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Abstract. Oral habits like chewing and smoking are main causes of oral cancer, which has a higher mortality rate than many other cancer forms. Currently, the long term survival rate of oral cancer is less than 50%, as a majority of cases are detected very late. The clinician's main challenge is to differentiate among a multitude of red, white, or ulcerated lesions. Hence, new noninvasive, reliable, and fast techniques for the discrimination of oral cavity disorders are to be developed. This study includes autofluorescence spectroscopic screening of normal volunteers with and without lifestyle oral habits and patients with oral submucous fibrosis (OSF). The spectra from different sites of habitués, non-habitués, and OSF patients were analyzed using the intensity ratio, redox ratio, and linear discriminant analysis (LDA). The spectral disparities among these groups are well demonstrated in the emission regions of collagen and Flavin adenine dinucleotide. We observed that LDA gives better efficiency of classification than the intensity ratio technique. Even the differentiation of habitués and non-habitués could be well established with LDA. The study concludes that the clinical application of autofluorescence spectroscopy along with LDA, yields spontaneous screening among individuals, facilitating better patient management for clinicians and better quality of life for patients. [DOI: 10.1117/1.3608923]

Keywords: oral habits; oral submucous fibrosis; collagen; flavin adenine dinucleotide; redox ratio; linear discriminant analysis; spectral intensity ratio.

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1 Introduction

Cancer of the head and neck are among the leading cancer types all over the world. Oral and pharyngeal cancer combined together is the sixth most common cancer. For oral cancer, annual estimated incidence is around 275,000 and two thirds of these cases occur in developing countries. Globally, the high incidence areas for oral cancer are South and Southeast Asia, parts of Western and Eastern Europe, parts of Latin America and the Caribbean and Pacific regions. Among these, South and Southeast Asia is reported to have the highest degree of occurrence among men. About 25% of male patients attending hospitals for cancer treatment are for oral cancer.[1,2]

Lifestyle factors, living environment, and patient's genetics play an important role in the occurrence and development of oral cancer. Among these, lifestyle factors that include alcoholism, smoking, and chewing of betel quid with or without tobacco are considered as the main etiological factors. Combination of any of the two habits further increases the risk for oral cancer.[3] Worldwide, 7 to 19% of oral cancers are associated with alcohol consumption, 25% with tobacco usage (smoking and/or chewing), 10 to 15% with micronutrient deficiency, and more than 50% with betel quid chewing in areas of high chewing prevalence.[4]

According to World Health Organization reports, almost two billion people over the globe consume alcohol and almost 80 million have diagnosable disorders related to alcohol consumption. Risk for the development of oral cancer among people who consume alcohol regularly is three to five folds higher than that among nondrinkers.[4–7] It has been reported that, risk of head and neck cancer increases when smoking duration is greater than 20 years and the daily frequency of smoked cigarettes is higher than 20.[8]

About 20% of human population consumes betel quid as part of their cultural fabric.[9] Due to cultural and religious tradition and lack of awareness, there is a large degree of betel quid chewing with and without tobacco seen in the regions of South and Southeast Asia. Especially in the Indian subcontinent, its

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consumption is drastically high. A higher rate of consumption of such products are also reported all over the world, where there are migrants from the Indian subcontinent. The main reason for a high occurrence of oral cancer in these areas is supposed to be the chewing habits.[1,10,11]

Oral submucous fibrosis (OSF) is a potentially malignant disease with malignant potential greater than 30%, which originates mainly due to areca nut chewing, especially the commercialized one available in packets. In India, incidence of OSF is devastating and termed as the prime symptom for oral cancer.[11–13] Earlier epidemiological studies described OSF as a precancerous condition,[14] whereas recent reports show OSF as a potentially malignant disorder with a precancerous nature.[15] OSF usually has a very slow onset, and if the use of areca nut continues it is irreversible and likely to become progressively more severe. At present, there is no curative treatment for OSF. The most serious aspect of OSF is its precancerous nature. Unlike other precancerous lesions, OSF does not regress either spontaneously or with discontinuation of habits. Slowly growing oral cancer is found in OSF patients and the transformation rate ranges from 7 to 13%.[10]

The oral epithelium becomes more vulnerable to carcinogens due to various types of oral habits. The increasing incidence of this potentially malignant condition necessitates the establishment of early diagnosis techniques for effective preventive measures. Biopsy followed by histopathology is the gold standard for oral cancer screening.[16] But this technique is error prompt due to manual or sampling mistakes. Moreover, by the time the patient reaches the clinic for a biopsy followed by histopathological evaluation the disease would have progressed beyond the treatment stage in most cases. Hence, alternative noninvasive methods that can provide molecular level information needs to be looked into for effective intervention for the management of oral cancer. Optical spectroscopic techniques such as fluorescence spectroscopy,[17–26] diffuse reflectance spectroscopy,[27] infrared spectroscopy,[28] and Raman spectroscopy[29] are the emerging *in vivo* diagnostic tool for cancer.

In vivo fluorescence spectroscopy is an emerging technique used for real time oral cancer screening. Such techniques are flexible and adequate to provide tissue characteristics at a molecular level. The biochemical alterations at the time of tissue transformation can be well differentiated from the variations in the autofluorescence spectral profile. Because of its simplicity in recording, minimal time is required for the study and comfort level of patients, fluorescence spectroscopic techniques have been emerged as a promising tool in the diagnosis procedure in oral oncology.[17–26]

In this study, we are presenting the autofluorescence spectral variations of a group of patients who had varying degrees of fibrosis of the oral cavity and a group of habitués and non-habitués. In order to analyze the damage caused by various oral habits, quantification of collagen level and redox ratio variation between non-habitués, habitués, and OSF patients were also performed. For exact differentiation of the spectra among different groups and to determine the performance level of the clinical trial, spectral intensity ratio analysis and linear discriminant analysis were also carried out.

2 Materials and Methods

2.1 *Study Population*

The study consisted of 30 volunteers without any habits (non-habitués), 25 volunteers with habits (habitués), and 15 OSF patients. Color of the mucosa appeared to be normal in all subjects. The fluorescence spectra were acquired from different sites of the oral cavity like right and left buccal mucosa, palate, and floor of mouth. In five cases of the habitués, spectra were also acquired from sites with any visual changes. Mild blanching and depigmentation was observed in these cases. In the case of OSF, blanching, depapillation of the tongue, and fibrotic bands were observed in all cases. The clinical details of OSF patients such as age, duration, and frequency of areca nut chewing, smoking and alcoholism, and clinical grading were collected (see Table 1). Spectra from 20 sites with a clinically observable lesion were acquired from 15 OSF patients. Habitués and OSF patients of this study had mainly chewing habits along with smoking or alcoholism or both together for a period of five or more years. They were using either betel quid or processed areca nut available in packets or both for chewing. Non-habitué volunteers were strictly selected from those who never had any such habits and had good oral hygiene and health.

The study was approved by the ethics committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The procedure was explained to the volunteers and patients and their consent letter in the regional language was obtained before the measurements.

2.2 *Study Protocols*

Before spectroscopic measurements, volunteers and patients were asked to thoroughly wash their mouth for 2 min in order to avoid the presence of food or any other intake that they recently consumed. After spectroscopic measurements, an incisional biopsy was taken from the respective sites of OSF patients, to confirm clinical findings. In the case of volunteers with and without habits, only visual inspection was conducted.

2.3 *Instrumental Setup*

The auto-fluorescence measurements were carried out using the Spectrofluorometer (Fluorolog-III; Jobin Yvon Inc., USA), which consisted of a fiber optic probe for *in vivo* applications. The emission spectra were recorded from the different sites of the oral cavity of the OSF patients and volunteers.

