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Development of an *in vivo* mouse model to study oral submucous fibrosis

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BACKGROUND: Epidemiological data have shown an association of areca nut chewing with oral submucous fibrosis (OSF). Experimental evidence to confirm this has been limited. Fibrosis-promoting activity of areca nut was tested in an animal model.

METHOD: Buccal mucosa of a group of 20 female BALB/c strain mice, 10–12 weeks of age, was treated twice daily 6 days per week with topical application of aqueous areca nut extracts for 300–600 days. A control group ($n = 20$) was treated with 50 mM NaCl. The influence of areca nut on the oral epithelium and connective tissue was recorded semiquantitatively by light microscopy.

RESULTS: The areca nut-treated oral epithelium showed progressive changes in epithelial thickness leading to atrophy, increased cellularity of fibroblasts, fibrosis of connective tissue, focal infiltration of inflammatory cells and muscle atrophy. On killing after 600 days of treatment, the scores on cellularity, inflammation and muscle atrophy were significantly different to the control group ($P = 0.03$).

CONCLUSION: The study provides further evidence that areca nut contributes to the development of OSF in treated animals. The model has the potential to test synergism of areca nut with other carcinogens and any therapeutic interventions.

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Keywords: areca nut; epithelial atropy; oral submucous fibrosis; mouse model

Introduction

Oral submucous fibrosis (OSF) is a potentially malignant disorder that primarily affects any part of the oral cavity and sometimes the pharynx. The disease is chronic, insidious and progressive in nature (1). This generalized condition of the mouth eventually becomes a debilitating disease with mucosal rigidity causing discomfort, burning and limitation of opening of the mouth. People with OSF carry a high risk for development of oral cancer.

Oral submucous fibrosis is predominantly found among the people of South Asia and is closely associated with the habit of betel-quid chewing. Several case–control studies show evidence that areca nut, a constituent of betel quid, is the major risk factor for OSF. Areca nut is chewed in various forms: raw, boiled or roasted. There are approximately 600 million people worldwide amounting to 10–20% of the world's population, who use raw areca nut or in any processed form as a chewing substance (2).

Areca nut, which is the endosperm of the fruit of *Areca catechu*, has carbohydrates, fats, proteins, crude fibre, polyphenols (flavonols and tannins), various alkaloids and mineral matter as its major constituents (3). Alkaloids are the most important chemical constituents that produce a range of physiological effects acting on cardiovascular and neurological systems when areca nut is chewed. These are arecoline, arecaidine, guvacine and guvacoline (4–6). Specific nitrosamines are also abundant in the nut, and contribute to its mutagenic effects (3).

It is generally observed that the onset of OSF in humans is insidious and takes about 2–20 years to present with symptoms. Submucosal fibrosis of different areas of the oral mucosa leads to difficulty of opening the mouth (trismus), while blanching of the oral mucosa is an important clinical feature at the early stage of OSF (7, 8).

Characteristic histological features of OSF include atrophic epithelium, juxta-epithelial hyalinization, collagen of different density and infiltration of inflammatory cells (7, 9). Although epithelial changes are variable, almost all cases show generalized and marked atrophy of the oral epithelium with the loss of rete

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processes. Pindborg and Sirsat (10) described four consecutive stages, namely very early, early, moderately advanced and advanced, in OSF by light microscopy.

There are no established animal models of OSF to date (3). However, Huang et al. (11) claimed to have produced a rat model of OSF in Hunan Medical University, China and the *in vivo* experiments of Khrime et al. (12) showed histopathological findings akin to OSF induced by 'pan masala' on the rat mucosa. The characterizations of these models are not complete and the experimental evidence is neither convincing nor reproducible (3).

The objective of the present study was to develop an *in vivo* mouse model that features characteristics of OSF.

Materials and methods

Ten- to twelve-week old adult female albino mice of BALB/c strain, weighing 28–30 g each, were randomly selected and grouped into control and test animals, each group comprising 20 animals. These groups were further divided into four subgroups of five animals, according to the duration of the treatment period, namely 300, 350, 450 and 600 days. The animals were segregated and housed in the same clean and ventilated environment of the Animal Facility, Faculty of Medicine, University of Peradeniya, Sri Lanka. They were provided with standard diet (CIC Feeds (pvt), Ekala, Sri Lanka) and water *ad libitum*, and maintained in constant 24-h alternate light (6.00 AM to 6.00 PM) and dark (6.00 PM to 6.00 AM) cycles.

The aqueous areca nut extract was prepared from fresh, mature endosperms of *A. catechu* by dissolving nuts in 0.9% normal saline (50 mM NaCl) while maintaining a constant concentration of 265 g of areca per litre of saline throughout the experiment. A drop (approximately 35 μl) of areca extract was applied to the buccal mucosa of each mouse in the test groups using a plastic transfer pipette and a similar amount of 50 mM NaCl was applied on control mice throughout the experimental period. After each application of areca extract/normal saline, any drinking water was withheld for 2 h. The treatment procedure was followed twice a day, morning and afternoon, for 6 days per week.

At the end of each treatment period, mice were killed with an overdose of chloroform ($CHCl_3$); their buccal mucosa were dissected out and immersed in ice-cold phosphate-buffered saline (pH 7.4). The harvested tissues were fixed in 4% parafomaldehyde at 4°C for 16–20 h and, following processing, wax embedded.

