Stromal changes in apparently normal mucosa of smokers and pan chewers – a multi-parametric approach

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Abstract

Aim: To compare and contrast the various changes in the connective tissue among patients with the habits of smoking, pan chewing and controls by using fluorescence spectroscopy, histopathology and transmission electron microscopy. **Method**: Thirty subjects were categorized into three groups: pan chewers, smokers and controls without any oral lesions. Fluorescence spectroscopy was carried out using FLUOROMAX-2. Excitation spectroscopy was performed at 280 and 320nm respectively and Emission spectroscopy was performed at 340 and 390nm excitation. Subsequently, histopathological evaluation and transmission electron microscopy was done for biopsies taken from test groups and controls. **Results**: The mean, standard deviation and test of significance of mean values between different groups for intensity-380nm, intensity–420nm and intensity – 460nm at 320 nm excitations, showed that the Mean values in group I and group II were significantly higher than the mean value in group III with a p-value of less than 0.001. Considering the histopathological parameters, the pattern of the sub-epithelial connective tissue, the presence of chronic inflammatory cells and lysis of connective tissue was significant. The ultrastructural analysis revealed the presence of epithelioid, spindle or elongated and stellate shaped fibroblasts in the connective tissue. **Conclusion**: In this pilot study, we could find variations in emission characteristics of various amino acids which correlated with histopathology and electron microscopy. Our study suggests that there are connective tissue changes in oral mucosa among smokers and pan chewers, though it is apparently normal in clinical presentation. These initial connective tissue changes could determine the progression of altered mucosa to a pre-cancer or cancer, which is further related to other complex interactions. Elaborate studies are required to evaluate the significance of our hypothesis.

Keywords:
Autofluorescence, normal mucosa, smokers, pan chewers

Introduction

Oral cancer is dominated by squamous cell carcinoma, which represents 90%-95% of all oral cancers. The role of tobacco in the development of oral squamous cell carcinoma is well recognized. The long-term prognosis is quite poor, and treatment can lead to further functional and cosmetic problems[1-2]. Tobacco use, including smoking of cigarettes, cigars and pipes, reverse smoking (smoking with the lit end inside the mouth), chewing of betel quid (a mixture of areca nut, slaked lime, and tobacco wrapped in betel leaf), and use of smokeless tobacco increases the risk of cancers of the upper aerodigestive tract[3-4]. On population-based case-control studies, cigarette smokers have risk from two to five times than that of non-smokers for developing oral cancer. The risk increases with rise in number of cigarettes smoked and duration of smoking[5].

Tobacco is chewed predominantly as an ingredient of betel quid or pan, which is a combination of betel leaf, areca nut, and lime. Based on analysis that excluded smokers, smokeless tobacco users experienced about four to six times the risk of oral and pharyngeal cancer than non-users[1-2]. In the Indian scenario, the proportion attributed to tobacco use in the form of smoking and chewing comprises about 61-70% of cancer incidence. Among Indians, alcohol does not emerge as a strong risk factor[6].

There is increasing evidence that sub-epithelial connective

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tissue can modify the phenotypic expression of the overlying epithelium. Various tissue culture studies suggest that the histodifferentiation of the epithelium, including its phenotype and keratin expression could be extrinsically modified by mesenchymal fibroblasts. It is possible to elucidate that the underlying connective tissue plays a pivotal role on maintenance of the integrity of overlying epithelium, as per several studies on precancer and cancer[7-9]. Thus this study was designed for evaluating different parameters of the stromal changes in apparently normal oral mucosa of smokers, pan chewers and controls using fluorescence spectroscopy. Moreover, histopathological examination using Hematoxylin and Eosin stain and Van Gieson stain was performed, as well as the evaluation of ultrastructural changes in fibroblasts using transmission electron microscopy (TEM).

Materials and methods

Thirty subjects aged from 16 to 60 years (mean age 45.6 years) without any oral lesions from the Department of Oral Medicine and Radiology, College of Dental Surgery, Saveetha University, Chennai were considered for the present study. The subjects were categorized into the following groups: GROUP I, with 10 subjects without the habits of smoking or alcoholism or pan chewing, as the controls, GROUP II: 10 subjects with the habit of pan chewing (10-15 quid's per day) for about 5-10 years; GROUP III: 10 subjects with the habits of smoking more than 10 cigarettes per day for about 5-10 years,.

