing to OSF, including activation of a TH2 type immune response dominated by B cells, with exacerbated TGF-beta 1 release (Feng and Ling, 2000; Snapper et al., 1993).

Malnutrition has been suggested as a contributing factor to OSF, as the disease presents in many countries where malnutrition is rise. A greater incidence of poor nutritional status is reported amongst people with OSF compared with controls (Wahi et al., 1966a). Vitamin and iron deficiency states have also been implicated in the
development of OSF. Marked iron and vitamin B complex deficiency has been observed in 77% of patients with OSF in Malaysia (Ramanathan, 1981). Nonetheless, the precise role played by these factors is unclear, while the possibility remains that an inability to eat because of OSF is the basis for the relative malnutrition, rather than malnutrition being causal of the disease.

Eosinophils, observed in connective tissues in OSF, have been suggested as indicating an allergic reaction (Pindborg and Singh, 1964), while an autoimmune basis for the disease has been further suggested by a number of workers (Canniff et al., 1985; Gupta et al., 1985; Haque et al., 1997; Pillai et al., 1992). However, some reject this concept as no specific allergen has been identified (Shah et al., 1994). Nonetheless, increased levels of both local and circulating immune complexes have been observed in OSF subjects, and these have included complexes with IgA, IgG, and IgM (Balaram et al., 1987; Remani et al., 1988; Shah et al., 1994). Also, elevated serum IgG levels were observed among 30 OSF patients in England, relative to normal individuals (Canniff et al., 1986), while others have found higher levels of circulating immunoglobulin G, IgM and IgA in OSF patients (Gupta et al., 1985; Shah et al., 1994). Further support for a possible role for autoimmunity, comes from the observation that HLA A10, DR3, and DR7 are MHC phenotypes more often present in OSF subjects (Canniff et al., 1985). Nonetheless, while haplotypic pairs have been identified, extended studies have failed to demonstrate statistical significance for such associations (Chen et al., 2004; Chiang et al., 2002a; Chiu et al., 2001). Nonetheless, a possible relationship between OSF and the MHC class related
gene A (MICA) is reported, with a significantly higher frequency of the allele A6 of MICA in OSF patients compared to controls (Liu et al., 2004).

Other features of OSF that are consistent with a possible auto-immune aetiology are the comparatively early age of onset, reported changes in circulating levels of serum immunoglobulins, the presence of autoantibodies, involvement of the DR locus in the genetic predisposition, and the female sex bias in many studies although this was not observed in Nepal (Adhvaryu et al., 1986; Borle and Jagtap, 1987; Canniff and Harvey, 1981; Canniff et al., 1985; Phatak, 1984; Rajendran et al., 1986).

Similar observations have been made in animals, with a possible immunological mechanism suggested by the observation that arecoline stimulates a delayed hypersensitivity response in mice (Selvan et al., 1991). Separately, a modulatory effect of arecoline on the B-cell mediated immune response is reported, with a reversible dose-dependent suppression of the immune response in rats (Selvan and Rao, 1993).

However, difficulties occur in interpreting some of these studies, in that controls were lacking in a study of serum auto-antibodies (Canniff et al., 1986), while circulating immune complexes noted as significantly increased in patients with OSF and oral cancer are also raised in healthy betel quid chewers compared with controls (Balaram et al., 1987). Similarly, elevated levels of circulating immune complexes, as well as of IgG and IgM, are observed in both oral cancer and OSF subjects (Remani et al., 1988).
4.1.2. **Histological features of oral submucous fibrosis**

4.1.2.i. **Leukocyte infiltrate associated with oral submucous fibrosis**

As mentioned above (4.1.1.ii.), four histopathological stages were originally described for OSF, and each of these has been characterized by a different profile of inflammatory cells (Sirsat and Pindborg, 1967). In this paradigm, the earliest presentation of the disease is accompanied by infiltrates of polymorphonuclear leukocytes, while lymphocytes and plasma cells become increasing prevalent as the disease progresses. The cellular infiltrate in OSF is composed primarily of T lymphocytes, and especially activated CD4+ helper/inducer T lymphocytes, while CD20+B lymphocytes and CD 68+ macrophages and Langerhans’ cells are only occasionally seen (Haque et al., 1997).