Sections (4 μm) were stained with haematoxylin and eosin, van Gieson for collagen fibres and Masson's trichrome for the young collagen (13). Tissue sections were examined by light microscopy and scored independently and blindly (by the pathologist P.A.) for: epithelial atrophy, connective tissue fibrosis, cellularity (population of fibroblasts) and vascularity (population of blood vessels), hyalinization of the connective tissue interface, inflammation and muscle atrophy. A grading system was introduced to semi-quantitatively assess the histopathological changes: normal [0–1], mild [2–5] and marked [6–10] to estimate the progression of the disease in mouse mucosa in different test groups. Five consecutive microscopic fields avoiding the margin of trimmed tissues were examined for each section.

The Wilcoxon test was used to compare data obtained from the histopathology grading of cellularity, inflammation and muscle atrophy. A computer-based statistical package (MINITAB®, version 11) was used in this analysis. $P < 0.05$ was regarded as statistically significant.

Ethical permission for undertaking this study was granted by the Committee on Research and Higher Degrees, Faculty of Medicine, University of Peradeniya, Sri Lanka.

Results

General observations

All mice appeared healthy during the entire experimental period with no noticeable loss of body weight in any of the groups. At the time of final killing of the group by 600 days, the body weight of mice in the experimental group averaged 44.5 g. ± 2.8, while the mean weight of control mice was 49.3 g. ± 4.7.

Although clinical observations of the mouth of the mice were restricted, towards the latter period of the treatment, test mice showed signs of restricted mouth opening. In the latter part of the study the insertion of the transfer pipette into the mouths of test mice was progressively more difficult.

Microscopic observations

Group 1 (300-day treatment)

Compared with controls (Fig. 1A) in test mice, orthokeratinized buccal epithelia were flattened in general (Fig. 1B). However, some pointed and long papillary epithelial compartments showed features akin to hypertrophy. Buccal epithelia were mildly inflamed and showed intercellular oedema. Infiltration of neutrophils within the epithelium was apparent (Fig. 3C). Interpapillary connective tissue compartments were broadened and collagen in the lamina propria showed noticeable changes. It was denser and less cellular ($*P = 0.03$) than the collagen in control animals and showed increased thickness. The scattered fibroblasts in the fibrous layer had plump nuclei. Neutrophils and eosinophils were the predominant inflammatory cells. There was a general reduction in the number of blood vessels and this was statistically significant ($*P = 0.03$). Collagen in the lamina propria had extended up to the underlying muscle layers and showed some muscle atrophy too. However, muscle atrophy was not statistically significant. Hyalinization was not prominent.

Group 2 (350-day treatment)

In comparison with the control buccal mucosa (Fig. 1C), it was evident that the buccal mucosae of the treated mice were thinner and atrophic (Fig. 1D). Intercellular oedema was observed in some sections. Connective tissue of the lamina propria was thickened