The fiber optic probe of numerical aperture, 0.22 and 1 cm outer diameter was used for the *in vivo* measurements. The bifurcated Y-type fiber optic probe originating from the spectrometer end merges to become a single fiber bundle as it comes in contact with the patient. The excitation source was a 450 W Xenon lamp. The desired excitation wavelength was selected and is transmitted to the site through one arm of the Y type cable and the received fluorescence signal was directed back to the spectrometer through the other arm. The distal end of the probe that comes in contact with the oral cavity was covered with a transparent test tube (Borosil) in order to avoid contamination. A correction factor was uniformly applied to all spectra to compensate for the changes made by the test tube. After spectral acquisition from

Table 1 Clinical report of OSF patients involved in this study (N: nil; O: occasionally).

		Arecanut chewing		Smoking		Alcoholism			
Number	Age/Sex	Duration in months	Frequency per day	Duration in months	Frequency per day	Duration in months	Frequency per week	Number of sites involved	Clinical grading
1	28/M	132	14	75	4	60	3	1	Moderate
2	32/F	216	3	N	N	N	N	1	Mild
3	69/F	624	2	N	N	N	N	1	Mild
4	74/F	660	2	N	N	N	N	1	Moderate
5	25/M	144	10	96	6	N	N	1	Severe
6	45/F	324	3	N	N	N	N	1	Mild
7	54/F	432	2	N	N	N	N	1	Moderate
8	55/F	444	3	N	N	N	N	3	Moderate
9	26/M	132	8	96	3	72	O	1	Severe
10	52/F	420	4	N	N	N	N	2	Moderate
11	33/M	216	5	N	N	156	4	1	Moderate
12	38/F	288	4	N	N	N	N	2	Moderate
13	52/M	432	2	456	8	408	O	1	Mild
14	23/M	120	12	108	5	N	N	1	Severe
15	50/M	436	3	276	3	360	4	2	Moderate

each patient, the test tube was cleaned and disinfected with 2% glutaraldehyde solution. The excitation wavelength of 320 nm is selected using Datamax™ software (Datamax, Round Rock, Texas, USA) and the in-built double-grating monochromator. All emission spectra were recorded in the 355 to 550 nm range in 1 nm increments.

2.4 *Data Processing and Analysis*

2.4.1 *Processing of spectra*

All spectra were baseline corrected and data values extracted. Data values of each spectrum were normalized with respect to maximum intensity wavelength in the 460 ± 10 nm region. From the normalized values, data corresponding to the 380 ± 10 and 530 ± 10 nm regions were also extracted, sorted, and the wavelength with maximum intensity value was chosen. Using the statistical software package SPSS-16, a one way analysis of variance (ANOVA) test was carried out on these maximum intensity values. Correlation between the groups, non-habitues versus habitués, non-habitués versus OSF, and habitués versus OSF was calculated using ANOVA.

2.4.2 *Redox ratio*

The redox ratio was computed on the basis of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) related signals using the following equation:

$$\text{Redox ratio} = \frac{\text{FAD}_{\text{intensity}}}{\text{FAD}_{\text{intensity}} + \text{NADH}_{\text{intensity}}}, \quad (1)$$

where $\text{FAD}_{\text{intensity}}$ and $\text{NADH}_{\text{intensity}}$ are the emission intensities at 530 and 460 nm, respectively.[17,30]

2.4.3 *Spectral intensity ratio analysis*

To get the variation in collagen level with respect to NADH, the mean fluorescence intensity ratio of both these fluorophores (NFI 460/NFI 380) was calculated. Variations in the collagen level in non-habitués, habitués, and OSF patients were found out using these values. Spectral ratio reference values were plotted as scatter plots. Sensitivity, specificity, accuracy, and positive and negative predictive values were also calculated from the observed variation in these values.

2.4.4 *Linear discriminant analysis*

Linear discriminant analysis (LDA) is a multidimensional statistical tool. It is used to investigate the dependence and connections among variables, helping to reduce and simplify data and to classify objects into groups.[31] In this study, we carried out LDA on the normalized spectra of each category using SPSS. Sensitivity, specificity, accuracy, and positive and negative predictive values corresponding to LDA results were also found out

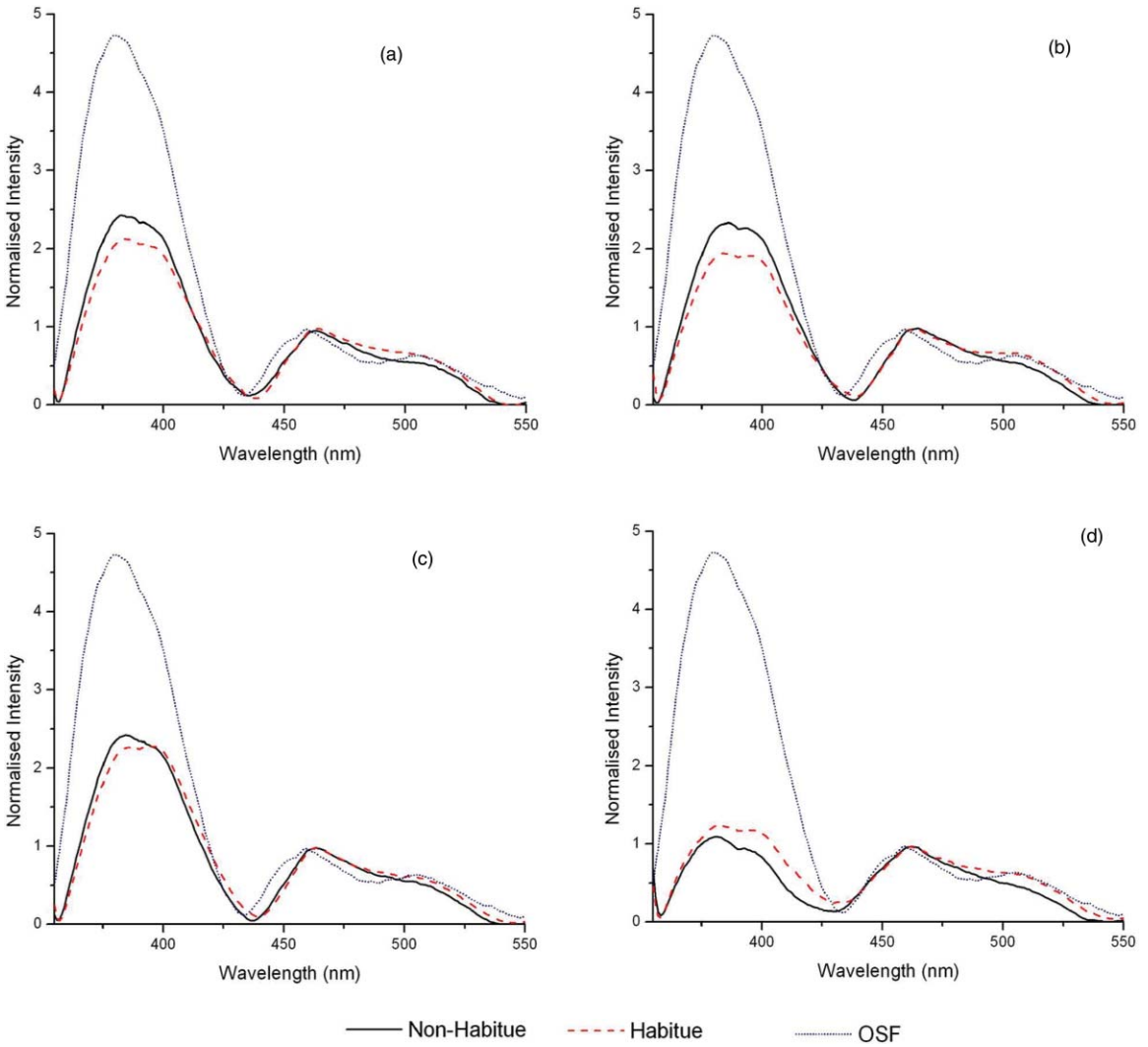

Fig. 1 Average fluorescence emission spectra from different anatomical sites non-habitués, habitues, and OSF patients. (a) Floor of the mouth, (b) left buccal mucosa, (c) right buccal mucosa, and (d) palate.