Of importance for the current study is that OSF is associated with the increased presence of antigen-presenting HLA-DR positive cells, as well as an increase in the ratio of helper to suppressor-cytotoxic T cells in both the epithelium and lamina propria (Haque et al., 1997). Similar observations are reported by others who note an increase in the numbers of T cells, B cells and CD68 positive macrophages with a predominance of CD4 lymphocytes over CD8 positive lymphocytes in the subepithelial connective tissue of OSF patients compared with controls (Chiang et al., 2002b). Interestingly, the infiltrate of T lymphocytes is reported to become less dense with progression of the disease from moderately advanced to advanced forms. However, when biopsies of moderately advanced and advanced OSF are compared,
there is no significant difference with regard to the infiltration of tissues by B-lymphocytes and macrophages (Chiang et al., 2002b).

4.1.2.ii. Epithelial changes associated with oral submucous fibrosis

Atrophy of the epithelium together with juxta-epithial hyalinization is reported in OSF (Daftary, 1992), and these features are important when considering data shown in this chapter. An early report demonstrates that 70% of OSF biopsies show atrophic epithelium relative to controls, while about 2% have hyperplastic epithelium and the remainder have epithelium of normal thickness (Pindborg et al., 1970). There is loss of rete ridges in the majority of cases with atrophic epithelium, while rete ridges appear normal in the remaining specimens. In addition, the buccal mucosa, which is not normally keratinized, is ortho-keratinized in 26% of OSF biopsies, while 22% are para-keratinized (Pindborg et al., 1970).

'Signet-ring' cells, primarily in the basal epithelial cell layer, are reported in approximately 13% of OSF biopsies (Pindborg et al., 1970). However, interpretation of this data is difficult due to the absence of a control group in the relevant study (Pindborg et al., 1970), while similar changes can result from nuclear shrinkage as a fixation artifact in normal mucosa (Pindborg and Sirsat, 1966).

Pindborg (1970) likened the appearance of a number of biopsies to lichen planus, referring to the presence of 'colloid bodies' in the epithelium, a change that is now thought to be due to apoptotic keratinocytes. Other lichen planus-like features, noted by Pindborg (1970), were a tendency to formation of pointed rete ridges surrounded
by lymphocytes and a marked lymphocyte infiltration with a band-like distribution in
the lamina propria. Nonetheless, on re-assessment, Pindborg et al. (1980) concluded
that the OSF cases involved had co-existent lichen planus superimposed on separate
and pre-existing OSF (Pindborg et al., 1980).

Signs of dysplasia in 7% to 25% of OSF biopsy specimens are important to note from
the perspective of the malignant potential of the disease (Pindborg et al., 1966;
Pindborg et al., 1970). It is interesting to note differences in the features of dysplasia
associated with OSF compared with those found in leukoplakia (Mehta et al., 1969).
In leukoplakia, the main dysplastic characteristics are increased mitotic activity,
hyperchromatism and basal cell hyperplasia, whereas in OSF, basal cell hyperplasia is
rare and irregular epithelial stratification, nuclear pleomorphism and a pronounced
intercellular oedema are the predominant dysplastic features (Mehta et al., 1969). This
is consistent with earlier findings in OSF where an increased number of mitotic
figures, nuclear hyperchromatism and loss of cellular polarity were noted as
prominent dysplastic features (Pindborg et al., 1970). In particular, emphasis has been
placed on the presence of prominent intercellular oedema, especially in the basal cell
layers, which is reported as occurring in 75% of epithelial dysplasias (Pindborg et al.,
1970), while it is unfortunate that there was no control group in this particular study.

4.1.2.iii. Change in the lamina propria in oral submucous
fibrosis

The first description of the changes in the lamina propria in OSF was published by
Sirsat and Pindborg (1967), who also proposed the four different histological stages
mentioned above. The first or 'very early' stage is characterized by finely fibrillar
collagen, marked oedema, large fibroblasts and blood vessels that are often dilated and congested. The inflammatory exudate consists mainly of polymorphs and occasional eosinophils. The 'early' stage follows and is associated with early signs of hyalinization in the juxta-epithelial region, in which collagen bundles are thickened and there are moderate numbers of fibroblasts. At this stage the inflammatory cells are mainly lymphocytes, eosinophils and plasma cells. In 'moderately advanced' and 'advanced' stages, there is a variable extent of hyalinization of the connective tissue. No oedema is reported for the 'advanced' stages, while the predominant inflammatory cells are lymphocytes and plasma cells and the state of the blood vessels in the 'moderately advanced' stage is reported as ranging from normal to being constricted, while there is obliteration of vessels in the 'advanced' stage (Sirsat and Pindborg, 1967).

4.2. Materials and Methods

This study was part of a prospective study developed to examine the success of various treatment regimes in the management of the loss of oral opening in patients with OSF.