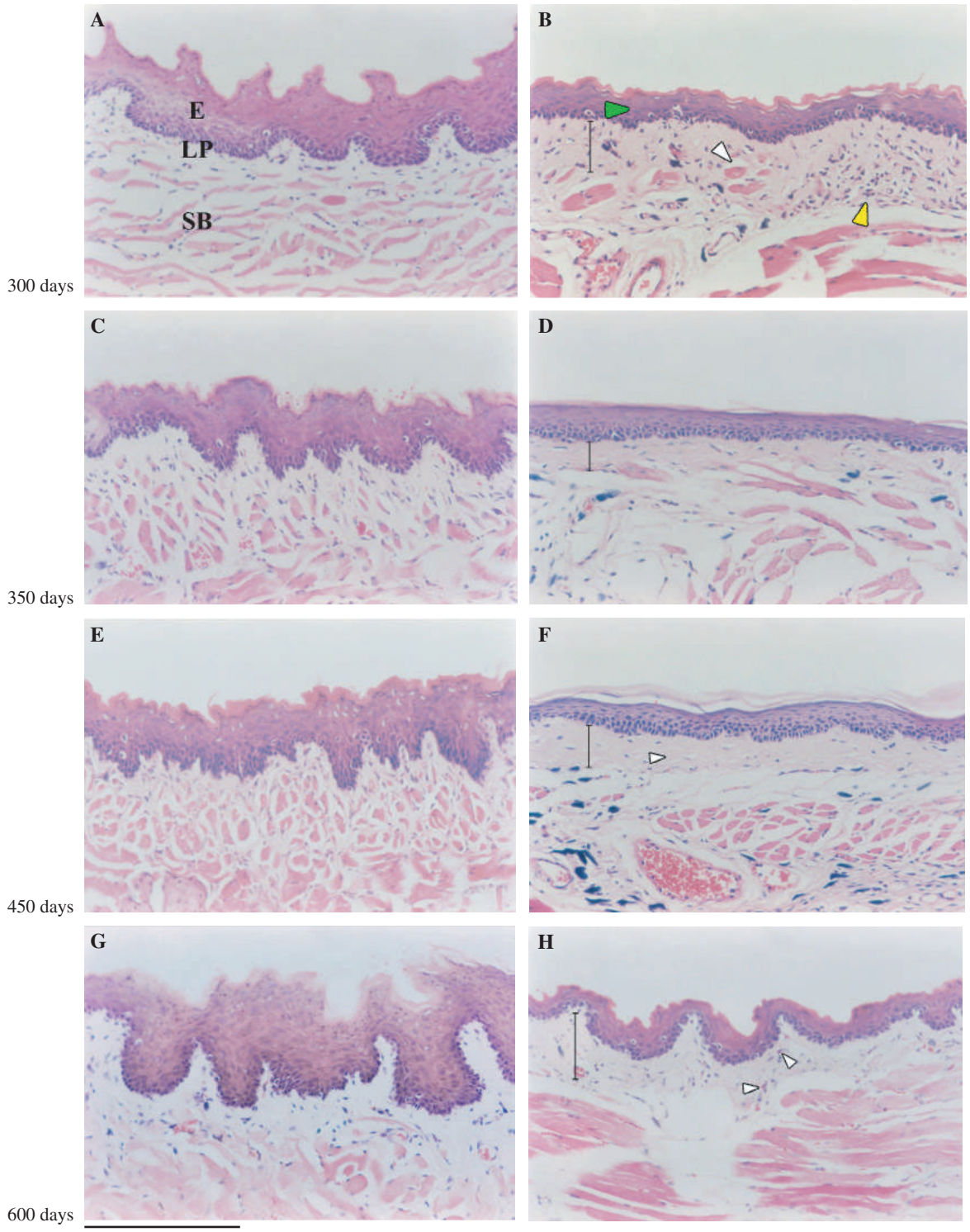

Figure 1 Photomicrographs showing the histopathological changes of the buccal mucosa during treatment intervals 300, 350, 450 and 600 days and from control mice at same time intervals. H&E × 20 (Bar = 200 μm). (B) 300-day areca-treated buccal mucosa. Compared with the corresponding control (A) markedly atrophic epithelium (green arrow), densely deposited oedematous collagen in the lamina propria, inflammatory exudate (yellow arrow), atrophy of the muscles (white arrow) and less vascular connective tissue are prominent. (D) 350-day areca-treated mucosa. Compared with the corresponding control (C) the epithelium shows marked atrophy. Densely deposited collagen is seen in the connective tissue. Both cellularity and vascularity are reduced. (F) 450-day areca-treated buccal mucosa. Compared with the corresponding control (E) it has an atrophic epithelium. Juxta-epithelial hyalinization is seen (arrow). Both cellularity and vascularity are considerably reduced. (H) 600-day areca-treated buccal mucosa. Compared with the corresponding control (G) epithelial atrophy is marked. Connective tissue shows densely deposited collagen. The obliteration of the blood vessels is shown (arrow). Chronic inflammatory cells are seen scattered in the connective tissue. In (A), E denotes buccal epithelium, LP, lamina propria and SB, submucosa.

with increased fibrosis. They appeared like dense bundles. The papillae of this area were broad and dense. Some areas of the connective tissue appeared like thin, long ribbon. Inflamed areas mainly contained neutrophils, lymphocytes and eosinophils. Cellularity of collagen and vascularity in the connective tissue were noticeably reduced. In the submucosal area muscle atrophy was noticeable but the differences compared with controls were not statistically significant.

Group 3 (450-day treatment)

Compared with controls (Fig. 1E) in test mice, the orthokeratinized epithelia of the buccal mucosa showed greatly reduced heights of rete processes (Fig. 1E,F). Therefore, most of the treated mucosa had an atrophic appearance. Basal cell destruction was apparent in some cases where subepithelial collagen was dense and acellular. Thickness of the lamina propria had increased considerably and the collagen showed a disordered, wavy appearance. Increase in the density of fibrous collagen produced ribbon-like areas in the connective tissue, devoid of any fibroblasts. One case showed histological features of hyalinization, just below the epithelium. Chronic inflammatory cells, predominantly lymphocytes and eosinophils, were seen in the subepithelial connective tissue. Cellularity had increased to a greater extent. Blood vessels were scanty and were surrounded by fibrous tissue. Increasing fibrous layers enclosed the underlying submucosal muscles. Muscle atrophy had increased in most of the cases.

Group 4 (600-day treatment)

Buccal mucosal epithelia showed atrophy and when the rete processes were present they appeared short and pointed. Thinning of the epithelium was prominent in the inter-papillary epithelial compartment. Widening of lamina propria was a significant feature of all sections (Fig. 1G,H). Thick, dense collagen devoid of plump fibroblasts was deposited in this area. Some tinctorial changes in collagen were observed by van Gieson and Masson's trichrome stains (Fig. 2). The van Gieson's stain showed densely-deposited collagen stained bright red. In contrast, the control tissues showed a webbed appearance of fibrillar collagen. Interestingly there were pale pinkish white amorphous areas immediately below the epithelium, which were not stained by van Gieson. The yellow areas stained by Masson's trichrome were shown corresponding to the pale pinkish areas of the van Gieson stain. Chronic inflammatory cells, predominantly lymphocytes and plasma cells, were present in the connective tissue. Some focal infiltrations of inflammatory cells were notable in the subepithelial tissue. These cells were aggregating at the apex areas of the

