Permeability characteristics of various types of areca nut preparations in Sprague–Dawley rat oral mucosa

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Background
Oral submucous fibrosis is a potentially malignant oral disorder causatively linked to the habit of areca nut consumption. The various types of preparation of the nut alter the properties and consequently its capability to diffuse through the oral mucosa. Permeability of the nut through the mucosal tissue is an important factor in the production of lesions.

Aims
The present study attempts to evaluate the permeability of various areca nut preparations standardized against arecoline in the buccal mucosa of Sprague–Dawley rats. Apart from normal mucosal permeability, we also aimed to assess the lesional tissues induced by the application of the areca nut solutions.

Materials and methods
Healthy in-bred Sprague–Dawley rats aged 3–4 months and weighing 100–200 g were randomly selected and divided into five groups: the control group, the raw areca nut group, the boiled areca nut group, the roasted areca nut group and the pan masala and pure arecoline groups. Permeability was assessed using a Franz diffusion chamber over a period of 24–72 h. Histological assessment to determine depth in the tissue was also done.

Results
The highest average permeation depths were recorded in the boiled areca nut group (1178.21 μm), followed by the raw areca nut (1157.50 μm), the pan masala (1110.34 μm) and the roasted areca nut (1072.36 μm) groups, as compared with controls (350.79 μm). Overall, there occurred a mild increase in the permeation depths of the solutions in all groups at 72 h compared with 24 h. Statistical analysis revealed that the permeation values had a significant negative correlation with epithelial and keratin thickness.

Conclusion
There seems to be a time-dependent and solute (areca nut)-dependent pattern in the permeability characteristics. Diffusibility is continuous, persistent and progressive, and tissue reaction in the form of epithelial changes and fibrosis does not appear to be a significant barrier in the process. This study strongly supports the pathological changes seen in the disorders caused by the consumption of areca nut in humans.

Keywords:
areca nut, oral submucous fibrosis, permeability, Sprague–Dawley rats

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Introduction

Oral submucous fibrosis (OSF) is a potentially malignant oral disorder that results in debilitating fibrosis of the oral tissues. It shows a clear-cut geographical and ethnic predisposition among the areca nut chewers of South-East Asia [1].

Areca nut chewing is considered to be causatively associated with the development of OSF [2–6]. Epidemiological estimates indicate that the habit of areca nut chewing is pursued by about one-tenth of the world's population. It is the fourth most common addictive psychoactive substance consumed after caffeine, nicotine and alcohol [7].

Consumption of areca nut occurs in various forms in the region, varying from raw, roasted and boiled to commercial varieties known as pan masala. The latter, a popular dispensation among the youth, is a concoction of areca nut powder with specified and unspecified additives. When combined with tobacco it is called gutka. Marketed aggressively, packaged intelligently and the pricing made affordable, its popularity has led to its widespread use [2–6].

The major constituents of areca nut include carbohydrates, fats, proteins, crude fibre, polyphenols (flavonols and tannins), alkaloids and mineral matter. Arecoline and its alkaloids are thought to play a major

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role in the development of OSF [2]. Arecoline penetrates the oral mucosa and is hydrolysed into arecaidine, which in turn induces fibroblast proliferation and increased collagen synthesis. The action of arecoline is thus dependent on the extent of its permeation in the oral mucosa [8-10].

The oral mucosal permeability is very complex and depends on the structure and pathologic status of the tissue as well as the physical and chemical nature of the penetrants. The degree of ionization of the penetrant and the pH of the solution are important factors governing the penetration of these compounds. The property of an unionized molecule that most influences its penetration is its relative solubility in nonpolar (lipid) and polar (aqueous) solvents. Substances that dissolve readily in both types of solvent pass rapidly across the mucosa, but maximum penetration occurs when the substance has slight preferential lipid solubility [18,19].

