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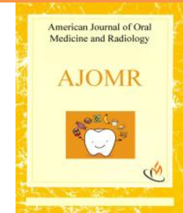

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AUTOFLUORESCENCE SPECTROSCOPY OF SMOKERS - AN IN VIVO STUDY

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ABSTRACT

Use of smoking tobacco is a major public health challenge in India. It requires a reliable technique that will useful for mass screening of the public and to detect the early changes before it clinically manifest. Aims to identify the differences in autofluorescence spectra of normal oral mucosa and smoker's oral mucosa. Group I consists of 20 individuals with normal mucosa and Group II consists of 40 individuals with smoking habit. Only males were included in this study and their age ranging from 25 to 35 years. *In vivo* fluorescence spectra from both groups were obtained by using hand held fiber optic probe attached to Varian Cary Eclipse fluorescence spectrophotometer. Independent-Samples T test was used for statistical analysis. *P* value was obtained to discriminate the statistical differences between the two groups. The averaged excitation and emission spectra of normal and smoker's mucosa showed significant differences statistically. Autofluorescence spectroscopy can be used effectively to differentiate the smoker's oral mucosa from normal oral mucosa.

INTRODUCTION

Portuguese introduced tobacco in India during Mughal era almost 400 years ago. Nicotine which present in tobacco is powerful addictive and it made tobacco usage a part of socio-cultural milieu in most parts of India. India is considered as third largest tobacco consumer and second largest tobacco producer in the world [1]. Almost two in five adults in rural areas and one in four adults in urban areas use tobacco either chewing or smoking form. The average age at initiation of tobacco use is 17 years and 8 months [2].

Tobacco is well-known etiological agent for the occurrence of precancerous lesion as well as oral squamous cell carcinoma (OSCC). Clinical changes with cellular alterations in the affected epithelial tissue are known as precancerous lesion. OSCC occurs as a result of several molecular, biochemical, cellular alterations and stroma changes like neovascularization. OSCC is mostly preceded by precancerous lesion [3,4].

One of the important ways to reduce morbidity and mortality is early detection of precancerous lesion or OSCC. Early detection needs a non-invasive technique which is simple, user friendly, cost effective and will help in the detection of tissue changes at molecular level before the clinical appearance of the lesion. Autofluorescence (AF) spectroscopy is one such emerging technique which might help to achieve above goals. It is an easy applicable tool for detecting the alteration in the structural and chemical composition of cells [5,6].

AF spectroscopic technique is based upon the principles of fluorescence. Oral tissue usually has biomolecules called fluorophores such as tryptophan and collagen. These fluorophores emit fluorescence when they are excited in the ultraviolet and visible spectral region.

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The changes of the surface epithelium from normal to malignancy, the fluorescence property of these fluorophores changes. These changes can be detected, which can further assist in the diagnosis of altered tissue or precancerous lesion or OSCC [7,8].

In the present study, the AF spectroscopic technique was used to detect the mucosal changes based on fluorescence property of the fluorophores in control group and smokers.

MATERIALS & METHODS

We had conducted the prospective study which carried out at Centre for Advanced Studies (CAS) in Marine Biology, Annamalai University, Tamil Nadu, India. 20 volunteers without the habit of smoking use were selected as controls [Group I]. 40 subjects with habit of smoking tobacco without any other habits were selected as study group [Group II]. Age distribution in both the groups was between 25 & 35 years old and all subjects were males.

Informed consent was obtained from each subject. Ethical clearance was obtained from Institutional Human Ethical Committee (IHEC) of Annamalai University. Subjects with habit of smoking tobacco, minimum of 5 years duration were included for this study Subjects with habit of chewing tobacco along with smoking tobacco, alcohol, clinically evident white/red lesions and any systemic illness were excluded from the study.

AF Spectroscopy [9]

The procedure of AF spectroscopy was explained to all the subjects. *In vivo* fluorescence spectra from both groups were obtained by using hand held fiber optic probe attached to Varian Cary Eclipse fluorescence spectrophotometer (Figure). Thoroughly disinfected fiber optic tip was placed in an arbitrary point of buccal mucosa which was formed by the intersection of the two imaginary lines, one at the occlusal plane and other line running down vertically in the upper second premolar-molar region.

Specific wavelength of light required for the study was obtained by the computer guided programme. The tissue was excited at 285 nm to obtain the emission spectra and the emission wavelength was fixed at 340 nm to obtain the excitation spectra. The spectroscopic analysis was carried out for each subject.