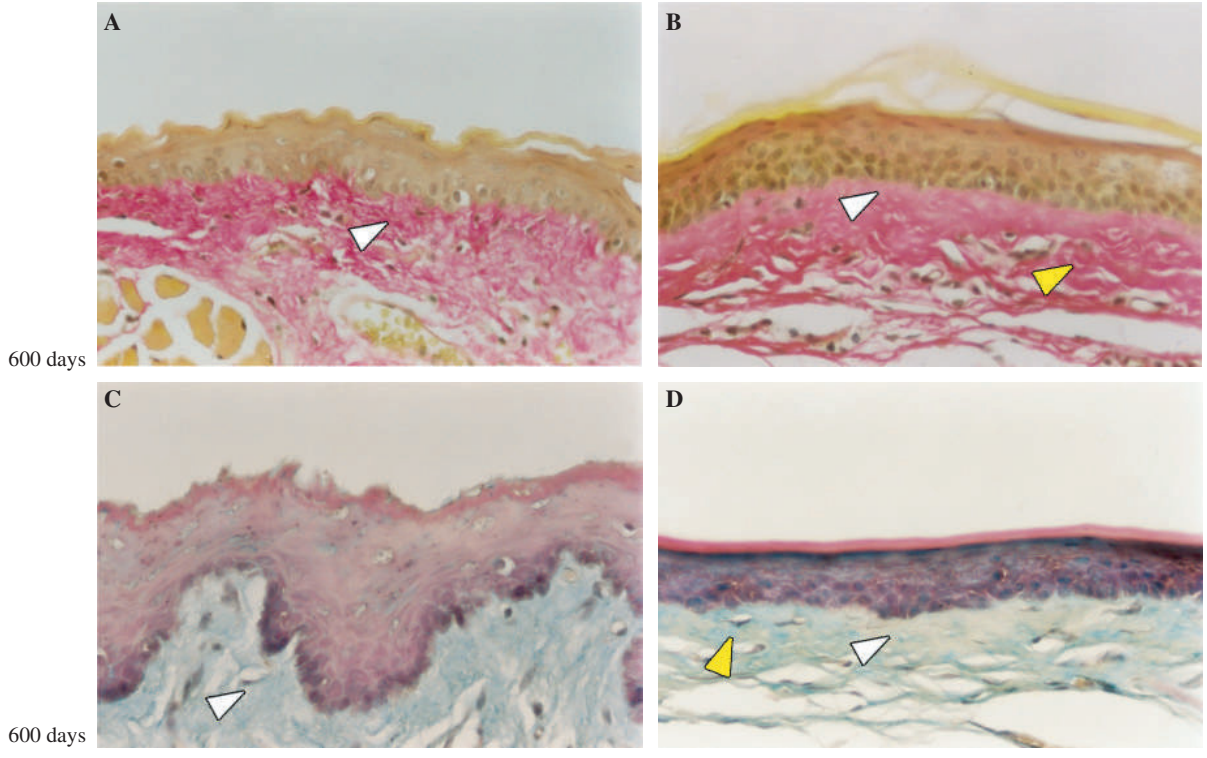

Figure 2 Photomicrographs of buccal mucosal specimens obtained from control (A, C) and areca-treated mice (B, D) highlight some tinctorial changes observed with van Gieson and Masson's trichrome stains, ×40 (Bar = 50 μm). (B) 600-day areca treated buccal mucosa (van Gieson). In comparison with its corresponding control (A) this photomicrograph shows highly dense collagen deposition in the connective tissue which is stained bright red with van Gieson (yellow arrow). An amorphous area (pinkish white) is seen just below the epithelium which is not stained by van Gieson (white arrow). (D) 600-day areca-treated buccal mucosa (Masson's trichrome). In comparison with its corresponding control (C) this photomicrograph shows some areas stained in green (yellow arrowhead) and yellow colours (white arrow). Yellow-stained areas are the corresponding amorphous areas of the van Gieson stain.