Fluorescence spectroscopy was carried out using FLUOROMAX-2 (USA, 1996) in the Department of Medical Physics, Anna University, Chennai, with a Fiber-optic probe attached to spectrofluorometer. The probe was placed in contact with the mucosa to record the data. For Group II, readings were taken from the area where the quid was placed and an area adjacent to the premolars at the occlusal level for groups I and III. Excitation spectroscopy was performed at 280 and 320nm respectively and Emission spectroscopy was performed at 340 and 390nm. Corrections were done for the non-uniform spectral response and for the variations in the intensity of illumination source of the spectrometer. The spot size of the illumination was approximately 1 cm in diameter and photo-bleaching was not observed in the test site after the procedure.

Subsequent to the autofluorescence spectroscopy, after taking informed consent, incisional biopsy was performed in the subjects of the three groups, under local anesthesia at the same sites used for fluorescent spectroscopy.

The biopsy samples were rinsed in saline and then fixed with 10% buffered formalin. The tissue was processed routinely and embedded in paraffin. 4µ sections were prepared with a Leica semi-automated microtome (RM 2245). Routine Hematoxylin and Eosin staining was done for microscopic examination of the sections. Sections were also stained with Van Gieson stain to demonstrate collagen. The Connective tissue in the sub-epithelial region and deeper region were assessed for the following parameters, Density – minimal/moderate/ increased, Pattern – wavy/ bundles/haphazard/parallel/stream, number of Fibroblasts - minimal/ moderate/ increased, number of Inflammatory cells - absent/minimal/moderate/ intense, Hyalinization– present/absent and amount of vascularity - minimal/ moderate/intense, Connective tissue status - homogenous/ lysis and Intensity of Van Gieson stain - minimal/ moderate/ intense. The epithelial parameters that were assessed include Keratinization – ortho/para/non-keratinized, Thickness– atrophy/hyperplastic/normal, Epithelium-connective tissue interface: normal/ thickened/discontinuous.

Samples were also fixed in 2.5% buffered glutaraldehyde, processed and subjected to transmission electron microscopy. All the parameters were tabulated and the statistical significance was assessed with SPSS Version 10.

Results

Native fluorescence spectroscopy of groups I, II and III at different wavelengths, corresponds to the native fluorophores present in cells and extracellular matrix, such as tryptophan, collagen, elastin and NADH.

The average emission spectrum was performed at 280 nm excitation corresponding to the amino acid residue, tryptophan. Normal oral mucosa and pan chewer's mucosa showed a prominent peak at 338 nm and a small peak at 440 nm. It is worth to mention that the emission at 280 nm mostly is due to tryptophan, as the emission efficiency (quantum yield) of other two amino acids, phenyl alanine and tyrosine is very minimal as that of tryptophan. Smoker's mucosa did not show any peaks at 338 nm and 440 nm.

In order to overcome the artifacts of instruments and tissue heterogeneity, normalization of spectra was performed in relation to its peak wavelength or any wavelength of interest. The averaged emission spectra of normal mucosa, mucosa of pan chewers and smokers at 280nm excitation showed maximum emission intensity at 340 nm (fig1). The normal tissues showed a lesser intensity than pan chewers and smokers, at higher wavelengths of 400-500 nm. It clearly indicates that NADH/NAD(P)H and collagen

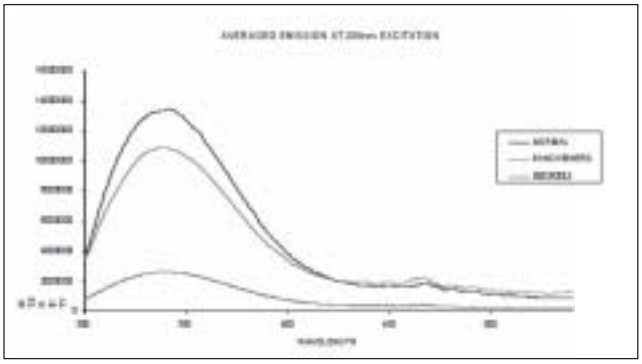

Fig. 1 - *Averaged fluorescence emission spectra of normal, pan chewers and tobacco smokers at 280nm excitation*

distribution may be altered under various tissue transformation conditions.