The roasted form of areca nut possesses the highest tannin content, followed by the raw and boiled types. The arecoline content is highest in the sun-dried raw variety followed by the roasted and the boiled variety of areca nut [2,8,9]. This alteration in the chemical composition of the nuts might result in variable permeability characteristics.

The initial stages of OSF are characterized by a hyperplastic epithelium and advanced stages by an atrophic epithelium. These changes in the epithelium architecture will transmute the permeability characteristics of the mucosa.

The present study was undertaken to evaluate the permeability of the oral mucosa of Sprague–Dawley (SD) rats that has been exposed to pan masala, pure arecoline and various forms of areca nut extracts for a period of 36 weeks and to correlate it with the various stages of development of OSF.

Materials and methods

Healthy in-bred SD rats aged 3–4 months and weighing 100–200 g were randomly selected and divided into five groups: the control group, the raw areca nut group, the boiled areca nut group, the roasted areca nut group, and the pan masala and pure arecoline groups. The rats were maintained under standard laboratory conditions at controlled temperature (25 ± 2°C) with 12 h light/dark cycle and humidity. The rats were provided with standard diet and water *ad libitum*.

The study protocol was approved by the Institutional Animal Ethics Committee.

Preparation of areca nut extracts/pan masala/pure arecoline solution

The extract was prepared by dissolving 0.2 g of powdered areca nut in 6 ml of distilled water, followed by centrifugation of the solution at 15 000 rpm for 30 min. The supernatant was collected and used for injection in the buccal mucosa of rats.

A similar protocol was followed for the preparation of pan masala and pure arecoline solution.

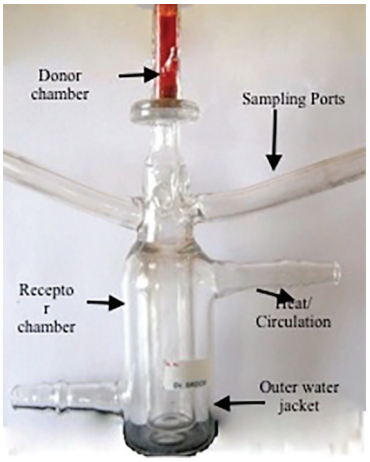
Development of an *in-vivo* model for oral submucous fibrosis in Sprague–Dawley rats

Experimental rats in the raw, boiled and roasted areca nut groups were injected with 0.2 ml of the respective solutions of the areca nut extracts using U–40 insulin syringes every alternate day for 36 weeks. Similarly, pan masala extract and pure arecoline solution were also injected. An untreated rat served as the control group.

Permeability experiment

Two animals each from the raw, boiled, roasted areca nut and pan masala groups and one animal from the pure arecoline group were killed at an interval of every 6 weeks. The left buccal mucosa was dissected out and trimmed to remove the skin and excess connective tissue. The tissues were oriented between the two halves of the Franz diffusion chamber (Fig. 1) with the epithelial surface facing upwards so that the solution could be dropped from the buccal mucosal surface to evaluate the penetration towards the submucosa. The joint of the chamber was sealed. A measure of 1 ml of pan masala solution was added to the donor compartment of the diffusion chamber. A few metal pins were placed in the receptor chamber and water was loaded in the water jacket of the

Figure 1



Franz diffusion chamber with labelled components.

diffusion cell and placed on a magnetic stirrer. The stirrer provided continuous stirring movement of the water, which ensured a moist humid environment simulating the effect of saliva in the oral environment. The tissues were left undisturbed for a period of 24 and 72 h, respectively. After the stipulated time period, the tissues were removed and fixed, followed by conventional processing, sectioning and staining with haematoxylin counterstain.

Images and image analysis

Images of the haematoxylin-stained sections were taken using microscope model LM 52-1711 (Both microscope and software from Olympus India Inc.) attached with DIGIvision 3 mp Scopeimage 9.0 (×3) software and subjected to image analysis with Lynx Biolux auto image analyser (ProReg Capture Pro 2.8.8, 2011) software.