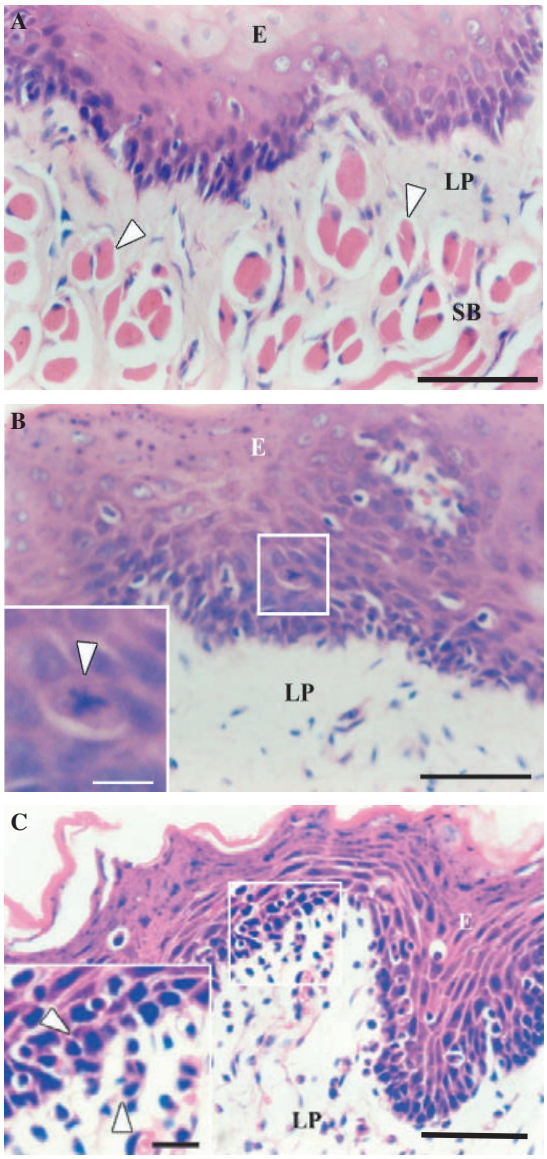

Figure 3 Photomicrographs illustrating histopathological changes in areca-treated mice (A), muscle atrophy (B), tripolar mitoses in buccal epithelium and (C) inflammatory changes in the lamina propria (H&E ×400). (A) 450-day areca-treated mice, muscle atrophy of the buccal mucosa showing atrophy of the striated muscles in the submucosal layer due to the invading collagen from the lamina propria (arrow). Muscles are surrounded by thick bundles of collagen. (B) 600-day areca-treated mice, tri-polar mitotic figure in the buccal mucosa in the supra basal layer of the buccal mucosal epithelium. (higher magnification insert, bar = 10 μm). (C) 300-day areca-treated mice, buccal mucosa showing inflammatory changes. Insert juxta-epithelial connective tissue area (higher magnification insert, bar = 20 μm).

inter-papillary connective tissue compartment. In some mice there were bizarre mitotic figures (tripolar) in the suprabasal layers of the buccal epithelium (Fig. 3B). Muscle atrophy was observed in the submucosal area to a greater extent (Fig. 3A).

Table 1 shows the progressive changes recorded on cellularity, inflammation and muscle atrophy at different time periods of killing, illustrating a gradual increase in severity of the changes observed.

Discussion

Epidemiological studies have established a strong causal relationship between areca nut use and the development of OSF (14, 15). Populations exposed to areca nut have odds ratios ranging from 60 to 132 to develop the disease, compared with non-users (14, 16). Evidence for genotoxicity of areca nut has been recently revealed (3).

Mice fed aqueous extract of nuts by gavage have shown to develop both benign and malignant tumours of the liver, and forestomach and stomach (17, 18). Most *in vivo* experiments on rat, hamster and baboons by administration of betel quid to the oral mucosa or cheek pouch have included tobacco in addition to nut. A few conducted with areca nut extract or pieces reviewed by International Agency on Research for Cancer have looked for its carcinogenicity and the ability to produce tumours (3). *In vivo* experimental data on the ability of the areca nut extract to produce OSF are meagre. There has not been any reproducible animal model of OSF to the best of our knowledge. Non-availability of an animal model of OSF limits further research into the understanding of the pathogenesis of this disease and furthermore, development of therapeutic agents to control the progression of this disease (19). Therefore, the present study was undertaken to develop an animal model of OSF with histopathological confirmation of the disease.

Being an insidious disease condition, OSF usually presents late in the life of an areca nut/betel quid chewer. The two distinct processes, ageing and maturation, occur as a consequence of time and involve qualitative and/or quantitative changes in the extracellular matrix (ECM). However, cellular ageing may be relative to the lifespan of the animal and thus the changes in matrix composition would be a function of time. Taking into account, the time taken for development of OSF in humans, the treatment periods in this study were allotted roughly to fall within the latter half of the life span of mice. This nature, we believe, would

Table 1 Summary of cellularity, inflammation and muscle atrophy in various test groups

		300 days		*350 days*		*450 days*		*600 days*[a]	
Parameters	*Animal no.*	*Median*	*Range*	*Median*	*Range*	*Median*	*Range*	*Median*	*Range*
Cellularity	5	2.5	2–3	3.0	2–4	3.5	3–6	3.0	2–4
Inflammation	5	4.0	1–4	2.0	1–4	2.5	1–4	3.0	3–4
Muscle atrophy	5	3.0	0–6	2.0	0–4	3.0	1–3	2.5	2–3

Grading system: normal [0–1], mild [2–5] and marked [6–10].
[a]600-day-treated mice compared with controls (Wilcoxon statistic 15; $P = 0.03$).

provide a close representation of the human disease condition.

The relevance of a particular animal model to a human disease rests on its ability to parallel the biological changes that characterize the disease in humans (20). Microscopically it is observed that BALB/c mice have, unlike humans, a sparsely distributed lamina propria below the epithelium. This morphological feature could be an added advantage, especially in relation to OSF. As a result, at the onset of fibrosis, the extension of collagen fibres deep into the tissues could affect striated muscles underneath. This phenomenon has important considerations to the mouse model described here. The small size of the oral cavity is a distinct drawback in monitoring clinical changes by visual investigation.

Atrophy of the oral mucosa is commonly associated with some conditions, which are considered precancerous in the oral cavity (21). It is frequently found among patients with micronutrient deficiencies such as iron (22), vitamin A (23) and its derivatives. Mucosal atrophy is a recognized feature of OSF after years of areca nut chewing. Pindborg and Sirsat (10) reported that in more than 90% of the cases, the oral epithelium of the clinically affected sites is markedly atrophic. This was confirmed by subsequent research (24–26). The present study was able to establish the characteristic atrophic epithelium in the buccal mucosa in all the experimentally treated animals at all treatment periods (Fig. 1).