4.2.1. Collection of clinical and demographic data

4.2.1.i. Development of OSF clinical questionnaire

A questionnaire was developed for the purposes of documenting the demographic data of patients with OSF, as well as the characteristic features for all patients presenting with OSF. Information on the maximal oral opening and clinical signs and symptoms was collected for all patients, although in some cases not all clinical
features of interest were recorded. A questionnaire (Figure 4.1) was designed to collect data in relation to four main areas relating to demographics, oral habits, the site of presentation of the disease and the clinical signs and symptoms associated with the disease.

Using a similar approach to that described in Chapter 2 (2.2.1.ii.), an earlier draft of the questionnaire was first tested with a number of clinicians and patients. This resulted in slight modifications, including provision for recording the presence of ulceration and ‘blistering’.

4.2.1.ii. Mucosal biopsies and histopathological confirmation of the disease

Permission for an incisional biopsy of the mucosa affected by OSF was sought from all patients who presented with the clinical signs of the disease. Informed consent was obtained only after appropriate information regarding the technique and negative and positive sequelae of the procedure were communicated to the patient. This included the information that the clinical symptoms would worsen for a limited period of from 4 to 12 weeks due to the surgical stimulation of fibrosis. Thirty-seven (61%) of the patients presenting consented to an incisional biopsy under local anaesthetic. All biopsies were taken at Patan Hospital, Kathmandu, from patients who were clinically diagnosed as having oral submucous fibrosis.

Blood was also collected from 34 patients (55.7%) consenting to this additional procedure, for determination of full blood count, haemoglobin and haematocrit values.
Figure 4.1

Form used to gather demographic and clinical data on subjects in Nepal who presented with signs and symptoms of OSF. The form allowed for the appropriate documentation of treatment as described in Chapter 5, and the progression of clinical signs and symptoms in a visual and numerical form. Symptoms as well as clinical signs were registered initially and on subsequent visits. Oral opening as measured in mm between two opposing central incisors using a vernier caliper was recorded. ‘Affected Areas’ related to the mucosal areas with fibrosis, while ‘pain’ was recorded as either being present all the time, or only while eating. ‘Blistering’ and ulceration related to the clinical presence of vesicles at the time of examination. ‘Active opening’ and ‘injections’ related to the treatment trialed as detailed in Chapter 5.
## Oral Submucous Fibrosis

**Name**: 
**Age**: 
**Sex**: 
**Address**: (Jilla) 
**Occupation**: 
**Diet**: Veg. / Non-Veg

### Habits:

<table>
<thead>
<tr>
<th>Smoking</th>
<th>Cig Bidi</th>
<th>Chewing Pan</th>
<th>Supari</th>
<th>Koine</th>
<th>Surti</th>
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<tr>
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<th>Weekly</th>
<th>Daily</th>
<th>Commenced</th>
<th>Ceased?</th>
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### Oral Opening

<table>
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<th>Year 1</th>
<th>Year 2</th>
<th>Year 3</th>
<th>Year 4</th>
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- **HCT**: 
- **WBC**: 
- **Hb**: 

### Affected Areas

- **Cervical Area**: 
- **Salivary Glands**: 
- **Entire Oral Cavity**: 

### Pain

- **Whole mouth**: 
- **Nose**: 

### Limitation

- **Masticatory Function**: 
- **Swallowing**: 

### Relevant History

- **Smoking**: 
- **Alcohol Intake**: 

### Observations

**NOTE**: 

- **Weight (Kg)**: 
- **BMI**: 
- **Blood Pressure**: 
- **Pulse Rate**: 
- **Temperature**: 
- **Sedimentation Rate**: 
- **Other Relevant Observations**:

### Conclusion

**Date**: 
**Relations**: Y/N
4.2.1.iii. Recording of clinical signs and symptoms

Patients presented to Patan Hospital (Kathmandu, Nepal) complaining of limited oral opening. The study extended over a period of 5 years and involved 61 patients. Ethical approval was from the Ethical Committee of Patan Hospital. Trained investigators interviewed patients and recorded: gender, age, areca nut habit, and occurrence of pain; and also examined patients for inter-incisal distance, identification of sites with OSF confirmed by biopsy, and oral vesicles and or ulcers. Blood tests for anaemia were offered, but accepted by only 34 participants. Unfortunately, in consequence of the difficulties associated with engagement of multiple examining clinicians, of the 61 participants, a pain history was only recorded for 40 subjects, while only 31 had further records made of the presence or absence of oral ulceration or vesicles. Nonetheless, sufficient data was collected to permit meaningful statistical evaluation by Wilcoxon’s Ranked sign, Mann-Whitney U, and Chi-square tests.