The averaged emission spectrums at 320 nm excitation corresponding to the collagen/elastin are shown in Fig. 2. The emission spectra corresponding to normal mucosa and pan chewer's mucosa showed two prominent peaks at 390 nm and 460 nm, respectively. The first peak which is at 390 nm corresponds to the collagen intensity (around 390) and the second peak corresponds to the NADH (around 460 nm), which are almost equal. However, surprisingly the smoker's mucosa did not show any increase in intensity at 390nm and 460nm.

The average excitation spectra at 340 nm emission corresponding to the tryptophan are shown in Fig. 3. The emission spectra corresponding to normal mucosa showed prominent intensity at 295 nm whereas the pan chewer's mucosa showed a prominent peak at 296 nm. Tobacco smoker's mucosa showed a small peak, with maximum intensity at 292 nm wavelength.

The normalized excitation spectra were performed at 34 0nm emission. Pan chewer's mucosa did not show significant peak shift compared to the normal individuals, both of them showed maximum intensity at 295nm. Surprisingly, smoker's mucosa showed maximum intensity at 292 nm showing a shift to the left, i.e., towards shorter wavelength. This shift to the left could probably attributed to any conformational changes in the tissue protein sequences and/or partial folding of protein structures.

The average excitation spectra at 390 nm excitation corresponding to the collagen are shown in Fig 4. The excitation spectra corresponding to normal mucosa and pan chewer's mucosa showed relatively a prominent peak at 295 nm, whereas smoker's mucosa showed a small peak at the same wavelength. The excitation intensity of pan chewer's mucosa was much less compared to normal at this emission spectrum.

The normalized emission spectra were performed at 390 nm emission. The emission spectral findings at 390 nm are similar to that of 340 nm emission. However, a slight shift to the left (towards shorter wavelength) was seen in pan chewer's mucosa when compared to the normal individuals. Smoker's mucosa showed a gross shift to the left, as seen in 340 nm emission. The maximum intensity values at this emission spectrum for normal, pan chewer's mucosa and smoker's mucosa are 298, 297 and 294 respectively. This clearly indicates that the absorption bands of collagen vary with tissue conditions.

In order to improve the diagnostic interpretation, different parameters were introduced at the emission peak of 320 nm excitation. We selected 320 nm excitation because the presence of fluorophores – collagen, hemoglobin and NADH that were altered in smokers. The wavelengths were selected at points where maximum intensity of corresponding fluorophores was observed. Based on these intensity values, we performed the statistical analysis.

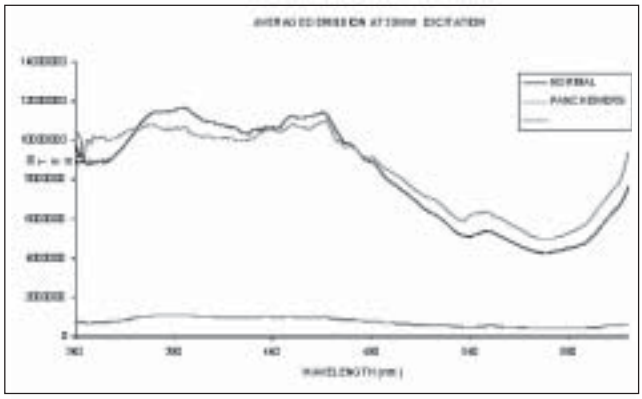

Fig. 2 - *Averaged fluorescence emission spectra of normal, pan chewers and tobacco smokers at 320nm excitation*

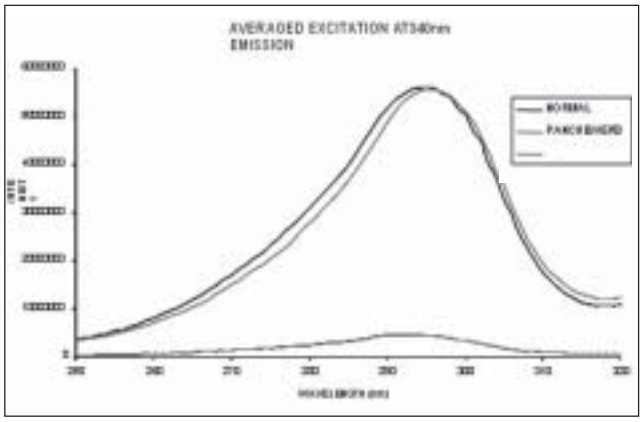

Fig. 3 - *Averaged fluorescence excitation spectra of normal, pan chewers and tobacco smokers at 340nm emission*