as per the method described by Jayanthi et al., and discriminant values and its averages were plotted.[19]

3 Results

3.1 *Autofluorescence Spectral Features*

In vivo autofluorescence measurements of different anatomical sites of oral cavity from non-habitués, habitués, and OSF patients were recorded. The averaged normalized fluorescence spectra of different anatomical sites of volunteers and OSF patients are shown in Fig. 1. Three broad peaks were mainly observed around 380, 460, and 530 nm in all the spectra. The peak around 380 nm is the most intense one in all cases except in the case of the spectra from the palate of habitués and non-habitués. The intensity of this peak varies considerably among different groups. Spectra from the floor of the mouth, left and right buccual mucosa shows a uniform pattern of intensity variation for this peak. The spectral feature from these sites shows that the intensity of this peak decreases from non-habitués to habitués. The spectral features from the palate shows nearly equal intensity for this peak for habitués and non-habitués, whereas in OSF patients this peak shows an intensity of nearly five times to that of habitués and non-habitués. The peak at 530 nm is well defined in the case of OSF and habitués, where as it appears as a very weak shoulder in non-habitués. This peak also shows comparatively high and equal intensity for both OSF and habitués.

The statistical test, ANOVA was performed on the maximum peak intensity of 380 and 530 nm between different groups. P values are given in Table 2. The peak intensity shows significant difference ($P < 0.05$) between non-habitués and habitués for different sites like the floor ($P = 0.002$), left buccal mucosa ($P = 0.024$), and right buccal mucosa ($P = 0.021$) of the oral cavity. But for the palate, the intensity variation is not statistically significant ($P = 0.818$). The peak intensity of both habitues and non-habitues showed significant variation when compared to that of OSF.

The range of values of a one way ANOVA analysis on maximum peak intensity of 530 nm was different from that of a 380 nm peak. The peak intensity of both habitués and OSF shows a significant difference with that of non-habitués. A significant

Table 2 One way ANOVA values on comparison of intensity values of different groups.

Wavelength (nm)	Locations in oral cavity	Non-habitué versus habitué	Non-habitué versus OSF	Habitué versus OSF
380 ± 10	Floor	0.002	0.000	0.000
	Right	0.021	0.000	0.000
	Left	0.024	0.000	0.000
	Palate	0.818	0.000	0.000
530 ± 10	Floor	0.100	0.180	0.506
	Right	0.006	0.069	0.682
	Left	0.015	0.047	0.682
	Palate	0.000	0.013	0.458

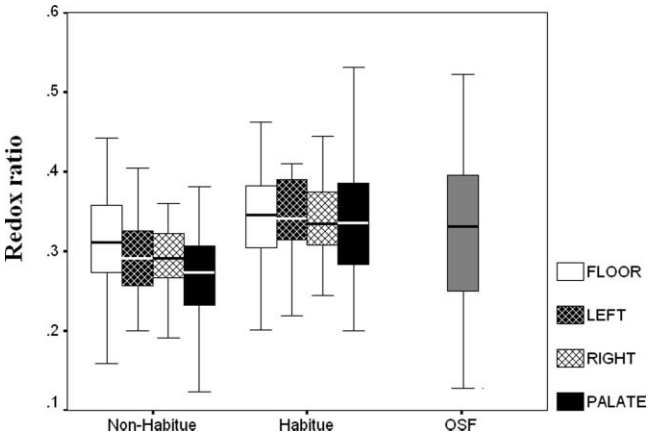

Fig. 2 Optical redox ratio for different sites of non-habitués, habitués, and suspected sites of OSF patients. Box plot includes median and standard deviation.

difference in the peak intensity was observed for the sites left ($P = 0.015$), right buccal mucosa ($P = 0.006$), and palate ($P = 0.000$) when non-habitués and habitués were compared. The intensity difference between these groups were not highly significant for the site floor ($P = 0.100$). A similar peak intensity variation was also observed between non-habitués and OSF. Here, significant difference in the peak intensity was observed for the left ($P = 0.069$) and right ($P = 0.047$) buccal mucosa, and palate ($P = 0.013$). For the floor of the mouth ($P = 0.180$), the peak intensity difference was not highly significant. No significant difference was observed between the peak intensity for any of the sites for habitués and OSF.

3.2 *Redox Ratio*

The calculated values of redox ratio for non-habitués, habitués, and OSF patients are given as box plot in Fig. 2. From Fig. 2 it is clear that there is a considerable increase in the value of redox ratio of non-habitués from both habitués and OSF. It is also observed that the redox ratio values of habitués and OSF are nearly the same.

A significant difference in the redox ratio value was observed between non-habitués and habitués irrespective of the sites. The calculated P values using ANOVA were 0.009 for left buccal mucosa, 0.000 for right buccal mucosa, and 0.012 for palate. For the site floor, ($P = 0.08$) redox value difference was not highly significant. When non-habitués and OSF are considered, only palate ($P = 0.014$) shows significant difference in the redox value. The redox value was not highly significant for floor ($P = 0.464$), left ($P = 0.181$), and right ($P = 0.124$) buccal mucosa. There was no significant difference between any of the redox values between habitués and OSF patients.

3.3 *Autofluorescence Intensity Ratio*

Figure 3 shows the scatter plot of the normalized fluorescence intensity ratio, NFI 460/NFI 380 from different anatomical sites of 30 non-habitués, 25 habitués, and 20 affected sites of 15 OSF patients. Exact positioning of the fiber probe at the anatomical sites of interest, especially palate, was difficult in the case of OSF. Hence, the average spectral data of different anatomical sites from each patient were used for further data analysis. The spectra from different anatomical sites of patients are expected to give similar features as most of the sites of oral cavity get affected by this disease. The cut off value of 0.7 and 0.3 chosen from the scatter plot of the fluorescence intensity ratio gives good discrimination between non-habitues, habitués, and OSF. A similar trend was observed for the spectral ratios for all sites except the palate. For non-habitués, the spectral ratio is between 0.7 and 0.3, and for OSF it is below 0.3. For habitués, the spectral ratio is observed as scattered throughout the entire region, but it predominantly appears as being focused above 0.7. The standard deviation with respect to the average spectral intensity ratio values are also plotted as a bar diagram (Fig. 4). It shows major variation between non-habitués and habitués for left and right buccal mucosa and floor of the mouth, but does not show variation for the palate of the oral cavity. The intensity ratio of all of the sites of non-habitués and habitués shows major variation compared to OSF. All of the results of binary classification using the spectral intensity ratio are given in Table 3.