The ECM is a dynamic, intricate network of macromolecules that plays an important role in regulating cellular function during normal and pathological remodelling processes, such as inflammation, tissue repair and tumour development (27). The major components of ECM are proteoglycans, collagens and glycoproteins, and the structural features of these components provide the basis for their involvement in a variety of interactions following cellular signalling (28). OSF has been described as a disorder of collagen metabolism in which there is an increase in production of highly cross-linked insoluble collagen (29). Cross linking of collagen plays a vital role in fibrosis. Fibrosis of the subepithelial connective tissue in OSF has been identified as the pathognomic feature which gives rise to limited mouth opening in humans (30, 31). It differs from pathological fibrotic conditions such as scleroderma, juvenile aggressive fibromatoses and abdominal desmoids (32). However, the nature and type of fibrosis in OSF have not yet been morphologically characterized. An electron microscopical study by van Wyk et al. (33) showed no abnormal collagen in OSF. Concerning the type of collagen involvement in OSF, whether it consists of specific subtypes or a mixture of collagen has not yet been resolved (29, 33, 34). Several studies have been carried out to elucidate the mechanisms underlying excessive deposition of collagen in the lamina propria in areca nut chewers who suffer from OSF (35–37). Based on the molecular pathogenesis, examining activation pathways of TGF-β and α, key enzyme lysyl oxidase tilting the balance of collagen metabolism towards fibrosis, OSF has been described as a collagen metabolism disorder (38).

The present study shows fibrosis as a continuous process occurring in the subepithelial buccal mucosal tissues of treated mice (Fig. 1). The amorphous areas confirmed by van Gieson and Masson's trichrome stains, an indication of early hyalinization, may reflect the presence of young collagen or altered ground substance or both (13). Hamner et al. (25) presented similar microscopic pictures in the oral biopsies of OSF patients. The findings confirming the excessive deposition of collagen in the treated animals reported here bear a close similarity to human OSF. It is possible that the excessive formation of fibrous tissue may be an attempt to repair the damage caused by irritation of tissue following application of the areca nut extracts. Sirsat and Pindborg (39) highlighted this situation of coexistence of defence and reparatory process or otherwise called 'the chronic productive response'. This kind of pathological situation is plausible with a mild irritant acting for long period that results in a chronic inflammatory condition in the affected tissues. However, application of a mild irritant such as capsaicin on rat palates (40) and hamster buccal mucosa (25) for long periods earlier failed to produce clinical or histological changes consistent with that of OSF. From our model it appears that the mild irritation caused by areca nut could produce tissue-specific responses linked to the chemical substances found in areca nut.

The progressive reduction in fibroblast density from 300 to 600 days of the experiment (Table 1) shows a strong relationship that exists with atrophy and fibrosis of the tissues. Recent *in vitro* tissue culture experiments have shown that areca nut alkaloids may have cytotoxic properties (36, 41). Thiol depletion in the fibroblasts could be a mechanism for arecoline-induced cytotoxicity (42). It is plausible to think that areca nut alkaloids may be cytotoxic to fibroblasts, eventually resulting in the reduction in cellularity. The atrophic epithelium may have facilitated the migration of areca nut alkaloids towards the connective tissue more readily.

Among several hypotheses that have been proposed for the development of OSF (43), cytokines and growth factors found in chronic inflammation are proposed to play a prominent role in the induction and progression of the disease (44). The group of animals killed at 300 days showed a higher inflammatory response compared with longer treatment periods. The predominance of neutrophils and eosinophils was observed, indicating a reaction to injury by the areca nut substances in a non-immune manner. The tissues seemingly have passed from an acute phase of inflammation, which usually present with a large number of neutrophil polymorphs, and have entered a chronic phase. Neutrophils showed strong phagocytosis and were seen infiltrating into the overlying epithelium (Fig. 3C). It is also interesting to note that the inflammatory cells were found as an infiltrate at the apex of the inter-papillary connective tissue compartment. Apparently this occurrence may have a strong relationship to the atrophy seen at the inter-papillary region. The inter-papillary epithelium

being thinner would potentially allow hazardous chemical substances of areca nut extract to permeate through this narrow epithelial strip into the connective tissue, resulting in the accumulation of neutrophils via chemotactic mechanism. Areca nut extract with alkaloids and polyphenols, being mild irritants, could also have stimulated an allergic response as evident by the presence of eosinophils. Infiltration of plasma cells, observed in the latter part of the treatment period, provides evidence for a chronic inflammatory process of the buccal tissues evoking an immune reaction in response to areca alkaloids.

The treated mice showed inflammatory responses akin to human OSF during the treatment periods. The presence of various forms of inflammatory cells suggests the chronicity of the inflammation. Inflammation is often correlated with the increase in fibrosis indicating immunological influences especially by the lymphocytes. The bizarre mitotic figures (tripolar) (Fig. 3B) found in the suprabasal layer of the epithelium of the buccal mucosa is evidence of cellular atypia contributing to later malignant transformation of OSF. Rajendran et al. (32) remarked that the characteristic fibrosis occurring with OSF may harbour a particular tendency to induce the overlying epithelium to undergo neoplastic transformation.