Inter-incisal distance was determined in two recordings per visit between left maxillary and mandibular central incisors, or the nearest appropriate teeth if the incisors were absent. Measurements were always passive without force applied. 32 mm was recognized as the lower limit of normal inter-incisal distance in the Nepali population (Cox and Walker, 1997).

Patients were encouraged to attend for review every month, and support of the patients. This regime was only occasionally adhered to, and then by those who lived within easy traveling distance of the hospital.
4.2.2. Biopsy materials and histological assessment

Biopsy specimens were placed in a 10% neutral buffered formalin solution (Shirdi Industries Ltd, Mumbai, Maharashtra, India) for 12 hours prior to dehydration with graded alcohols and histoclear (Apratin International, New Delhi, India), followed by infiltration with paraffin (Unique India, New Delhi, 110026 India) for section microscopy. 5µm sections were prepared with an ‘Advanced rotary’ microtome (Rattan Sales Corp., Model Basti, New Delhi 5. India), and collected onto glass slides (Proteq Systems, Shahdara, Delhi, 110032 India) before rehydration with histoclear and graded alcohols, and subsequent staining with haematoxylin and eosin (Belami Fine Chemicals Pvt. Ltd. Mumbai, India). Sections were examined using a Coslab Binocular microscope (Coslab, Ambala Cantt, Haryana India), and histopathological features recorded. To help control for possible observer bias, sections were examined by a separate pathologist on two different occasions.

The histological reports were undertaken by Assoc Prof Hedley Coleman and Assoc. Prof Hans Zoellner.

4.2.3. Statistical analysis

The statistical software package SPSS Graduate Pack Version 13.0 for Mac OS X was used to analyze the data. The Chi-square test was used to evaluate the statistical significance of differences in proportion, while the Mann-Whitney U Test was also used where appropriate. Two-tailed tests were used throughout with statistical significance being accepted in instances where p < 0.05.
4.3. Results

4.3.1. Oral Submucous Fibrosis achieved Peak

Prevalence by 20 to 40 Years of Age

Sixty-one patients were included in the study, being 19 females and 42 males. The participants in this study were not included in the epidemiological study described in Chapter 2. The age of participants ranged from 6 to 77 years, with an average of 35.5 ± 14.9 years, females and males being 33.4 ± 14.1 years and 36.7 ± 15.2 years respectively, while the slight difference between sexes was not statistically significant. Figure 4.2 demonstrates the sex distribution according to age group of the participating OSF patients. Fifty-one percent (31/61) of the OSF cases in this study fell in the 20-40 year age group.
Figure 4.2

Histogram demonstrating the proportion of males (black bars) and females (white bars), according to decade, presenting with OSF in the current study. While the male to female ratio for OSF was 2.2:1 the relative incidence of the disease across age groups was similar. The age-group of 20-29 years had the highest proportion of OSF cases amongst both males and females.
4.3.2. Regular areca nut use was prevalent but not universal amongst people with oral submucous fibrosis

The majority of the patients with oral submucous fibrosis, 91.8% (56/61), indicated they used areca nut with or without tobacco, with 78.9% of females and 82.1% of males reporting an areca nut habit. Importantly, this left 8% (5/61) of patients, comprising 4 females (mean age of 42.8 ranging from 32 to 52 years) and 1 male aged 40 years, indicating they did not use the areca nut and had not done so in the past. Although there were only five people in this group, the average oral opening for this group was 32.7 mm (SD 13.8 mm), so that oral opening was less restricted than amongst those patients reporting areca nut use, and this difference was significant (p<0.04).

The majority of the 61 OSF patients (63%) indicated they had commenced use of the areca nut during childhood, with only 3 subjects (5%) indicating they commenced the habit in their teens or later. Separately, there were a large number of individuals (32%) who were unable to recall when they commenced using the areca nut, consistent with likely use during earlier childhood.
4.3.3. Presentation of clinical signs associated with OSF

4.3.3.i. Oral submucous fibrosis affected different mucosal surfaces in a predictable order and this correlated with reduced oral opening

OSF presentation in Nepal appeared to affect discrete mucosal surfaces in a predictable way, such that the soft palate was first affected, with progressive disease involving the fauces, the buccal mucosa unilaterally and then bilaterally and then eventually the floor of mouth. In extremely advanced disease with severely restricted oral opening, the labial mucosa was affected as well. Figure 4.3 shows the way that increased oral mucosal involvement correlated with reduced oral opening. When the oral opening of patients with relatively early disease affecting the soft palate, fauces or unilateral buccal mucosa, was compared with that in patients with more advanced disease, a strong relationship between advanced disease and reduced oral opening was seen (p < 0.006). The disease always appeared to progress in this order in the Nepali population studied. However, in no situation was the disease actually observed to progress in any of the patients during the period of the study.