The permeability of the mucosa was evaluated by measuring the depth of permeation (in μm) of the brownish colouration of the pan masala solution in the tissue against a haematoxylin background. The depth of permeation was correlated with the keratin layer thickness [measured from the highest point to the lowest point of the cornium layer in three different areas of the section (edges and middle), and the average value recorded] and the thickness of the epithelium [measured from the lowest point of the cornified layer to the basement membrane in three different areas of the section (edges and middle), and the average recorded]. Also, the depth of collagen fibres – that is, the depth of fibres from the basement membrane to the deepest level of the fibrosis in the submucosa – was measured and correlated with the depth of permeation. The results were statistically analysed using Pearson's correlation test.

Results

The raw areca nut group

The raw areca nut-treated group showed a marked increase in fibrotic tissue deposition as early as the 6th week, yielding a value of 192.79 μm on the image analyser against 84.78 μm in the control group. The maximum width of fibrotic bands was obtained on the 36th week, measuring 376.92 μm in the image analyser (Table 1). The other significant tissue changes were an abnormally low epithelium thickness on the 6th week (31.25 μm) as compared with the control (228.50 μm). The average epithelium thickness was lower compared with the other groups, except for the pan masala group, which showed the most atrophic epithelium (Table 2). The keratin thickness followed an irregular trend with minimal values noted for the 18th-week tissue and highest values for the 24th-week tissue (Table 3). An almost three-fold increase in permeation depth (1218.8 μm) as compared with the control (408.73 μm) was noted. In addition was a consistent increase in the depth of permeation from the 6th to the 36th week, except for a slight decrease in the 12th and 24th week (Tables 4 and 5).

Boiled areca nut group

A continuous upsurge was seen in the depth of permeation from the 6th to the 36th week, with the highest value being noted on the 36th week

Table 1 Comparison of the depth of collagen fibres (μm) in the various groups with the subsequent weeks

Weeks	Control	Raw areca nut	Roasted areca nut	Boiled areca nut	Pan masala	Pure arecoline
6	84.78	192.79	161.31	140.72	165.69	157.25
12	84.78	145.74	175.82	151.22	174.63	150.93
18	84.78	203.37	166.93	267.87	182.73	214.69
24	84.78	204.56	261.81	247.87	216.90	273.72
30	84.78	329.51	258.56	281.94	286.34	314.04
36	84.78	376.92	336.88	342.53	341.18	337.49
Average depth of collagen fibres	84.78	242.15	226.89	238.69	227.91	241.35

Table 2 Comparison of epithelium thickness (μm) in the various groups with the subsequent weeks

Weeks	Control (untreated)	Raw areca nut	Roasted areca nut	Boiled areca nut	Pan masala	Pure arecoline
6	228.50	31.25	182.99	252.38	76.28	271.82
12	228.50	104.50	132.10	241.19	74.78	166.97
18	228.50	90.44	95.38	88.11	71.23	104.2
24	228.50	72.72	85.99	76.04	77.78	86.49
30	228.50	83.72	59.82	72.56	73.11	83.61
36	228.50	70.33	56.49	44.67	47.62	55.20
Average epithelium thickness	228.50	75.49	102.13	129.16	70.13	128.05

(1697.00 μm) (Tables 4 and 5). Keratin thickness was found to decrease with increase in the duration of exposure to the areca nut. The lowest average keratin thickness (24.84 μm) was obtained in this group in comparison with the other groups (Table 3). The most atrophic epithelium was seen on the 36th week (44.67 μm). In comparison with the other test groups the boiled areca nut-treated group had the least changes in epithelium thickness values (Table 2). The 18th-, 30th-, and 36th-week tissue had fibrosis values in the upper range (Table 1).