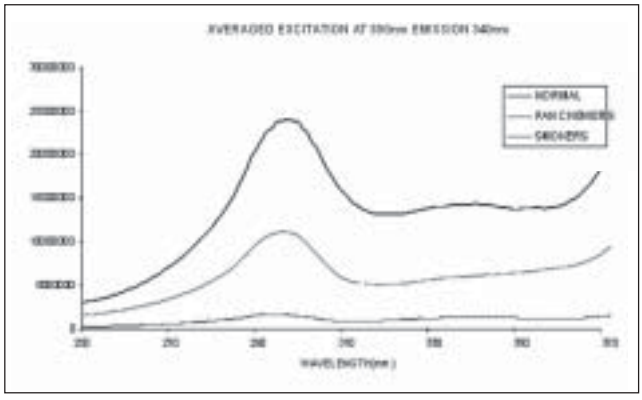

Fig. 4- *Averaged fluorescence excitation spectra of normal, pan chewers and tobacco smokers at 390nm emission*

There was a considerable difference in the fluorescence intensity at I-380 between normal mucosa and smoker's mucosa (Fig. 5, Table 1). It was also observed that this intensity value discriminated normal from smoker's mucosa with a sensitivity of 100% and specificity of 100%. There was a considerable overlap in the fluorescence intensity at I-380 between normal and pan chewer's mucosa. This intensity value discriminated normal from pan chewer's

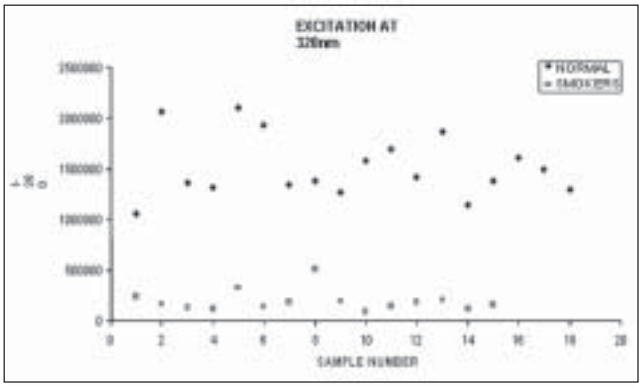

Fig. 5 - *Scatter plot of intensity value at 380nm Vs sample number for normal and tobacco smokers from fluorescence emission at 320nm excitation*

Table 1 – Fluorescence intensity at I-380

Groups	Sensitivity	Specificity
Group I vs.Group III	100	100
Group II vs. Group III	100	100
Group I vs. Group II	78	12

mucosa with a sensitivity of 78% and specificity of 12%. There was considerable difference in the fluorescence intensity at I-380 between pan chewer's mucosa and smoker's mucosa (Fig. 6). This intensity value discriminated smoker's mucosa from pan chewer's mucosa with a sensitivity of 100% and specificity of 100%.

The data were compared between groups for intensity-380 nm, intensity–420 nm and intensity – 460 nm at 320 nm excitations, showing that the mean values in GROUP I and GROUP II were similar ($p > 0.05$) and both significantly higher than GROUP III ($p < 0.001$).