3.4 *Linear Discriminant Analysis*

Discrimination scores calculated using statistical analysis provides the characteristics of each spectrum. The pair wise discriminant score for the site floor of the mouth, right, and left buccal mucosa, and palate are shown as Figs. 5–8, respectively. The first two discriminant functions obtained from the linear discriminant analysis for different sites are also shown as scatter plots in Figs. 9–12. The resultant discriminant score 1 is plotted along x and score 2 along the y axis. Each group centroid is also shown. The centroid of each group gives the average of the discrimination value with respect to the concerned site. For all of the sites, group centroids are differentiable from each other and have nearly an equal distance between groups. Pair wise correlation of the groups (non-habitués versus habitues, non-habitués versus OSF, and habitués versus OSF) give the number

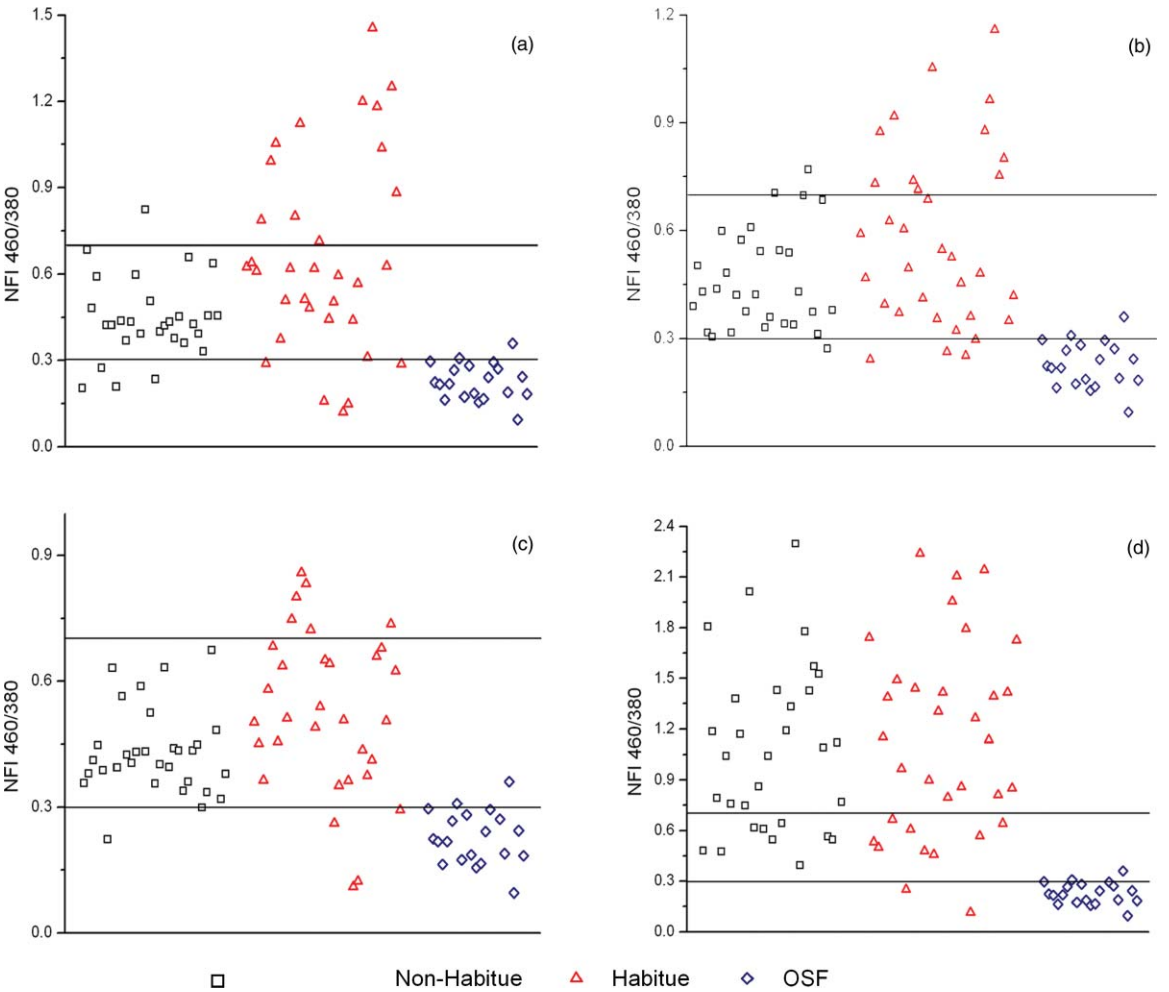

Fig. 3 Spectral intensity ratio plot (NFI 460/380) of different anatomical sites. (a) Floor of the mouth, (b) left buccal mucosa, (c) right buccal mucosa, and (d) palate.

of positive and negative predictive values, which were utilized for binary calculations used to analyze the performance level of this test. All of the results of binary classification using LDA are also given in Table 3.

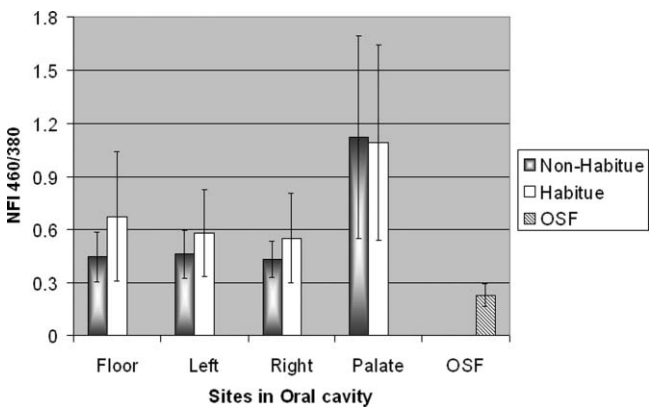

Fig. 4 Mean and standard deviation of the spectral intensity ratio (NFI 460/380) for different anatomical sites of non-habitués, habitués, and OSF.

4 Discussion

Even though there are many reports available on the variation in the fluorescence spectra of both malignant and normal tissues, the exact structural and biochemical alterations occurring in the tissues during tissue transformation, especially in the premalignant condition, is not well understood. The main cause for the spectral variation among different groups can be due to the variation in the concentration of endogenous fluorophores like tryptophan, collagen, NADH, FAD, porphyrin, lipids, etc. In this study, we have attempted the *in vivo* analysis of biochemical changes caused by lifestyle oral habits, which leads to the development of fibrosis in the oral mucosa and restricts the movement of jaws and leads to oral cancer at a later stage in many cases. Hence, effective and real-time diagnosis of a potentially malignant condition, like OSF and mucosal alteration leading to OSF, is important.

The fluorescence emission spectra from the tongue are not included in this study because of the observed uneven spectral profiles. Moreover, it is reported that the fluorescence spectra from the tongue is difficult to discriminate due to the presence of emission peaks of bacteria.[18,19,24] Pre-processing methods like baseline correction and normalization of spectra were done in all cases in order to get better efficiency in the

Table 3 Overall diagnostic accuracies obtained for the discrimination of non-habitués, habitués, and OSF using spectral intensity ratio and linear discriminant analysis.

Test conducted	Loca- tions in oral cavity	Non-habitué versus habitue					Non-habitué VS OSF					Habitué versus OSF				
		Se (%)	Sp (%)	ACC (%)	PPV (%)	NPV (%)	Se (%)	Sp (%)	ACC (%)	PPV (%)	NPV (%)	Se (%)	Sp (%)	ACC (%)	PPV (%)	NPV (%)
Spectral ratio NFI 460/380	Floor	42.86	96	67.92	92.31	60	95	86.21	89.80	82.61	96.15	95	84.85	88.68	79.17	96.55
	Left	37.93	93.10	65.52	84.62	60	95	93.55	94.12	90.48	96.67	95	87.88	90.57	82.61	96.67
	Right	21.43	100	60.71	100	56	95	93.33	94	90.48	96.55	95	87.5	90.38	82.61	96.55
	Palate	74.19	29.03	51.61	51.11	52.94	95	100	98.04	100	96.86	95	93.94	94.34	90.48	96.86
Linear discriminant analysis	Floor	78.79	78.57	78.69	81.25	75.86	95	100	97.92	100	96.55	95	100	98.08	100	96.97
	Left	69.7	86.21	77.42	85.19	71.43	100	96.55	97.96	95.24	100	100	100	100	100	100
	Right	78.13	75.86	77.05	78.13	75.86	100	100	100	100	100	100	96.77	98.04	95.24	100
	Palate	69.7	83.34	76.19	82.14	71.43	95	100	98	100	96.77	100	96.88	98.08	95.24	100