Roasted areca nut group

The average permeation values noted were three-fold (1144.81 μm) that of the control tissue (408.73 μm). The permeation values were the least compared with the other test groups. The highest value was noted for the 30th-week (1707.49 μm) and the least for the 6th-week tissue (514.13 μm) (Tables 4 and 5). An increase in keratin thickness was seen from the 6th to the 24th week, with a decrease in the further weeks. The average keratin thickness value was highest in this group (45.22 μm) compared with the other test groups, nearing the values of the control untreated rat (52.83 μm) (Table 3). A continuous decrease in epithelium thickness and an increase in the depth of fibrosis were observed during the experiment (Table 2). The average fibrosis levels were the lowest in the roasted group as compared with the other groups (Table 1).

Pan masala group

The highest permeation was noted for the 36th-week tissue (1576.67 μm) and the lowest for the 6th-week tissue (667.17 μm) with an irregular trend in between (Tables 4 and 5). As compared with the areca nut extract-treated and the pure arecoline-treated group, the histopathological changes in the pan masala-treated group in the 6th to the 36th week were restricted to epithelial atrophy (70.13 μm), decreased keratin thickness, and minimal fibrous deposition (Tables 2 and 3). The maximum extent of fibrosis of 341.18 μm obtained from the image analyser for this group was in the 36th week (Table 1).

Pure arecoline group

The depth of permeation value recorded showed the highest peak in the 36th (1517.59 μm) and the 30th week (1475.09 μm) and the lowest value in the 6th week (687.75 μm) (Tables 4 and 5). The average keratin thickness was found to be slightly higher (56.32 μm) than in the control group (52.83 μm), with the lowest value being obtained in the 30th week (14.73 μm) (Table 3). A consistent decrease in epithelium thickness with the passage of weeks was noted, with the average value higher (128.05 μm) than in the other test groups, except in the boiled areca nut group (129.16 μm), which had values in the similar range (Table 2). The level of fibrosis increased from the 6th to the 36th week (Table 1).

Overall, there was a mild increase in the permeation depth of the solutions at 72 h compared with 24 h. A three-fold increase in permeation depths was recorded compared with the control. However, there was little variation in the average permeability ratios of the various forms of areca nut, pan masala and arecoline solutions (Tables 4 and 5) (Figs 2–5 and Graphs 1–5).

Table 3 Comparison of keratin thickness (μm) in the various groups with the subsequent weeks

Weeks	Control (untreated)	Raw areca nut	Roasted areca nut	Boiled areca nut	Pan masala	Pure arecoline
6	52.83	23.08	73.81	50.89	56.41	56.32
12	52.83	38.92	44.86	28.05	33.46	19.85
18	52.83	21.37	23.67	24.62	46.45	26.28
24	52.83	40.46	91.95	15.39	65.16	34.10
30	52.83	26.26	13.05	17.61	41.81	14.73
36	52.83	22.59	23.96	12.49	23.22	24.04
Average keratin thickness	52.83	28.78	45.22	24.84	44.42	29.22

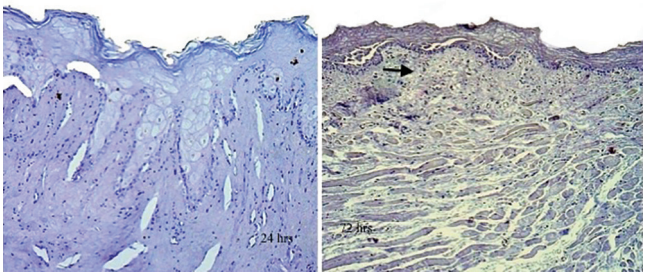
Table 4 Comparison of the depth of permeation at 24 h (μm) in the various groups at subsequent weekly intervals

Weeks	Control (untreated)	Raw areca nut	Roasted areca nut	Boiled areca nut	Pan masala
6	350.79	1231.26	412.54	610.66	592.21
12	350.79	846.58	829.93	1009.05	1208.99
18	350.79	1246.84	1205.25	1153.29	1128.21
24	350.79	1011.87	946.91	1334.46	1003.79
30	350.79	1249.01	1649.43	1264.82	1189.37
36	350.79	1359.29	1389.50	1697.00	1539.44
Average permeation depths	350.79	1157.50	1072.26	1178.21	1110.34