In the histopathological assessment (Table 2) all the subjects of GROUP I and II did not show lysis in the connective tissue. From GROUP III, 6 cases showed lysis

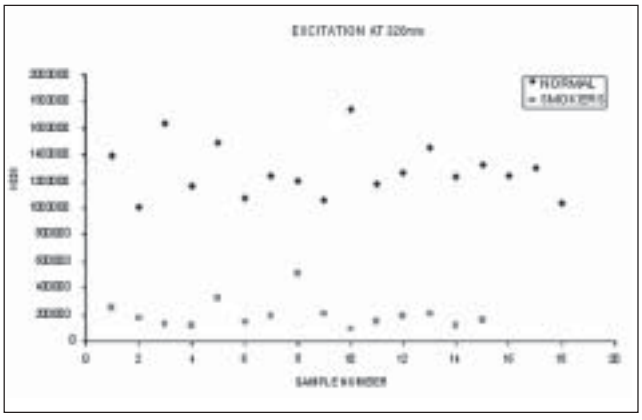

Fig. 6 - *Scatter plot of intensity value at 380nm Vs sample number for pan chewers and tobacco smokers from fluorescence emission at 320nm excitation*

Table 2 – Histopathological assessment

Criteria	Group I	Group II	Group III
Lysis in the connective tissue	0/10	0/10	6/10
Intense inflammatory cell infiltrate	0/10	0/10	2/10
Moderate inflammatory cell infiltrate	0/10	8/10	6/10

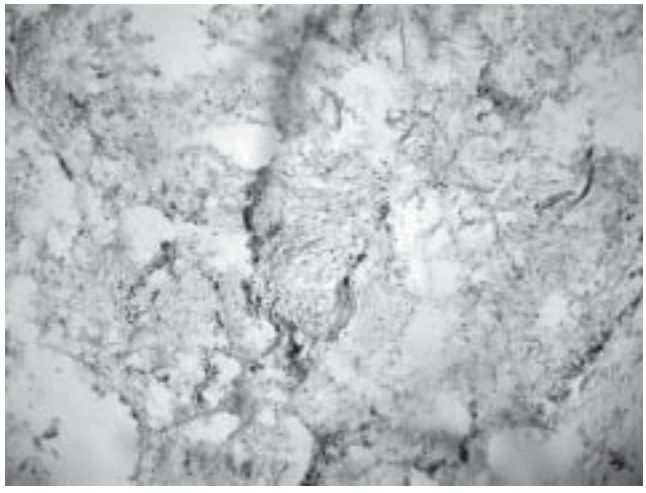

Fig. 7 - *Lysis of connective tissue H&E – 10x*

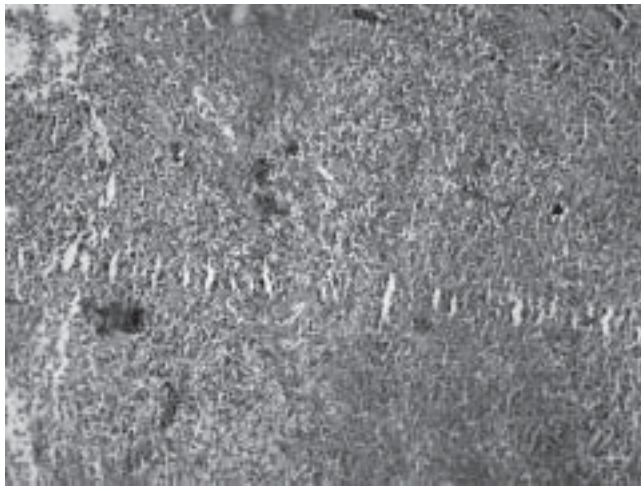

Fig. 8 - *Inflammatory cells in the connective tissue H&E – 10x*

(Fig. 7) and 4 cases did not show. Presence of lysis in the connective tissue and presence of inflammatory cells in the deeper region of the connective tissue were significant ($p = 0.024$ and $p = 0.036$, respectively). It was observed in GROUP I 6 cases with minimal inflammatory cells and 4 cases with mild inflammatory infiltrate, whereas in GROUP III, 2 subjects presented mild inflammatory infiltrate and 8 subjects moderate inflammatory infiltrate (Fig. 8). In GROUP II, mild inflammatory infiltrate was observed in 2 subjects, moderate inflammatory infiltrate in 6, and intense inflammatory infiltrate in 2. The other histopathological parameters did not achieve statistical significance.