Muscle atrophy observed in the treated animals correlated well with the concomitant cellular changes occurring in the tissues affected by fibrosis and atrophy. It has been noted in human OSF that as fibrosis extends into the submucosal layer, atrophic and degenerative changes appear in the muscles (34). Some ultrastructural findings by El-Labban and Canniff (45) showed that the muscle fibres contained large pools of homogeneous material which caused compression of the sarcomeres along with other alterations in the cellular structure. Electron microscopic studies of *in vivo* experimental tissues and collagen subtyping of different stages of development of fibrosis may further advance our understanding of these reported changes.

Conclusion

The present study examined in detail the development of an animal model for OSF based on light microscopy findings. The *in vivo* model shows its consistency and stability through all the treatment periods. We demonstrated in this *in vivo* mouse model the effects of areca nut extract on epithelial thickness leading to atrophy, connective tissue fibrosis, progressive reduction in fibroblasts, and inflammatory changes, all found in human OSF. The experimental data presented further support areca nut contributing to the causation of OSF. The model allows further studies to test syngerism of areca nut with other substances such as tobacco, and examining nutritional interventions that might further influence the progression of fibrosis.

References

1. International Agency for Research on Cancer. WHO classification of tumours: pathology and genetics of head and neck tumours. Eds: Barnes L, Eveson JW, Reichart PA, Sidransky D. Lyon: IARC Press, 2005; 181.
2. Gupta PC, Warnakulasuriya S. Global epidemiology of areca nut usage. *Addict Biol* 2002; **7**: 77–83.
3. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. *Betel-quid and Areca-nut Chewing and Some Areca-nut-derived Nitrosamines*, Vol. 85. Lyon: International Agency for Research on Cancer, 2004.
4. Raghavan V, Baruah HK. Arecanut: India's popular masticatory- History, chemistry and utilization. *Econ Bot* 1958; **12**: 315–25.
5. Huang JL, McLeish MJ. HPLC determination of the alkaloids in betel nut. *J Chromatogr* 1989; **475**: 447–50.
6. Lord GA, Lim CK, Warnakulasuriya S, Peters TJ. Chemical and analytical aspects of areca nut. *Addict Biol* 2002; **7**: 99–102.
7. Daftary DK, Murti PR, Bhonsle RB, Gupta PC, Mehta FS, Pindborg JJ. Oral precancerous lesions and conditions of tropical interest. In: Prabhu SR, Wilson DR, Daftary DK, Johnson NW, eds. *Oral diseases in the tropics*. Delhi: Oxford University Press, 1993; 402–28.
8. Zain RB, Ikeda N, Gupta PC et al. Oral mucosal lesions associated with betel quid, areca nut and tobacco chewing habits: consensus from a workshop held in Kuala Lumpur, Malaysia, November 25–27, 1996. *J Oral Pathol Med* 1999; **28**: 1–4.
9. Johnson NW, Maher R, Trivedy C, Warnakulasuriya S. The clinical condition and pathology of oral submucous fibrosis (OSF). *Oral Dis* 1997; **3**: 278–9.
10. Pindborg JJ, Sirsat SM. Oral submucous fibrosis. *Oral Surg Oral Med Oral Pathol* 1966; **22**: 764–79.
11. Huang S, Ling T, Wu H. Experimental study on aqueous areca nut extracts inducing oral submucous fibrosis in rats. I. Observations of histomorphometry. *HuaXiKouQiangYi XueZaZhi* 1997; **15**: 91–3, 96.
12. Khrime RD, Mehra YN, Mann SBS, Mehta SK, Chakraborti RN. Effects of instant preparation of betel nut (*pan masala*) on the oral mucosa of albino rats. *Indian J Med Res* 1991; **94**: 119–24.
13. Bancroft JD, Cook HC. *Manual of histological techniques and their diagnostic application*. New York: Churchill Livingstone, 1994.
14. Maher R, Lee AJ, Warnakulasuriya KAAS, Lewis JA, Johnson NW. Role of areca nut in the causation of oral submucous fibrosis – a case control study in Pakistan. *J Oral Pathol Med* 1994; **23**: 65–9.
15. Gupta PC, Sinor PN, Bhonsle RB, Pawar VS, Mehta MC. Oral submucous fibrosis in India: a new epidemic? *Natl Med J India* 1998; **11**: 113–6.
16. Sinor PN, Gupta PC, Murti PR et al. A case-control study of oral submucous fibrosis with special reference to the etiologic role of areca nut. *J Oral Pathol Med* 1990; **19**: 94–8.
17. Bhide SV, Shivapurkar NM, Gothoskar SV, Ranadive KJ. Carcinogenicity of betel quid ingredients: feeding mice with aqueous extract and the polyphenols fraction of betel nut. *Br J Cancer* 1979; **40**: 922–6.
18. Bhisey RA, Ramchandani AG, D'Souza AV, Borges AM, Notani PN. Long-term carcinogenicity of pan masala in Swiss mice. *Int J Cancer* 1999; **83**: 679–84.
19. Mehta AR, Bokil KP, Chandavarkar RY. Pre-cancerous lesion in the oral cavity. In: Rao RS, Desai PB, eds. *Oral cancer*. Bombay: Tata Memorial Centre, 1991; 24–40.
20. Roach HI, Shearer JR, Archer C. The choice of an experimental model: a guide for research workers. *J Bone Joint Surg Br* 1989; **71**: 549–53.