STATISTICAL ANALYSIS

Average & Normalized emission and excitation spectra

The individual scans of various wavelengths from each site of the patients were averaged and averaged emission and excitation spectrum was obtained. In order to eliminate the discrepancy due to interpatient variation of the fluorescent intensities, normalization of each emission and excitation spectra of its maximum intensity was done. Normalization was done by dividing the intensity amplitude for each emission wavelength to the peak intensity in the total spectra.

Ratio parameters

The ratio of intensities at two different wavelengths was computed for each scan. These wavelengths were selected where maximum spectral differences were noted. Using these ratio parameters, mean and standard deviation were calculated and Independent-Samples T test was done using IBM SPSS statistics version 20.

RESULTS

The average emission spectrum of the normal mucosa and smoker's mucosa at 285 nm excitation showed a prominent peak at 330 nm. The normalized emission spectra of normal mucosa, smoker's mucosa also showed the similar findings at 285 nm excitation, which corresponds to the amino acid tryptophan. In graph, wavelength is shown in X axis and fluorescence intensity in Y axis (Graph 1 & 2). Statistical analysis of Independent-Samples T test showed high statistical significant ($P < 0.005$) between normal and smokers (Table 1).

The average excitation spectrum of the normal mucosa and smoker's mucosa at 340 nm emission showed a prominent peak at 290 nm. The normalized emission spectra of normal mucosa, smoker's mucosa also showed the similar findings at 340 nm emission (Graph 3 & 4). Statistical analysis showed significant statistical differences ($P < 0.05$) between these two groups (Table 2).

Table 1. Mean standard deviation and significant of normal and smokers group at 285 nm excitation

Ratio	Groups		t value	F ratio	Significance
	Normal (20) M ± S.D	Smoker (40) M ± S.D			
$I_{330/400}$	5.8450 ± 0.12	5.3795 ± 0.47	4.307	13.007	0.001
$I_{332/400}$	5.8675 ± 0.11	5.4080 ± 0.45	4.476	13.631	0.000
$I_{334/400}$	5.8690 ± 0.14	5.4202 ± 0.44	4.403	11.724	0.001
$I_{336/400}$	5.8725 ± 0.14	5.4233 ± 0.43	4.519	13.155	0.001
$I_{338/400}$	5.8155 ± 0.17	5.4033 ± 0.40	4.396	8.804	0.004

$P < 0.005$

Table 2. Mean standard deviation and significant of normal and smokers group at 340 nm emission

Ratio	Groups		t value	F ratio	Significance
	Normal (20) M ± S.D	Smoker (40) M ± S.D			
$I_{260/300}$	0.4285 ± 0.02	0.4650 ± 0.05	-2.608	5.093	0.02
$I_{260/310}$	1.1245 ± 0.11	1.3090 ± 0.22	-3.376	5.818	0.01
$I_{270/300}$	0.6230 ± 0.04	0.6810 ± 0.07	-2.996	5.432	0.02

$P < 0.05$

Figure 1. ***In vivo*** **spectral recording with the fiber optic probe placed over the desired area**

Figure 2. Averaged fluorescence emission spectra of normal and smokers at 285 nm excitation

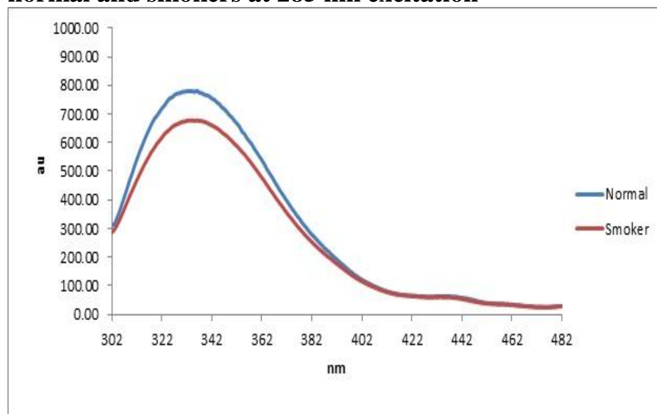


Figure 3. Normalized fluorescence emission spectra of normal and smokers at 285 nm excitation

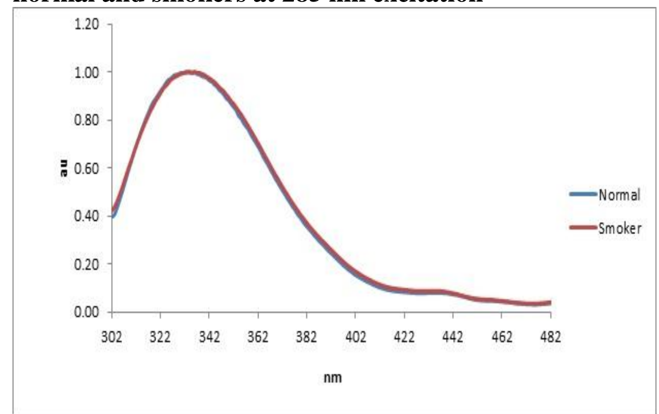


Figure 4. Averaged fluorescence excitation spectra of normal and smokers at 340 nm emission