The age and maximal inter-incisal distance of individual patients with OSF is plotted and marked according to sex in Figure 4.4. The mean value for the inter-incisal distance for these 61 individuals was 26.9 ± 10.2 mm. Females presented with an oral opening of 23.4 ± 10.8mm, and males 28.4 ± 9.8 mm, while the difference between means for both sexes was not statistically significant. No clear relationship between
A Clinical and Histopathological Investigation of OSF in Nepal

age and reduced oral opening was found.

4.3.3.ii. The major clinical symptoms of oral submucous fibrosis were restricted oral opening and pain

While all 61 OSF patients reported a subjective history of reducing oral opening, 51 (82%) presented with an inter-incisal distance of less than 32 mm considered the lower limit of normal opening in the Nepali population. Twenty-one out of the 40 patients for whom a pain history was available reported that they had oral mucosal pain, comprising a pain incidence of 53% of patients with OSF. Although it might be expected that there would be a correlation between the severity of pain and degree of restriction in oral opening, no clear relationship between these two clinical features of OSF was seen (Figure 4.5). Similarly, there was no statistically significant difference between the sexes with regard to the incidence of pain or opening less than 32mm. Nonetheless, 15 out of these 40 patients (37%) reported they had pain during eating, while 6 out of 40 patients recorded indicated almost constant pain (15%). It was not possible to clinically distinguish between the painful symptoms described by the OSF patients and those symptoms associated with ‘burning mouth’ syndrome.

Interestingly, not all patients with vesicular or ulcerative lesions reported a history of pain, such that 6 out of 10 people with vesicular lesions, comprising 2 females and 4 males, did not volunteer a history of oral pain. There was no statistically significant difference between the sexes with regard to the incidence of pain or distribution of vesicular or ulcerative lesions.
The presence of vesicular or ulcerative oral lesions was recorded for only 31 of the OSF patients studied, but of these 11 (35%) presented with one or other of these lesions. Vesicles were seen in 3 of 7 females (42%), and 7 of 24 males (29%), so that considering both genders together 10 of the 31 OSF patients for whom the presence or absence of ulcerative or vesicular lesions was recorded (32.3%), had vesicular lesions on examination. Ulceration was seen in 1 of 7 females (14%) and 6 of the 24 males (25%) recorded, so that between the two sexes 7 out of 31 OSF patients had ulcerative lesions (22.6%). While vesicular lesions were more frequent compared with ulcers, the two types of lesions usually occurred together, such that only one of the patients with oral ulceration did not also have vesicular lesions.
Figure 4.3

Scattergram showing oral opening according to the progressively increasing oral mucosal surfaces affected with OSF. OSF appeared to affect the oral mucosa in a highly predictable way, such that fibrosis affected first the soft palate, and then the fauces, unilateral buccal mucosa, bilateral buccal mucosa, floor of mouth and lips in that order. This was accompanied by progressive reduction in the inter-incisal distance ($p < 0.006$), with most patients presenting with an oral opening less than 32 mm (dashed line) being the lower end of the normal range of opening in Nepal.
Figure 4.4

Scatter diagram indicating the age, sex and oral opening (mm) in individuals at the time of presentation relative to the lower limit of normal opening in Nepal (dotted line). Eighty four percent of patients presented with an inter-incisal distance below the lower limit of normal opening in Nepal. There was no clear relationship between the age of presentation or oral opening, while there was similarly no clear difference between the sexes.
4.3.3.iii. An absence of correlation between haematocrit and oral opening in oral submucous fibrosis

Of the 61 participants, 34 agreed to have blood tests being 10 females and 24 males, of whom 6 females (60% of those with blood tests) and 7 males (29% of those with blood tests) were found to have anaemia. Females had mean values for packed cell volume of 33.5%, with a range range of 17% to 43% (normal 35% to 45%), and males had a mean packed cell volume of 40%, ranging from 13% to 50% (normal 40% to 50%). Figure 4.6. illustrates the haematocrit values obtained for individual males and females, relative to oral opening. No clear association between haematocrit and oral opening or gender was seen.
Figure 4.5

Scattergram showing the inter-incisal distance of individual OSF patients grouped according to whether they had no pain, pain on eating or constant pain. Although there was substantial variation in oral opening, with many patients having an inter-incisal distance of less than 32 mm considered the lowest opening in the normal range, there was no convincing correlation between limited oral opening and the severity of pain suffered by patients.
Figure 4.6