Table 5 Comparison of the depth of permeation at 72 h (μm) in the various groups with the subsequent weeks

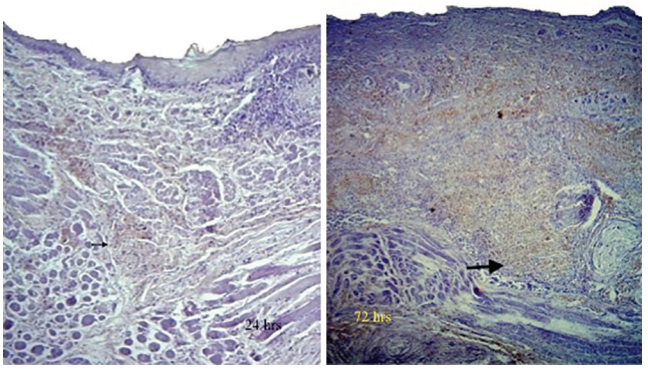
Weeks	Control (untreated)	Raw areca nut	Roasted areca nut	Boiled areca nut	Pan masala	Pure arecoline
6	466.67	1339.92	615.71	737.26	742.13	687.75
12	466.67	1071.96	1075.01	1165.47	1290.64	1220.31
18	466.67	1350.53	1297.55	1213.65	1168.99	1134.59
24	466.67	1134.39	1090.25	1443.96	1093.79	943.82
30	466.67	1354.93	1765.55	1338.87	1296.98	1475.09
36	466.67	1428.90	1460.07	1770.17	1613.90	1517.59
Average permeation depths	466.67	1280.10	1217.36	1278.23	1201.07	1163.19

Figure 2



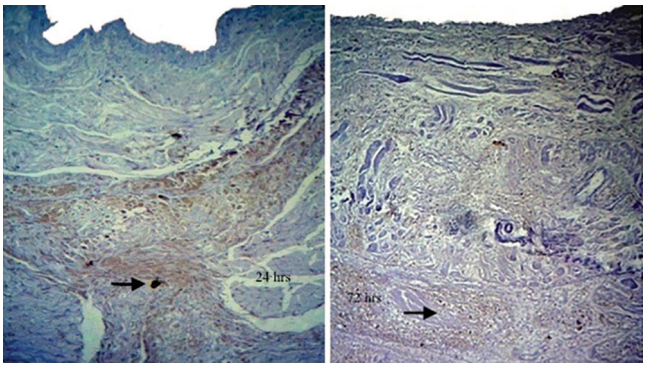
Composite photomicrograph of a tissue section from a control rat showing the permeation at 24 and 72 h. Arrows denote the depth of permeation of the test solution into the tissue (indicated by the brownish discoloration of the test solution).

Figure 3



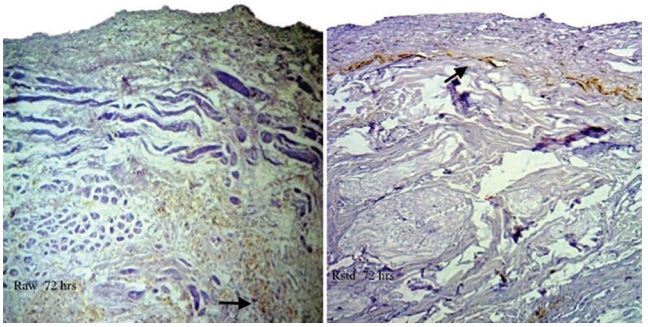
Composite photomicrograph of permeation of boiled areca nut solution at 24 and 72 h. Note the increasing depth of permeation reaching deeper layers of the connective tissue. Arrows denote the depth of permeation of the test solution into the tissue (indicated by the brownish discoloration of the test solution).