21. Warnakulasuriya KA, MacDonald DG. Epithelial cell kinetics in oral leukoplakia. *J Oral Pathol Med* 1995; **24**: 165–9.
22. Ranasinghe AW, Warnakulasuriya KAAS, Tennekoon GE, Seneviratna B. Oral mucosal changes in iron deficiency anaemia in a Sri Lankan female population. *Oral Surg Oral Med Oral Pathol* 1983; **55**: 29–32.
23. Jafarey NA, Zaidi SHM. Carcinoma of the oral cavity and oropharynx in Karachi (Pakistan). An appraisal. *Trop Doct* 1976; **6**: 63–7.
24. Pindborg JJ, Mehta FS, Daftary DK. Occurrence of epithelial atypia in 51 Indian villagers with oral submucous fibrosis. *Br J Cancer* 1970; **24**: 253–7.
25. Hamner JE, Mehta FS, Pindborg JJ, Daftary DK. Altered staining reaction of connective tissues in 53 submucous fibrosis patients. *J Dent Res* 1971; **50**: 388–92.
26. Pindborg JJ, Bhonsle RB, Murti PR, Gupta PC, Daftary DK, Mehta FS. Incidence and early forms of oral submucous fibrosis. *Oral Surg* 1980; **50**: 40–4.
27. Ioachim E, Charchanti A, Briasoulis E et al. Immunohistochemical expression of extracellular matrix components tenascin, fibronectin, collagen type IV, and laminin in breast cancer: their prognostic value and role in tumour invasion and progression. *Eur J Cancer* 2002; **38**: 2362–70.
28. Boudreaw NJ, Jones PL Extracellular matrix and integrin signaling: the shape of things to come. *Biochem J* 1999; **339**: 481–8.
29. Reichart PA, van Wyk CW, Becker J, Schuppan D. Distribution of procollagen type III, collagen type VI and tenascin in oral submucous fibrosis (OSF). *J Oral Pathol Med* 1994; **23**: 394–8.
30. Pindborg JJ. Oral submucous fibrosis: a review. *Ann Acad Med Singapore* 1989; **18**: 603–7.
31. Warnakulasuriya S. Semi-quantitative clinical description of oral submucous fibrosis. *Ann Dent* 1987; **46**: 18–21.
32. Rajendran R, Sunil A, Twinkle SP, Anilkumar TV, Anne J. Cell death does not herald epithelial involution (“atrophy”) in oral submucous fibrosis: a TEM study. *Ind J Dent Res* 2004; **15**: 13–9.
33. van Wyk CW, Seedat HA, Phillips VM. Collagen in submucous fibrosis: an electron-microscopic study. *J Oral Pathol Med* 1990; **19**: 182–7.
34. Utsunomiya H, Tilakaratne WM, Oshiro K et al. Extracellular matrix remodelling in oral submucous fibrosis: its stage-specific modes revealed by immunohistochemistry and *in situ* hybridisation. *J Oral Pathol Med* 2005; **34**: 498–507.
35. Canniff JP, Harvey W. The aetiology of oral submucous fibrosis: the stimulation of collagen synthesis by extracts of areca nut. *Int J Oral Surg* 1981; **10**: 163–7.
36. van Wyk CW, Oliver A, Hoal-van Helden EG, Grobler-Rabie AF. Observations on the effect of areca nut extracts on oral fibroblasts proliferation. *J Oral Pathol Med* 1994; **23**: 145–8.
37. Trivedy C, Baldwin D, Warnakulasuriya S, Johnson NW, Peters TJ. Copper content in *Areca catechu* (betel nut) products and oral submucous fibrosis. *Lancet* 1997; **340**: 1447.
38. Rajalalitha P, Vali S. Molecular pathogenesis of oral of submucous fibrosis – a collagen metabolic disorder. *J Oral Pathol Med* 2005; **34**: 321–8.
39. Sirsat SM, Pindborg JJ. Mast cell responses in early and advance oral submucous fibrosis. *Acta Pathol Microbiol Scand* 1967; **70**: 174–8.
40. Sirsat SM, Khanolkar VR. Submucous fibrosis of the palate: induction by local painting of capsaicin in Wistar rats treated with desoxycorticosterone acetate – an optical and electron microscopic study. *Arch Pathol* 1960; **70**: 180–7.
41. Jeng JH, Lan WH, Hahn LJ, Hsieh CC, Kuo MY. Inhibition of the migration, attachment, spreading, growth and collagen synthesis of human gingival fibroblasts by arecoline, a major areca alkaloid, in vitro. *J Oral Pathol Med* 1996; **25**: 371–5.
42. Jeng JH, Kuo ML, Hahn LJ, Kuo MY. Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. *J Dent Res* 1994; **73**: 1043–9.
43. Tilakaratne WM, Klinikowski MF, Saku T, Peters TJ, Warnakulasuriya S. Oral submucous fibrosis: review on aetiology and pathogenesis. *Oral Oncol* 2006; **42**: 561–8.
44. Haque MF, Harris M, Meghji S, Barrett AW. Immunolocalization of cytokines and growth factor in oral submucous fibrosis. *Cytokine* 1998; **10**: 713–9.
45. El-Labban NG, Canniff JP. Ultrastructural finding of muscle degeneration in oral submucous fibrosis. *J Oral Pathol* 1985; **14**: 709–17.

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