Scatter diagram plotting haematocrit for male and female participants with OSF relative to oral opening. The lower limits of the normal ranges of haematocrit for Nepali males (dotted line) and females (dashed line) are shown. The lower limit of the normal range of oral opening is also indicated (bold arrow). No clear relationship between haematocrit and oral opening was seen.
4.3.3.iv. Four OSF patients also presenting with oral squamous cell carcinoma (OSCC)

Of the 61 patients in the study, four patients (one female and three males) were identified with oral carcinomas, with the mean age being 46.3 ± 9.9 years. Three of these presented with OSCC at the time of first examination, while one patient developed OSCC fourteen months after initial presentation and clinical confirmation of OSF. This last patient was female, did not report any oral habit and was the youngest presentation of OSCC at 33 years, while the other three were males and reported regular use of the both areca nut and tobacco in the form of cigarettes or bidis. The four OSCC cases all had moderately to well-differentiated squamous cell carcinomas. Two of these presented on the buccal mucosa, another on the posterior maxillary gingiva, and the fourth occurred in the vestibule of the lower lip, where a preparation of areca nut, tobacco and spices was habitually held. Figure 4.7 shows the clinical appearance of one of these patients, as well as the attendant histopathology of the invasive tumour.
Figure 4.7

Clinical photograph and histopathological images of a male patient, 60 years of age, with OSF and OSCC, and who smoked tobacco and chewed areca nut. (A) The carcinoma occurred in the buccal mucosa but was largely obscured by a dense fibrous band (FB) stretching between the attached gingiva of the maxilla and mandible. (B) Histopathology of the malignancy revealed invasive islands of well differentiated keratinocytes (arrows) below the level of the epithelium (Ep), associated with a dense inflammatory infiltrate (In). (C) Mild dysplastic changes were seen in adjacent epithelium, with drop shaped rete ridges (arrow heads) and infiltrates of lymphocytes and plasma cells (In) together with early juxta-epithelial hyalinization characteristic of OSF. (Bars, B = 250 μm, C = 50 μm)
4.3.4. **Histopathological features of oral submucous fibrosis observed in the Nepali population**

4.3.4.i. **Confirmation of the clinical diagnosis of oral submucous fibrosis**

Thirty-seven formalin fixed, and paraffin embedded biopsy specimens were available for analysis, with 57% of patients in the 20 to 40 year age group. The characteristic features of OSF are sub-epithelial deposition of dense and hypovascular collagenous connective tissue, chronic inflammatory cells to a varying degree, epithelial atrophy or hyperkeratosis in early lesions, and epithelial dysplasia. These features were seen in all biopsies studied, although not all features were seen in all cases. Interestingly, there was little correlation between clinical symptoms and the histological stage of the disease as defined by Sirsat and Pindborg (1967). Further, in the five cases where multiple biopsies were obtained from the same patient, histopathological diagnosis was of different stages on the basis of the published staging method (Sirsat and Pindborg, 1967).

The appearance of collagen was variable, particularly in the sub-epithelial area. In 2 out of 37 specimens (5%), collagen had a finely fibrillar appearance and there was no hyalinization of collagen while in a further 12 cases (32%), thickened bands of collagen with early signs of hyalinization in the juxta-epithelial area were seen. Five of the remaining 23 cases demonstrated a complete band of hyalinized collagen in the juxta-epithelial region where no clearly separate bundles of collagen could be discerned (Fig. 4.8). Six percent of the biopsies revealed all three collagen patterns in different parts of biopsies, often less than 8mm apart. Surprisingly, there was no clear
association between the histopathological presentation, and age, nor the length of time that subjects reported exposure to areca nut.

Inflammatory cells were present in all specimens studied, with 4 cases of 37 cases having at least some neutrophilic polymorphonuclear leukocyte infiltrates with occasional eosinophils, while the remaining 33 cases had infiltrates comprising primarily lymphocytes and some plasma cells. In 13 cases, infiltrates of lymphocytes were diffuse, while in 3 cases were patchy. The remaining 12 cases had a dense band-like infiltrate of lymphocytes, reminiscent of lichen planus (Figure 4.8).