The ultrastructural analysis of the oral mucosa of controls by TEM showed presence of continuous, homogeneous,

thick collagen bundles with occasional blood vessels and numerous fibroblasts. The fibroblasts were spindle / elongated in shape with elongated nucleus, dense chromatin, numerous endoplasmic reticulum and extended cisternae. The smoker's mucosa showed the presence of numerous discontinuous bundles and few dense granules. There were few fibroblasts actively secreting collagen with increased bundles of collagen showing prominent banding. The fibroblasts were elongated to spindle shaped, with elongated nucleus pushed to one end, numerous endoplasmic reticulums, extended cisternae, granules filled with proteinaceous material. The mucosa of pan chewers showed the presence of immature, thin filaments and the banding was not prominent. The fibroblasts were epithelioid in shape with a round nucleus, prominent nucleolus and endoplasmic reticulum along with increased cellularity. Highly vacuolated cells containing some debris, degenerating cells or apoptotic cells with fragmented organelles and apoptotic bodies were seen. Inflammatory cells, predominantly granulocytes and mast cells were also evident in both the study groups.

Discussion

Autofluorescence spectroscopy is a non-invasive and easily applicable tool for the detection of alterations in the structural and chemical compositions of cells, which may indicate the presence of diseased tissue[10].

When tissue is illuminated with specific wavelengths of ultraviolet (UV) or visible (VIS) light (excitation), fluorescent biological molecules (fluorophores) will absorb the energy and emit it as fluorescent light at longer wavelengths (emission). Furthermore, there are non-fluorescent light absorbers (such as hemoglobin) and scatterers (cells and sub-cellular organelles) in tissues that modulate the tissue fluorescence intensity at the excitation and emission wavelengths. Endogenous fluorophores in tissue include amino acids, structural proteins, enzymes and coenzymes, vitamins, lipids, and porphyrins. Each of these molecules has unique excitation and emission spectra in the UV/VIS spectral region. The excitation wavelengths used, ranging from 300 to 460 nm, allow for characterization of a number of biologic fluorophores, including tryptophan, nicotinamide adenine dinucleotide, flavoproteins, and collagen, all of which are present in tissue systems. Tryptophan shows maximum intensity at 280nm, collagen at 320nm, NADH at 340nm and endogenous porphyrin at 405nm excitation[11].

Accordingly, autofluorescence spectra were performed corresponding to these wavelengths for normal mucosa and mucosa of pan chewers and smokers. Though there are studies on autofluorescence spectra in sub mucous fibrosis, leukoplakia and squamous cell carcinoma[10-13], our pilot study is the first of its kind in English literature to assess the autofluorescence in apparently normal mucosa of smokers and pan chewers.

In the present study, the averaged fluorescence emission spectrum of normal mucosa, mucosa of pan chewers and smokers, at 280 nm and 320 nm excitation wavelengths were performed. Although the normalized spectral signature of smoker's mucosa is similar to that of pan chewers and normal mucosa, the absolute intensity is 8 folds lesser than that of normal mucosa. The major peak around 338 nm may be attributed to tryptophan emission. Most proteins are endowed with an intrinsic UV fluorescence because they contain aromatic amino acids, particularly phenylalanine, tyrosine and tryptophan. Of these three aromatic amino acids tryptophan has the highest fluorescence quantum yield overshadowing markedly the emissions of the other two. Tryptophan emission maxima in proteins can vary from 332 nm to 342 nm depending on the protein. Free tryptophan has a characteristic fluorescence emission at 350–360 nm[14]. This decrease in tryptophan levels may be attributed to the sparse cellularity or it may also be due to the distortion of fluorescent intensity.

Analysis using fluorescent excitation spectra is a complimentary technique which is sensitive to any conformational changes that take place during the process of tissue transformation. It will provide the changes in absorption band of molecules. In order to get the emission spectrum, the molecule of interest should be excited at its exact absorption wavelength. Generally it is very difficult to measure absorption spectra of turbid media like cells and tissues. Under such conditions, fluorescence excitation spectra can indirectly measure the absorption band. The peak emission of the fluorophores is monitored at different excitation wavelengths. The maximum intensity corresponding to a particular wavelength of excitation is the absorption wavelength of the molecule. Hence, we also monitored the fluorescence excitation spectra for all the tissues, for their emission at 340 nm and 390 nm to confirm whether the emission at 340 nm is due to tryptophan and to check whether there is any change in the absorption band, when normal tissues undergo various transformations.