Sensitivity (Se) = True positive/ (true positive + false negative)
Specificity (Sp) = True negative/ (true negative + false positive)
Accuracy (Acc) = (true positive + true negative) / (positive + negative)
Positive predictive value (PPV) = True positive/ (true positive + false positive)
Negative predictive value (NPV) = True negative/ (true negative + false negative)

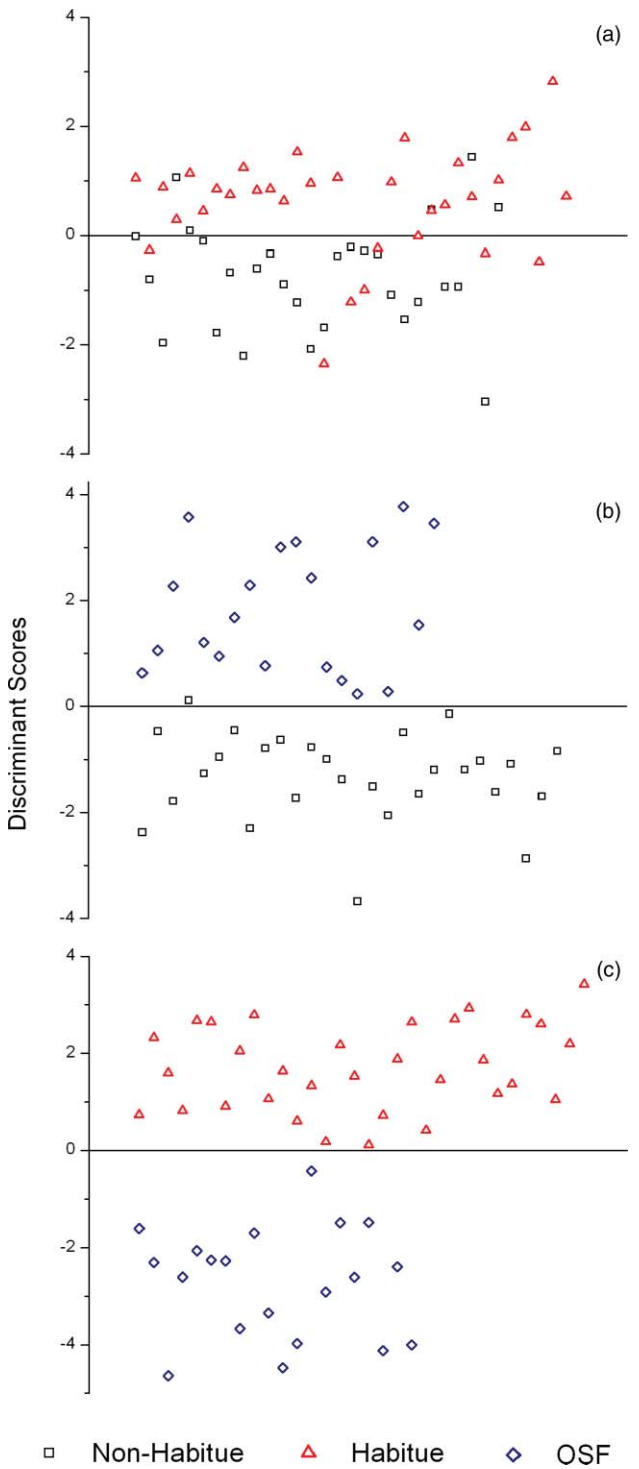

Fig. 5 Pairwise scatter plot based on discriminant function score obtained for the site floor of the mouth. (a) Non-habitué – habitué, (b) non-habitué – OSF, and (c) habitué – OSF.

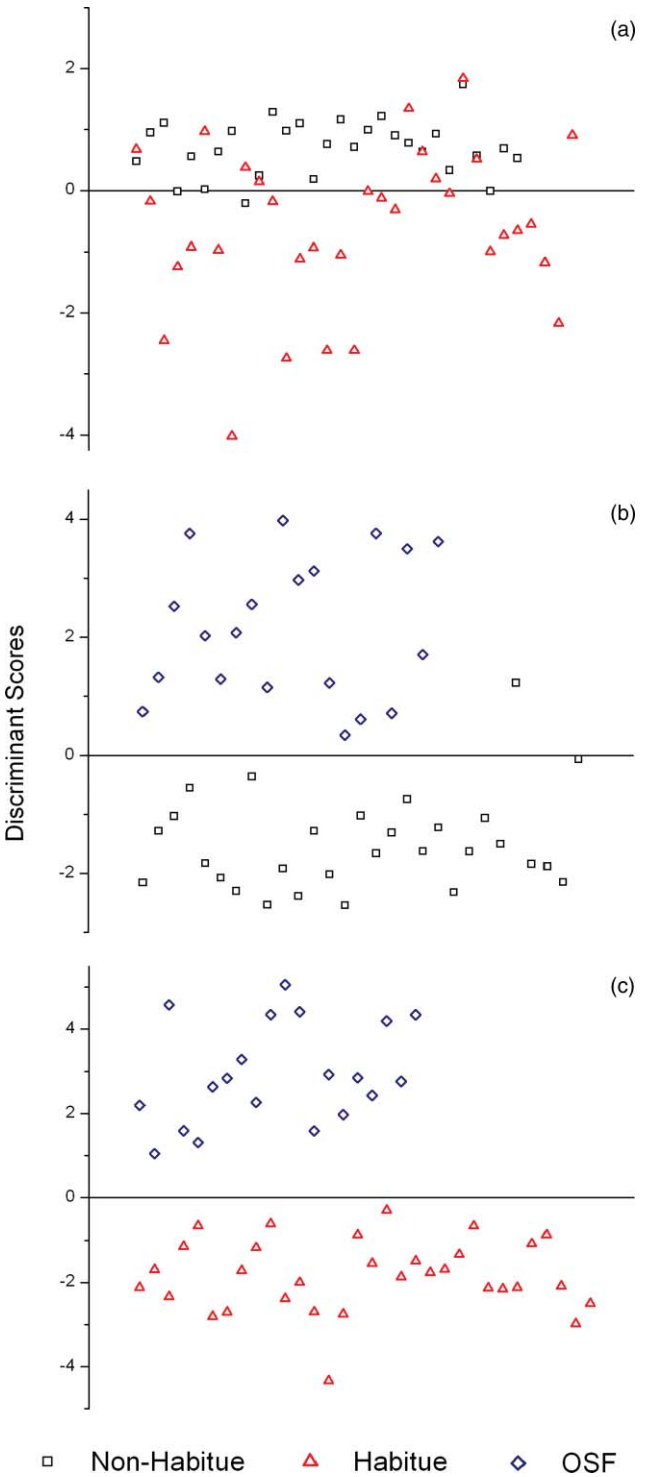

Fig. 6 Pairwise scatter plot based on discriminant function score obtained for the site left buccal mucosa. (a) Non-habitué – habitué, (b) non-habitué – OSF, and (c) habitué – OSF.

classification. Normalization with respect to specific autofluorescence peak intensity gives better results in classification compared to the mean scaling of normal data, and hence, we have used the normalized spectra throughout this study.

4.1 *Autofluorescence Spectral Features*

At 320 nm excitation, the autofluorescence peaks due to the emission from endogenous fluorophores like collagen, NADH, and FAD are observed at wavelengths 380, 460, and 530 nm, respectively.[17,32] Many research groups have reported that excitation around 320 nm is the ideal wavelength to study the variation of collagen, which is the main fluorophore involved in the pathogenesis of OSF.[17,20–25] In the present study, the collagen peak around 380 nm shows considerable intensity variation among the different groups considered.