Figure 4



Composite photomicrograph of permeation depth of pan masala solution at 24 and 72 h. Note the depth of permeability lesser than that of boiled areca nut. Arrows denote the depth of permeation of the test solution into the tissue (indicated by the brownish discoloration of the test solution).

Figure 5



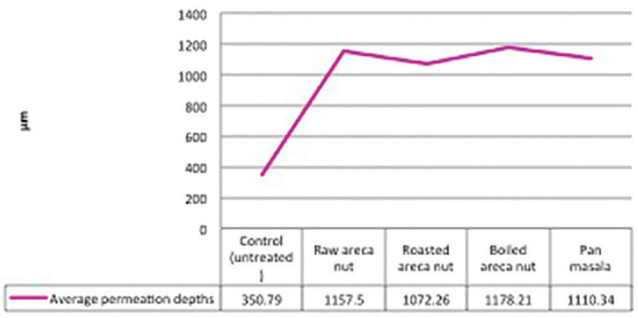
Composite photomicrographs of permeation depth in tissues of raw areca nut (left) and roasted areca nut (right) solutions at 72 h. Variable depths of permeation but lesser than levels in boiled areca nut. Arrows denote the depth of permeation of the test solution into the tissue (indicated by the brownish discoloration of the test solution).

Discussion

Areca nut is perused in many forms in the Indian subcontinent and South-East China region and range from raw to commercial varieties. Processed forms of areca nut are also an important and common form of consumption, of which the more common forms include boiled, roasted and baked nuts. The roasted variety of nut possesses the highest tannin content (5–41%), followed by the raw (25%) and boiled (17%) varieties. The arecoline content is highest in the sun-dried raw variety (1.35%) followed by the roasted (1.29%) and the boiled variety (0.1%) [2,8,9].

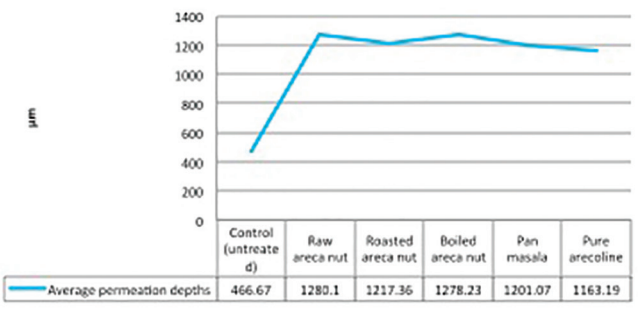
Permeability plays an important role in the aetiology of certain oral mucosal diseases, including premalignant conditions and cancer. OSF is characterized by specific epithelial changes of which a hyperplastic epithelium in the initial stages of the

Graph 1



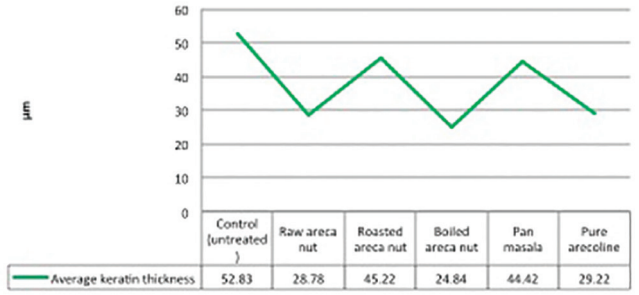
Comparison of the average depth of permeation at 24 h (μm) in the various groups.

Graph 2



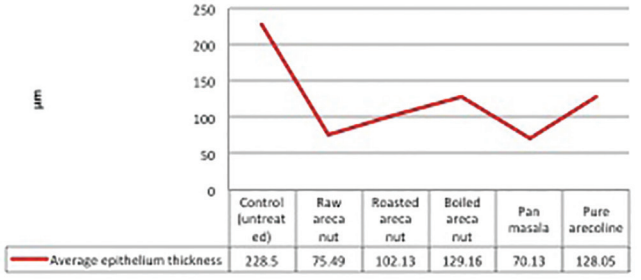
Comparison of the average depth of permeation at 72 h (μm) in the various groups.