A shift to the right, i.e., to higher wavelength is called Red shift. Similarly a shift to the left is called Blue shift. It is found that normal and pan chewer's mucosa have similar absorption bands at 300nm. Howeve,r smoker's mucosa had very minimal absorption, which was 30 times lesser than normal. This may be due to the scattering or reflection of light due to decreased blood supply. Though there are many controversies behind the decrease in fluorescence, our results are in line with Tsai et al.[12], who attributed the decrease in fluorescence to the distortion of fluorescence caused by collagen excess in the sub-mucosa.

The average excitation spectra of normal, mucosa of pan chewers and smokers at 340 nm emission corresponding to tryptophan, smoker's mucosa showed the left shift compared to normal, indicating that there is a considerable rearrangement in the protein sequences of amino acids.

The autofluorescence emission spectra at 320 nm excitation, corresponding to collagen, revealed a first peak around 390 nm, which corresponds to emission from collagen and elastin. The second peak at 460 nm corresponds to NADH present in epithelial cells of the surface mucosa. The valley between the two peaks corresponds to the hemoglobin absorption of

the tissues. The emission spectra of normal mucosa and pan chewers showed two prominent peaks centered at 390 nm and 460 nm with a valley around 420 nm. However, the relative intensity of emission of pan chewers is slightly lower than that of normal individuals. The smoker's mucosa had only one broad peak in the region from 370 nm to 530 nm without any dip at 420 nm, indicating less vascularity. It is surprising to observe that smokers displayed decreased fluorescent intensity compared to that of normal mucosa and pan chewer's mucosa at this particular wavelength. This observation is contradictory to that of Chen et al.[13] who found increased intensity for collagen in oral sub mucous fibrosis. However, our results are similar to findings of Tsai et al.[12], since decreased intensity for collagen in sub mucous fibrosis was observed. The changes in epithelium may result in increased NADH intensity. However, the NADH intensity in pan chewers was slightly less compared to that of normal. This might be due to minimal scattering caused by collagen that is beginning to accumulate in the sub-mucosa. In smoker's mucosa, no peak was observed at 440 nm, which probably could be attributed to the distortion of fluorescence caused by collagen. The valley that is observed around 420 nm corresponds to hemoglobin absorption. The valley indicates the amount of vascularity in the tissues. Normal mucosa and pan chewer's mucosa showed a prominent dip around 420 nm. But smokers did not show any dip at this wavelength. It is also interesting to note that spectral signature of smokers was entirely different from normal and pan chewers. The spectral band of normal and pan chewers showed a valley around 420nm and 580nm and this indicates a considerable vascularity in these tissues. However, the valley at 420nm and 580nm are completely absent in smokers indicating minimal vascularity.

Liang et al.[15] explained that the left shift by photo-physical characteristics of tryptophan depends on the micro-environmental conditions. In particular, the emission of tryptophan depends upon its solvent polarity. The shift to shorter wavelength occurs as the solvents surrounding the tryptophan residues decrease[13]. This explanation could be applied to smoker's mucosa, as there is stabilization of collagen, making them less soluble to collagenases when compared to normal collagen. Another explanation for the left shift or the decrease in tryptophan intensity could be due to the sparse cellularity in the dense, collagenized sub mucosa. Another reason for the decrease in collagen fluorescence, in spite of having more amounts of collagen and elastin, is that there may be a reflection of light at the epithelium-connective tissue interface without any absorption. This may be due to the change in the refractive index of the tissues. Fluorescence intensity can be influenced by inter-subject variability in the amount of blood, with absorption leading to a wavelength-dependent decrease in fluorescence intensity. Besides the biological variation, varying experimental circumstances can influence the total fluorescence intensity[10].

The present non-invasive in-vivo autofluorescence spectroscopy helps to differentiate smoker's mucosa from normal with a significant difference. However, further studies with more samples are necessary to identify the exact cause of decreased fluorescence in smokers, though we attributed it to the distortion of fluorescence as mentioned by Tsai et al.[12]. The attempt to differentiate pan chewers from normal was also successful, since early changes with decreased fluorescent intensity compared to normal were noted.