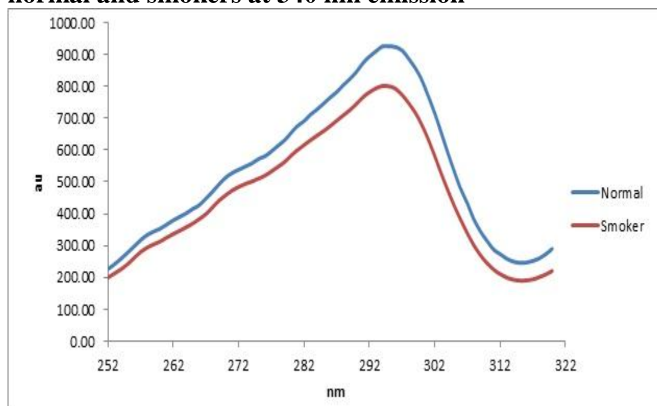
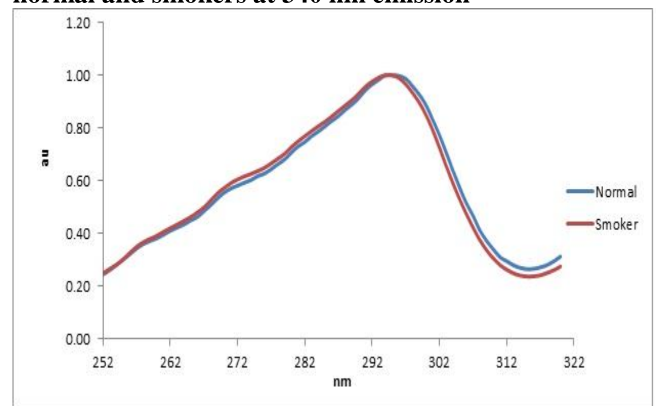


Figure 5. Normalized fluorescence emission spectra of normal and smokers at 340 nm emission



DISCUSSION

The incidence of OSCC in India is on the rise with increase in the usage of tobacco. Elimination/reduction of tobacco use will reduce the occurrence of precancerous lesion, thus will further prevent the development of OSCC [10]. Various diagnostic modalities are available for oral cancer detection. Visual screening lacks accuracy, reproducibility and needs experience even though it decreases the mortality in heavy smokers. Toluidine blue (TB) staining is said to be an inexpensive and sensitive adjunct tool to identify high-grade dysplasia and early OSCC. It is less sensitive for premalignant lesions with 58% false negative rates for identifying mild to moderate dysplasia [11]. Brush cytology is non-invasive, inexpensive and almost painless and needs minimal technical skills. It has certain disadvantages of false-negative results and error in sampling [12]. Optical based diagnostic aids are promising new technology for detection of early epithelial changes [13]. AF spectroscopy is one such emerging technique to detect the early changes even before the clinical manifestation. During carcinogenesis, the optical properties of tissue are altered. AF spectroscopy can quantitate these changes and gives information about the biochemical changes of the tissue in a non-invasive manner by using a simple fiber optic instrument [14].

The fluorescence emission and excitation spectra of smoker's mucosa had showed decreased intensity compare with normal mucosa. Our results were consistent with the findings of Kolli *et al* (1995) [15], Wang CY *et al* (1999) [16]. This decrease in the intensity was reflected as decreased synthesis of amino acid tryptophan. It can be attributed to the altered cellular metabolism because of lethal effects of tobacco [17].

Tobacco contains N-nitrosonornicotine, polycyclic aromatic hydrocarbons that act on the keratinocytes and enter into the nucleus, where they were metabolized by glutathione S transferase and cytochrome P450. The intermediate thus produced will bind with DNA by direct stimulation of heat of cigarette which causes DNA damage and altered cell proliferation [18].

CONCLUSION

AF spectroscopic technique can serve as a reliable diagnostic tool and to detect the mucosal changes before the lesion appears clinically due to smoking. It can lead to possible low-cost mass screening for precancerous lesions and OSCC in primary health care centers. It can also aid in improving our understanding of the biological changes which will give a new impetus and better perception of cancer biology.

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