There was some degree of correlation between leukocytic infiltrate and clinical symptoms, in that oral opening was in the normal range for those cases where primarily polymorphonuclear cells were present. Oral opening of less than 32mm was seen in all cases where collagen hyalinization occurred, and this was typically associated with lymphocytic infiltrates, though one case was markedly acellular with severe hyalinization. A further feature expected from the literature, was melanin incontinence in 13 out of 37 cases studied (Figure 4.8).
Figure 4.8

Paraffin sections of specimens from patients with OSF, displaying features characteristic of the disease. Stratified squamous epithelium (Ep) was present in all biopsies examined, while normal delicately reticular juxta-epithelial collagen was present in parts of individual biopsies from most patients (A). (B) Partial hyalinization (PH) was seen in many specimens with apparently advanced disease. Infiltrates (In) of primarily lymphocytes with plasma cells and occasional eosinophils (Eo) were seen in the lamina propria of all specimens studied, often beneath areas of juxta-epithelial hyalinization (JEH), while the extent of these inflammatory cell infiltrates varied from small clusters (Cln) to extensive broad sheets of cells (C,D,E,F): (G) Melanin incontinence was frequently seen (arrow heads). (H) In apparently advanced disease, partially hyalinized collagen extended beneath broad zones of juxta-epithelial hyalinization, often associated with a thin and atrophic epithelium. (H&E, Bars = 40 μm)
4.3.4.ii. Novel histopathological features observed in oral submucous fibrosis

In addition to the histopathological features expected for oral submucous fibrosis outlined above, a number of features were noted which had not apparently been described in the earlier literature.

In 4 out of 37 cases, inflammatory infiltrates had clearly perivascular and perineural distributions, while in 3 specimens a mixed inflammatory cell response containing neutrophilic polymorphonuclear leukocytes was seen in minor salivary glandular tissue (Figure 4.9).

An unusually close association between capillaries and the overlying epithelium was noted in 5 out of 37 cases, so that the endothelial lining of capillaries were apparently in direct contact with the epithelium, and in 2 further cases appeared to enter the epithelium from the underlying lamina propria (Figure 4.9). This last feature was apparent only in cases with features histologically defined as 'early' OSF, where there was increased vascularity and minimal reduction in oral opening. In addition, perivascular collagen appeared at least partially hyalinized in 33 cases (Figure 4.9).

Infiltration of the epithelium by lymphocytes was sometimes very extensive, so that the epithelium became heavily vacuolated and indistinct (Figure 4.9). At times, this appeared very similar to the basal cell liquefaction, characteristic of lichen planus, and this was noted in 30 out of 37 cases studied, together with infiltration of lymphocytes into the basal cell area (Figure 4.10). Importantly, despite this appearance, as well as the frequent but usually not coincident presence of a lichenoid band-like infiltrate of lymphocytes, no patients presented with the clinical signs of lichen planus.
Intra-epithelial vesicles were noted in specimens of 3 of the 37 cases biopsied. These were often associated with intra-epithelial infiltrates of lymphocytes, while occasional rounded Tzank-like cells were seen (Figure 4.10). It was of note that nine out of 61 patients studied reported vesicles in the mouth, usually after eating spicy foods.
Figure 4.9

Paraffin sections of OSF specimens displaying apparently novel histopathological features. Vessels (V) were readily identified and often contained erythrocytes while these were occasionally surrounded by dense inflammatory infiltrates of lymphocytes (In) (A), as were some nerves (N) (B). (C) Minor salivary gland tissue with mucous acinar cells (Ac) occasionally had a mixed inflammatory infiltrate with numerous neutrophilic polymorphonuclear leukocytes (PMN). (D) An unusually close relationship between superficial vessels (V) and the stratified squamous epithelium (Ep) was seen in some specimens, such that there was intimate contact between endothelium and epithelium (D, G). (E) Collagen about some vessels (V) was hyalinized. (F) Heavy epithelial lymphocytic infiltrates were associated with vacuolation of the epithelium (VEp), and this may have been the origin of intraepithelial vesicles (Ve) (G,H), where an acantholytic process appeared to result in the appearance of occasional free-floating Tzank-like cells (arrows) (H). (H&E, Bars = 40 μm)
Figure 4.10

Paraffin sections of OSF biopsies demonstrating basal cell liquefaction and collagen hyalinization. (A) Heavy infiltrates (In) of lymphocytes with plasma cells and occasional eosinophils (Eo) were seen. (A,B) At times, the lymphocytic infiltrate extended into the adjacent epithelium (Ep), with the appearance of basal cell liquefaction (BCL) similar to that seen in lichen planus. The vacuolation and infiltration was not always confined to the basal layers, with spinous cells also sometimes involved (C). (D) Despite the unexpected epithelial degeneration seen, other areas of these biopsies had the partially hyalinized collagen (PH) expected of OSF. (H&E, Bars = 40 μm)
4.4. Discussion