Subsequently, biopsy samples were taken from the representative site as detected by autofluorescence. Keratinization of mucosal epithelium is believed to be inter-related with the inflammatory cell infiltrate. In our study, the samples predominantly showed hyper-parakeratinization. The epithelial thickness may depend on the tobacco habit and the location. The epithelial hyperplasia could be an adaptive response to local irritants, to provide a greater degree of protection to the underlying connective tissue.

The collagen fibers were wavy among the controls and majority of the pan chewer's mucosa. Considering the amount and nature of collagen in the sub-epithelial region and deeper region, the haphazard and thickened arrangement of connective tissue seen in smokers along with lysis, correlates with the connective tissue changes seen in the early stages of sub mucous fibrosis. The presence of inflammatory cells also has a role to play in the fibrosis of connective tissue stroma. The production of inflammatory mediators is controlled by various enzymes such as cyclo-oxygenases. Increased expression of fibrogenic cytokines like TGF-beta, PDGF, bFGF and pro-inflammatory cytokines like IL-1 & 6 plays a role in fibrosis[16].

Subsequently, to confirm to our histopathological findings, TEM was performed. In smoker's mucosa, numerous discontinuous bundles were present along with few dense granules. There was an increase in collagen bundles and prominent banding was also evident. The fibroblasts were elongated to spindle shaped, elongated nucleus pushed to one end along with numerous endoplasmic reticulums, extended cisternae and granules filled with proteinaceous material. Mast cells were also present. There were few fibroblasts actively secreting collagen.

Our study also revealed the presence of epithelioid, spindle or elongated and stellate shaped fibroblasts In accordance with literature, Mollenhauer and Bayreuther[17] described three distinct fibroblast cell forms in rat connective tissue that can be identified on the basis of their morphology. They can also be distinguished from one another by the amount and type of collagen synthesized. The F1 fibroblast is spindle shaped, highly proliferative and secretes low levels of type I and III collagen. The F2 fibroblast is more epitheloid, less proliferative and synthesizes relatively more collagen. The F3 fibroblast is a large stellate cell and the least proliferative, produces four to eight times more types I and III collagen than F1. According to these workers, F2 cells sequentially arise from F1 cells and F3 cells sequentially arise from F2 cells. The lysis and fragmentation of collagen fibres was also considered as one of the change especially authenticated as an ultrastructural change in sub mucous fibrosis.

Binnie and Cawson[18] found an excess of fine, immature fibrils and inter-fibrillar matrix in sub mucous fibrosis. The collagen fibrils were observed to be fragmented, some of them showed frayed and bent ends with apparent partial degeneration into amorphous material. Rajendran et al.[19] also reported similar ultra-structural findings in sub mucous fibrosis. Thus the ultrastructural changes of increased fibrosis in smoker's mucosa, is in accordance with the findings of autofluorescence and histopathology and these changes are comparable to oral sub mucous fibrosis.

Our study showed that smokers had connective tissue changes similar to sub mucous fibrosis more than that seen in pan chewers. Literature states that sub mucous fibrosis is more prevalent in pan chewers and smokers have increased tendency to develop leukoplakia, though both are caused by tobacco products.

Hence, we have hypothesized that the response of connective tissue in relevance to smoking differs from pan chewing. In smoking, apart from the tobacco component, heat also acts as an additional source of insult and may aid in faster diffusion. The initial response of collagen fibres in smoking could be due to the synergistic effect of heat and tobacco. The collagen response would prevent the further diffusion, resulting in changes in epithelium and may thereby progress to leukoplakia and other changes. Tilakaratne et al.[16] have hypothesized that dense fibrosis and less vascularity of the corium, in the presence of altered cytokine activity creates a unique environment for the carcinogens of tobacco and areca nut to accumulate over a long period of time either on or below the epithelium.

But in pan chewing, diffusion may be slow in comparison to smoking, thereby producing an initial, protective inflammatory response in the connective tissue. The fibroblasts would initially produce collagen as a defensive reaction and subsequent insults would result in the morphologic change of fibroblasts and thus progress to sub mucous fibrosis.

Hence, our study suggests that there are connective tissue changes in oral mucosa among smokers and pan chewers, though it is apparently normal in clinical presentation. These initial connective tissue changes could determine the progression of altered mucosa to a pre-cancer or cancer, which is further related to other complex interactions. Elaborate studies are required to evaluate the significance of our hypothesis.

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