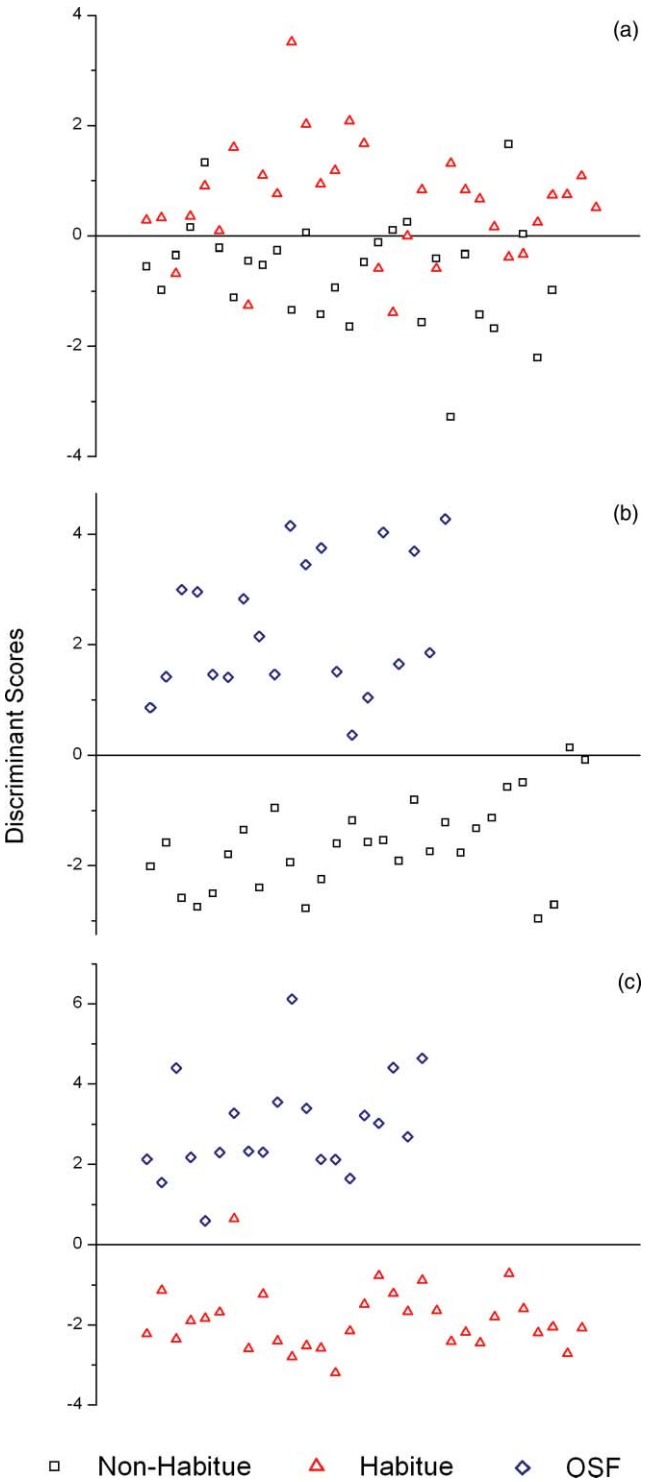

Fig. 7 Pairwise scatter plot based on discriminant function score obtained for the site right buccal mucosa. (a) Non-habitué – habitué, (b) non-habitué – OSF, and (c) habitué – OSF.

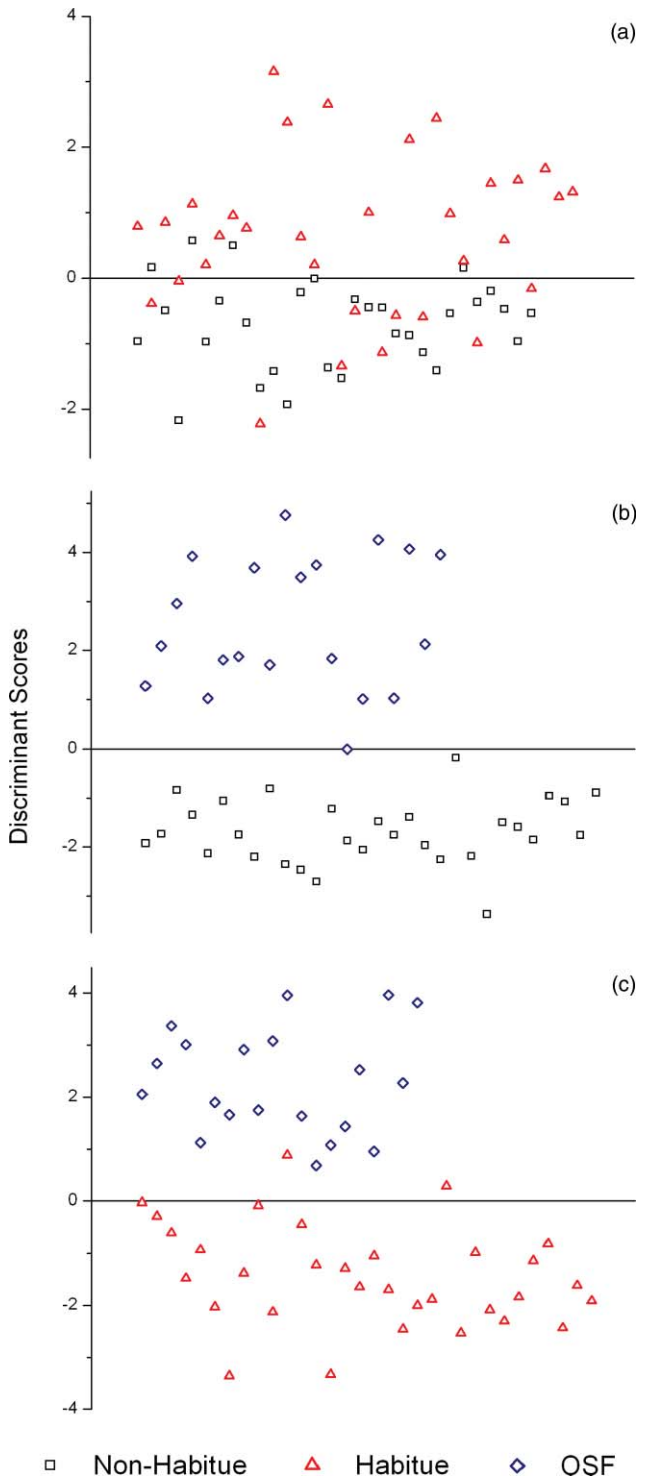

Fig. 8 Pairwise scatter plot based on discriminant function score obtained for the site palate. (a) Non-habitué – habitué, (b) non-habitué – OSF, and (c) habitué – OSF.

In this study, it is observed that the intensity of the collagen peak is less in habitués than in non-habitués except in the case of the palate. Tsai et al. have reported reduced levels of collagen fluorescence as an indicator for epithelial hyperkeratosis, epithelial dysplasia, and squamous cell carcinoma.[25] But according to Chen et al., the level of collagen is equal in normal, hyperkeratosis, and leukoplakia patients.[23] Both of these groups have carried out their work without normalization of the spectra and, hence, would have definitely considered arbitrary intensity values, which considerably varies on normalization. In our study, habitués who had no clinically observable lesions showed lower levels of collagen. This has not been observed or earlier reported

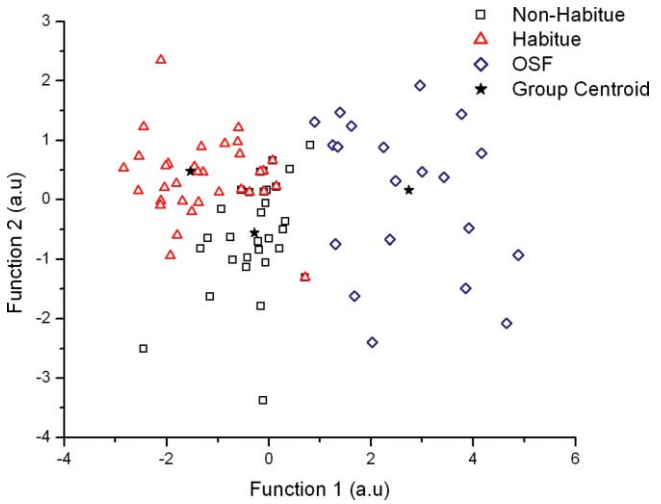

Fig. 9 Linear discriminant analysis scatter plot of non-habitués, habitués, and OSF patients plotted for floor of the mouth.