Graph 3



Comparison of the average keratin thickness (μm) in the various groups.

Graph 4



Comparison of the average epithelium thickness (μm) in the various groups.

lesion is considered to be a protective response against the constituents of the nut penetrating the mucosa. The advanced stages of OSF are characterized by an atrophic epithelium. These changes in the epithelium architecture will alter the permeability characteristics of the mucosa.

In a previous study to determine the permeability of arecoline and arecaidine in the presence of areca nut extracts it was found that arecoline shows greater permeability than arecaidine and that the permeability decreases in the presence of areca nut extracts. This decrease was probably due to the high molecular weight tannins in areca nut that bind to the cell membrane proteins to form stable complexes and hinder the further permeation of the solution through the mucosa [11,12].

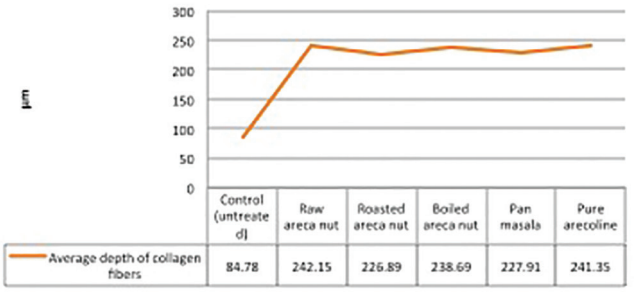
Our study was designed to determine the permeability of the rat buccal mucosal tissues treated with areca nut, pan masala and pure arecoline solution over a period of 36 weeks and to correlate it with the histological changes in the tissues as a continuum. A literature review showed that no studies of similar design have been reported.

The study used buccal mucosa obtained from SD rats rather than human samples mainly because of the hesitancy among patients and doctors for a biopsy procedure. Moreover, it has been proved that SD rats are a sustainable and reproducible model for OSF because of their ability to parallel the biological changes that characterize the disease in humans and give appreciable results within a reasonable period of time [13,14]. Moreover, these animals are easy to handle, inexpensive and universally available at all animal laboratories.

The use of the pan masala solution as the standard indicator for the permeability experiment was due to two reasons. First, this commercial form of areca nut preparation is the most commonly used in the Indian subcontinent. Second, the arecoline content of the pan masala solution was found to be in the higher level among the groups assayed, and a direct correlation of the permeability results with arecoline could thus be evaluated.

The results obtained from our study showed OSF-like lesions in different degrees in the areca nut, pan masala and pure arecoline-treated groups as compared with the control (untreated animal). The histological changes observed included an atrophic epithelium with partial or complete loss of rete ridges, accumulation

Graph 5



Comparison of the average depth of collagen fibres in the various groups.

of dense bundles of collagen fibres, juxta-epithelial hyalinization and decrease in vascularity. This is in accordance with previous studies that reported similar findings on exposure to areca nut [13,15,16].

A characteristic atrophic epithelium was obtained in the buccal mucosa of all experimentally treated animals at all treatment periods especially in the pan masala and the raw areca nut-treated group starting from the initial 6th week to the 36th week. The early manifestation of an atrophic epithelium in the pan masala-treated group could be due to the combined effects of areca nut and other additives like lime, catechu and other unspecified ingredients in pan masala and also due to a slower rate of diffusion of pan masala solution compared with that of the areca nut solution as reported by a previous study in our laboratory [17]. The slow diffusion of the solution may account for the solution being limited to the epithelium for a longer duration and hence may cause more deleterious effects on the epithelium in comparison with the effects on the submucosa. The more damaging effects of the raw areca nut solution can be explained by its higher arecoline and tannin concentration. The least damaging effect on the epithelium was noted for the boiled areca nut group, which again can be correlated to the lower concentration of the active constituents – that is, arecoline and tannin – in it.