The present study included histo-pathological confirmation of OSF, while the main clinical and histopathological features found were consistent with those reported by others (Pindborg and Sirsat, 1966; Pindborg et al., 1980; Seet and van Wyk, 1988a; Sirsat and Pindborg, 1967). Nonetheless, several novel observations were made in the Nepali population studied. Of some interest, was that there was no clear relationship between the clinical signs noted and the histopathological presentation, with different biopsy sites in the same patients displaying features considered representative of different stages of the disease (Sirsat and Pindborg, 1967) (Section 4.3.4). This suggests that the 'staging' proposed by Sirsat and Pindborg (1966) may require revision, although a separate interpretation consistent with the literature is that the disease progresses at different rates to achieve different stages in different parts of the same mouth. This would be consistent with the apparent progressive involvement of consecutive mucosal tissues. However, this can only be confirmed with a longitudinal study and while notably the current work describes, in part, such a progression a study of a longer duration is necessary to confirm these initial observations.

The photomicrographs and descriptions of OSF published by various authors including Sirsat and Pindborg (1966), do not seem to completely encompass the histopathological presentation observed in Nepal and it is possible that this reflects geographic variations of the disease. Such geographic variations could be due to multiple factors including genetic variations, diet, other immunological challenges to the patient from parasitic infections, or malnutrition, as well as differences in customs for areca nut use. It would be interesting to establish a further multi-centre study to extend the current observations with larger numbers of patients from different areas,
including communities with known different patterns of areca nut use. The use of standard protocols would facilitate identification of true regional variations in disease presentation, and potentially lead to a greater insight into possible aetiological factors.

The current study was hampered by a suboptimal number of patients for study, and this was particularly so with regard to patients who denied areca nut use. These patients challenge the general assumption that areca nut is the primary cause of OSF, and raise the possibility that areca nut may accelerate OSF initiated by some other as yet unidentified cause, or alternatively that the disease may be caused by multiple independent agents. This may be consistent with an immunological basis for OSF, as areca nut may in some way promote an immune response to separate directly causal agents, despite the nut being perhaps not directly causal of the disease.

Several workers have suggested an immune basis for OSF (Canniff et al., 1985; Chang et al., 2005b; Liu et al., 2004). The novel observations made in this thesis, of histopathological features similar to the well recognized immune disease of lichen planus (Section 4.3.4.), support a role for the immune system in OSF. With regard to this, it is important to note that despite some histological similarities with lichen planus, none of the patients studied had the clinical features of lichen planus, while none of the biopsies studied had the classical features of this immune disease but were instead more consistent with the well accepted features of OSF (4.3.4.i.). In light of this, it seems possible that Pindborg’s earlier report of patients suffering with both OSF and lichen planus may not have represented two super-imposed pathologies in identical sites (Pindborg and Sirsat, 1966), but instead simply reflected similar underlying pathological processes in both diseases, as suggested in the current study.
Similarly, the perivascular and perineural lymphocytic infiltrates observed in this thesis, raise the possibility that vascular and or neural immune mediated damage may play a central role in the avascular nature of OSF, as well as the pain suffered by these patients. It is interesting that there was no clear correlation between pain and oral opening in the current study, suggestive that perhaps pain is elicited by mechanisms independent of the fibrotic response.

One benefit of the current study was that it provided the opportunity to examine the histopathological features of OSF in individuals from a relatively limited ethnic community. The geo-political features of Nepal, with its high mountain ridges running east-west across the country, sandwiched between the ice covered Tibetan plateau, and the Gangetic plains of India, has limited the free movement of the population.

It would be very interesting to develop a longitudinal study to examine whether OSF progresses in a predictable manner, as was suggested by the data in this cross-sectional study. An expanded multi-centre study would be able to provide adequate information.

It would also be interesting to characterize the specific sub-sets of lymphocytes in the biopsies studied. An attempt to do this was made in the current thesis, however, the cellular antigens were insufficiently preserved in biopsies for proper characterization of lymphocytic infiltrates. Nonetheless, it would be interesting to perform further work, using frozen section immuno-histochemistry to better define the cellular populations involved.
Finally, the data presented here for Nepal does not support the reported female predisposition to OSF (Gupta et al., 1980; Maher et al., 1994; Murti et al., 1990) reported in many centres, although there are studies which also go against this trend (Hazare et al., 1998). The male to female ratio of 2.2:1 amongst those seeking assistance and attending Patan Hospital is consistent with the epidemiological data presented in Chapter 2, where all the individuals with OSF were male.