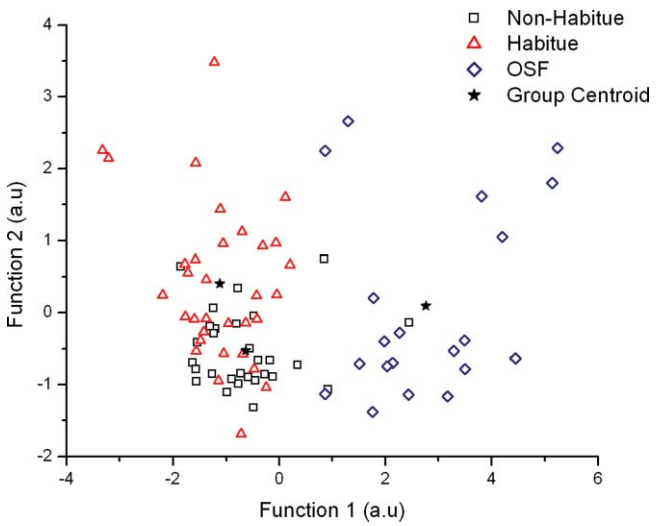

Fig. 10 Linear discriminant analysis scatter plot of non-habitués, habitués, and OSF patients plotted for left buccal mucosa.

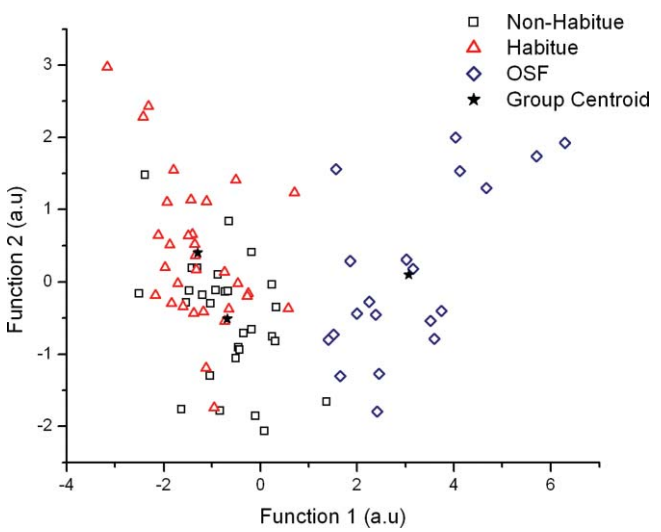

Fig. 11 Linear discriminant analysis scatter plot of non-habitués, habitués, and OSF patients plotted for right buccal mucosa.

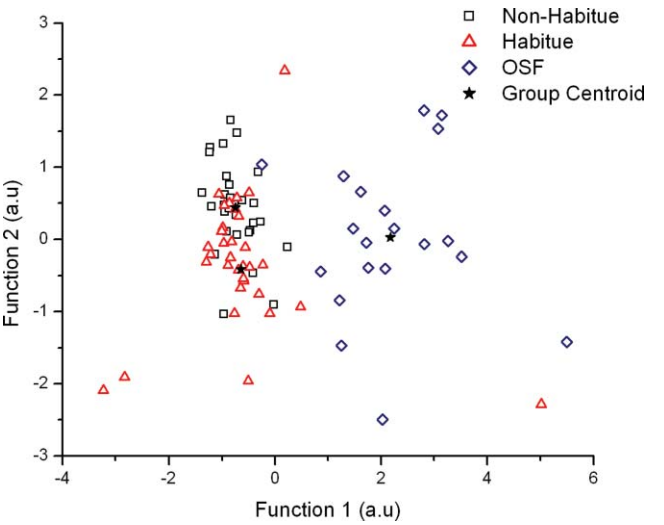

Fig. 12 Linear discriminant analysis scatter plot of non-habitués, habitués, and OSF patients plotted for palate.

in any of the studies and may be explained by the hypothesis that the collagen component of the epithelium and underlying connective tissue gets distorted due to the inflammatory changes that occur due to various oral habits.

Collagen is the most abundant protein in human body. It undergoes a wide variety of post-translational modification due to internal and external factors. These factors directly influence its structural and functional changes, which leads to serious diseases.[33] Continuous contact to the rough-surfaced area of areca nut and caustic lime during chewing can cause damages to the oral epithelium, which easily facilitates exposure of the inner connective tissue to external conditions like variation in *p*H and temperature, which promote the degradation of collagen.[34–36] An alkaline *p*H condition that occurs in the oral cavity due to the presence of caustic lime in pan and ghutka, may be one of the causative factors.[37] The frictional effect with slaked lime due to chewing can induce a temperature relatively higher than body temperature, which may cause a burning sensation in the oral cavity and can also act as a contributing agent promoting degradation. Continuous exposure of high temperature smoke due to smoking habits can also be considered as a reason for variation in the collagen level in habitués[38] Chronic exposure of alcohol can also make disturbances in the extracellular matrix in the oral cavity tissues. Alcohol has the capacity of eliminating the lipid component of the barrier present in the oral cavity that surrounds the granules of the epithelial spinous layer and results in increased basal cell layer density[6,7,39,40] All of these factors end up in the degradation of collagen, which is well demonstrated by the reduced level of collagen in the fluorescence spectra of habitués in this study. All of the above-mentioned factors will have less impact on the site palate because of the special anatomic positioning of the site. Hence, the degree of initial disruption of epithelium and related mechanisms are expected to be minimum with respect to the site. This can be the reason for the minor change observed in the intensity of this peak toward the higher side.

Another finding in the present study is the elevated level of collagen in the fluorescence spectra of OSF patients. This

observation is in line with the findings of Sivabalan et al.,[17] Haris et al.,[20] Vedeswari et al.,[21] Chen et al.,[23] and Tsai et al.[25] An elevated level of collagen is due to changes in the extracellular matrix occurring due to the collagen over production and reduced degradation of the structure; stable collagen type I trimer synthesized by OSF fibroblasts, which might contribute to the collagen accumulation in OSF.[41] Oral submucous fibrosis is probably the consequence of a disturbance in the hemostatic equilibrium between synthesis and degradation of extracellular matrix and/or altered fibrolysis, which may result in the fibrosis during betel-quid and areca nut chewing.[42] Presence of alkaloids such as arecoline, arecaidine, guvacine, guvacoline, tannins, and catchins in areca nut cause fibroblast proliferation and increased collagen synthesis in the oral cavity. Occurance of higher tissue concentrations of the trace element copper due to arecanut chewing may increase the activity of the enzyme lysyl oxidase, an extracellular copper-dependent enzyme that catalyses the cross-linking of elastine and soluble collagen to form insoluble collagen in the extracellular matrix. Lysyl oxidase is implicated in the pathogenesis of several fibrotic disorders including oral submucous fibrosis.[43,44] In normal conditions, epithelium would act as an effective barrier to such alkaloids and trace elements, by preventing their accumulation in sub epithelial lamina. But in OSF, epithelial damage caused by factors like chewing and smoking habits leads to penetration of such alkaloids and elements, which promotes the collagen proliferation.[10,44,45]