The rat buccal mucosal tissues initially showed hyperorthokeratosis on exposure to areca nut and pure arecoline irritants but later showed decreased keratin thickness because of the constant irritating effect on the tissue.

The increased amount of fibrotic tissue was found in the test groups as compared with the untreated animal and is attributable to the chemical irritation caused by the constituents of the nut. The fibrosis levels recorded in this study using image analysis revealed maximum fibrosis at 36 weeks in the raw areca nut group measuring ~376.92 μm. The least amount of fibrosis was seen at the 6th and 12th week for all experimental groups that increased until the 36th week, explaining the role of time in the development of OSF. This is in accordance with the previous studies that evaluated the effect of areca nut/pan masala extracts in rats/mice, and reported an increased fibrosis level in the tissues as compared with the controls [13,15,16]. The roasted and pan masala-treated tissues show the minimum fibrosis levels and the raw areca nut-treated and pure arecoline-treated tissues show the maximum fibrosis. These variations are probably because of the differences in the constituents of each form of areca nut caused by the processing procedures.

Overall, there occurred a mild increase in the permeation depths of the solutions in all groups at 72 h compared with 24 h, explaining the time factor as an important determinant of permeability. The values recorded for the depth of permeation were noted to be the highest for the 36th- and 30th-week tissues and lowest for the 6th- and 12th-week tissues. An increase in permeability of the tissues with time can be attributed to the tissue changes caused by the consumption of areca nut and its related compounds over a period of time. In our animal model, thinning of the epithelium and keratin layer was one consistent feature noted with the passage of time. The reduced epithelium and keratin thickness would amount to inferior barrier properties of the tissue and thereby to increased permeability.

The statistical analysis of our results revealed that the permeation values had a significant negative correlation with the epithelium and keratin thickness; that is, a thin keratin layer and an atrophic epithelium showed increased permeability of the tissues and vice versa. Although previous studies state the keratin layer to be an incomplete barrier, the depth of permeation values in this work was more closely and significantly related to the keratin thickness of the tissue.

A significant positive correlation was obtained between the depth of fibrosis and permeation values; that is, increased fibrosis levels were associated with an increased depth of permeation. The dense collagen bundles deposited in the connective tissue in OSF have probably limited the diffusion of the permeant into the tissue. However, our results reveal an increased permeation with increase in severity of the grades of OSF. This can be probably explained by the fact that the thick collagen fibre bundles are ineffective as a barrier and allow the permeant to pass through unhindered.

On extrapolation of the data obtained from our animal study to humans it was found that OSF-like changes

were seen in rats as early as 6 weeks, which corresponds to 2.5 years of areca nut consumption in humans. This is consistent with previous studies that state that it takes around 2–4 years for OSF lesions to develop in habitual areca nut users [18]. The average life span of humans is about 70 years, which is equivalent to 3640 weeks. SD rats have an estimated life span of 3 years, corresponding to 156 weeks. On calculating the ratio of the life spans of SD rats and humans a value of 1 : 23 was obtained; that is, 6 weeks in a rat corresponds to 2.5 years in humans. Prolonged intake of areca nut and pan masala leads to higher grades of OSF and a concurrent increase in permeability of the tissue [18].

Conclusion

Consumption of areca nut and its derivatives in any form has deleterious effects on the oral mucosa leading to development of OSF-like lesions characterized by epithelial atrophy, reduced keratin thickness, fibrosis and reduced vascularity. These alterations are concomitantly associated with an increased permeability in the tissues. This increased permeability brought about by the tissue changes would consolidate the fibrosis in the connective tissue, further aggravating the harmful effects and in turn leading to more advanced stages of OSF.

Also noted was the fact that the destructive potential of raw areca nut and pan masala solution is more than that of the other test solutions (roasted and boiled areca nut), which is reflected by the capacity of the former solutions to induce more fibrotic, epithelial and vascularity changes in a shorter time interval, in turn leading to a highly permeable tissue.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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