4.2 *Redox Ratio*

NADH and FAD are the metabolic co-enzymes that are considered as the primary electron transport chain in the cellular metabolism. NADH is the electron donor and FAD is the acceptor. Relative change in such oxidation and reduction rate is termed as redox ratio. Variation in the redox ratio of the cells is used to monitor the metabolic activity level at different conditions. Cancerous cells usually have increased metabolic activity and decreased blood flow due to the rapid cell division, alteration in oxygen demand, and supply and genetic variation.[46,47]

A decrease in redox ratio usually indicates an increase in metabolic activity, as observed in cancerous cells by various research groups. Upon treatment of the cancer cell lines with drugs, an increase in the redox ratio was observed by Kirkpatrick et al.[30] Skala et al. have reported a considerable difference in redox ratio values with respect to the level of malignancy using fluorescence lifetime studies in *ex vivo* and *in vitro* conditions.[48,49] Ostrander et al. reported differentiation of breast cancer cell lines using confocal fluorescence imaging based on estragen receptor status using an optical redox ratio as a tool.[50]

But so far, only one report is available on redox ratio variation in any precancerous or cancerous condition of oral cavity using *in vivo* fluorescence spectroscopy.[17] They have reported an increase in the redox ratio in the pretreated condition of OSF from that of the normal and post-treated condition. Our finding of increased redox ratio for OSF with respect to non-habitués clearly agrees with this result. Though OSF is premalignant in nature, a decrease in the redox value as reported in a neoplastic condition cannot be expected since OSF is primarily due to collagen disorder, and only a small percentage of OSF cases become malignant. The occurrence of malignancy in OSF would probably give a reduced value of the redox ratio as the metabolic activity further increases. Another finding of the present study is that the habitués whose collagen level is low compared to non-habitués, also show redox ratio values similar to that of OSF patients. Biochemical alterations other than changes in the collagen level could have caused the increase in the redox ratio value.

Earlier reports suggest changes in reactive oxygen species in the oral cavity tissue due to chewing and alcoholism. Nair et al. demonstrated that chewing stimulates phenomena like autoxidation and redox cycling due to a high *p*H level in the oral cavity, which endorses the production of reactive oxygen species.[51,52] Aqueous extracts of areca nut and catechu of high *p*H (> 9.5) generate superoxide anion and hydrogen peroxide in the oral cavity, which is supposed to be the main cause for the production of the reactive oxygen species change. Fe^{2+}, Fe^{3+}, and Cu^{2+} ions in areca nut also promote changes in the reactive oxygen species. Alcoholism also produces reactive oxygen species and promotes oxidative stress in the oral cavity tissues as a result of oxidation of ethanol to acetaldehyde associated with the induction of the microsomal ethanol-oxidizing system and cytochrome P450 2E1 enzyme. Under such an excessive oxidative stress condition, lipid peroxidation, which is referred to as oxidative degradation of lipids, becomes very significant and results in the production of aldehydic end products and corresponding protein adducts.[6,7] Based on these results, we presume that the changes that occur in the reactive oxygen species due to various oral habits can be the main reason for the observed increase in the redox ratio value of both habitués and OSF.

4.3 *Diagnostic Accuracies using Spectral Intensity Ratio and Linear Discriminant Analysis*

In this clinical trial, we have obtained an overall sensitivity of 21 to 74%, 95%, and 95% with corresponding specificity of 29 to 100%, 86 to 100%, and 84 to 93%, respectively, for discriminating non-habitués from habitués, non-habitués from OSF, and habitués from OSF using spectral intensity ratio values. Linear discriminant analysis provides a better sensitivity and specificity value than spectral intensity ratio analysis. It gives sensitivity of 69 to 78% and specificity of 76 to 86% for discrimination of non-habitués and habitués. It also gives a sensitivity of 95 to 100% and specificity of 96 to 100%, respectively, for discrimination of both non-habitués and habitués from OSF.

Using pair wise discriminant analysis, Sivabalan et al. obtained a sensitivity of 89% with a specificity range of 96 to 100% to differentiate between pre- and post-treated OSF and normal oral mucosa.[17] Tsai et al. reported a sensitivity and specificity of 100 and 93% for the discrimination of OSF from normal and obtained a total sensitivity and specificity of 81 and 87% to differentiate between premalignant epithelial hyperkeratosis, epithelial dysplasia, and squamous cell carcinoma from normal using receiver operating characteristic curve method classification.[25] Similarly, Wang et al. obtained sensitivity and specificity of 86.2 and 84.3% using a receiver operating characteristic curve classification method to differentiate normal mucosa from OSF, epithelial hyperkeratosis on OSF, and epithelial dysplasia on OSF.[22] As is clear from Table 3, the sensitivity, specificity, positive and negative predictive values, and accuracy values for habitués versus OSF and non-habitués versus OSF are very high in this study. The values for non-habitués to habitués are also good when LDA is applied.

Other than the studies on OSF, Jayanthi et al. have reported sensitivity of 92, 78, and 86% and specificity of 100, 100, and 90% in the differentiation of normal from benign, benign from premalignant, and premalignant from malignant oral cavity tissues using pairwise discriminant analysis.[19] Malliya et al. have reported a sensitivity of 89 to 100% and specificity of 74 to 100% for discrimination of normal, benign, premalignant, and malignant oral cavity tissues using fluorescence intensity ratios analysis.[18]

In this study, binary classification values of discrimination from non-habitués to habitués using intensity ratio for the sites floor of the mouth, right and left buccal mucosa provides low sensitivity with high specificity values. Here, damage caused by habits is not correctly identifiable due to the variation of collagen level in the habitués. This is an indicator that the level of collagen in the oral cavity tissues tends to vary with respect to the degree of usage and duration of habits. But for the site palate, a reverse result is observed. It shows high sensitivity with a low specificity value. As this site is always in contact with the tongue, the bacterial emissions from the tongue may also have an impact on masking the collagen peak. Moreover, considering the anatomy of the site palate, one can expect variation in the acquired spectra due to technical limitations. As the site is difficult to access, the fiber optic probe will always have an angle at the point of contact and this can vary from patient to patient resulting in the nonuniformity of the acquired spectral data. Hence, the case of the palate needs to be studied further using a modified probe, as the spectra collection is limited by many technical issues and, hence, lacks consistency in the findings.

Linear discriminant analysis gives good differentiation between all of the groups irrespective of the sites in the oral cavity. This technique gives better differentiation even for the site palate, which gives poor discrimination from non-habitués to habitués using spectral intensity ratios. This is in consideration of the explanation given for the poor specificity and sensitivity obtained for the intensity ratio analysis for the palate, where we consider only the intensity ratio from two emission peaks and not the whole spectrum. Moreover, as explained earlier, minor patient to patient spectral variation is expected due to the technical errors caused by the variation in the angle between the fiber probe and the surface of the palate.

5 Conclusion

From the results of this study, we arrive at the conclusion that autofluorescence spectroscopy along with linear discriminant data analysis is an efficient tool to diagnose oral cavity disorders caused by oral habits at a very early stage. The study also demonstrates that the data analysis based on a linear discriminant method is superior to the commonly used intensity ratio analysis in arriving at a classification style with very good sensitivity and specificity values. If this method can be adopted as a screening procedure in primary health centers and health awareness programs of urban and rural areas, the observation of a lower level of collagen and increased level of redox value if observed can be considered as a warning situation in the case of the habitué. These subjects who have a high chance of development of OSF or other oral cavity disorders may be separately categorized for further invstigation. Special attention could be given to these subjects for effective and better management as the treatment at this very early stage of transformation will be easy and effective.

Follow up study of a bigger population is in progress and is expected to give more specific biochemical changes that occur in the oral cavity tissues during tissue transformation. As lifestyle behaviors are difficult to eradicate, casualties due to oral cavity cancer can only be reduced by giving proper awareness to those ethnic groups who have the tendency of such habits.

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