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Fluorescence spectroscopy for the detection of potentially malignant disorders of the oral cavity: analysis of 30 cases

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Abstract

Oral cancer is a major health problem worldwide and although early diagnosis of potentially malignant and malignant diseases is associated with better treatment results, a large number of cancers are initially misdiagnosed, with unfortunate consequences for long-term survival. Fluorescence spectroscopy is a noninvasive modality of diagnostic approach using induced fluorescence emission in tumors that can improve diagnostic accuracy. The objective of this study was to determine the ability to discriminate between normal oral mucosa and potentially malignant disorders by fluorescence spectroscopy. Fluorescence investigation under 408 and 532 nm excitation wavelengths was performed on 60 subjects, 30 with potentially malignant disorders and 30 volunteers with normal mucosa. Data was analyzed to correlate fluorescence patterns with clinical and histopathological diagnostics. Fluorescence spectroscopy used as a point measurement technique resulted in a great variety of spectral information. In a qualitative analysis of the fluorescence spectral characteristics of each type of injury evaluated, it was possible to discriminate between normal and abnormal oral mucosa. The results show the potential use of fluorescence spectroscopy for an improved discrimination of oral disorders.

Keywords: squamous cell carcinoma, fluorescence spectroscopy, early diagnosis, potentially malignant disorders, oral cavity

(Some figures may appear in colour only in the online journal)

1. Introduction

Squamous cell carcinoma is the most frequent cancer type of the oral cavity, corresponding to over 90% of all malignant tumors [1, 2]. Unfortunately, most potentially malignant disorders and initial oral cancers are usually missed or misdiagnosed and not treated until they are at advanced stages. At advanced stages, the effectiveness of therapeutic modalities are low and result in high mortality and morbidity rates [3]. Moreover, the long-term results of oral cancer therapy have

been significantly hindered by the development of second primary tumors. Therefore, early detection of neoplastic changes in the oral cavity is essential to improve survival rates [4].

In clinical practice, an accurate clinical examination and biopsy of a suspect lesion must be done to determine the diagnosis [5]. However, most oral cavity squamous cell carcinomas do not originate from potentially malignant disorders nor from other noticeable clinical changes in the oral mucosa [6, 7]. Most potentially malignant disorders are clinically present as leukoplakia or erythroplakia, but histologically they may have a wide range of phenotypes such as hyperkeratosis or dysplasia [8]. On the other hand, similar clinical features of carcinomas at the initial stage and benign lesions are major factors making early cancer diagnosis a difficult task. Incisional biopsy remains as the gold standard diagnostic method for the detection of oral neoplasia, but the choice of a proper biopsy site in a large non-homogeneous potentially malignant disorder or malignant lesion is not simple and can result in misdiagnosis. Recently, optical techniques have been developed, aiming to became auxiliary tools to address these challenges [9–12]. Due to its sensitivity to tissue alterations, optical diagnosis has been indicated, not only for cancer detection, but also for other diagnostic applications, such as dental calculus and demineralization [13, 14]. Among those techniques, fluorescence spectroscopy is a noninvasive, accurate and fast diagnostic method that can be potentially used for the early detection and diagnosis of cancer in real time [15]. Its principal feature relies on the fact that several changes taking place along tumorigenesis alter light/tissue interactions, including tissue fluorescence, and these normal oral mucosa and potentially malignant or malignant lesions present distinct fluorescence spectra [16, 17].

Pavlova *et al* studied a Monte Carlo model that showed variations in optical parameters associated with neoplastic development that influence the intensity and shape of the fluorescence spectra. Changes associated with dysplastic progression were associated with a decreased fluorescence intensity and an emission shift to longer wavelengths [17]. Several *in vitro* and *in vivo* studies have shown the efficiency of fluorescence spectroscopy for oral cancer discrimination with high rates of sensitivity and specificity [10, 16]. Clinical studies have also presented the potential of this technique to improve cancer diagnosis, even though the discrimination of potentially malignant disorders versus cancer is still contradictory in the literature [18, 10].

In this study, we report the results obtained using a double excitation fluorescence spectroscopy system in 30 patients presenting oral leukoplakia and/or erythroplakia and in 30 volunteers with normal oral mucosa. Spectral analysis was performed using principal component analysis combined with clinical impression and histopathological diagnosis.

2. Patients and methods

The subjects included in this prospective clinical study were 30 volunteers with normal oral mucosa and no history of malignancy in the upper digestive tract, and 30 patients with potentially malignant disorders clinically detectable at various stages of development. All subjects were over 18 years old, both genders, smokers and nonsmokers. The investigated lesions were located in the oral cavity and a biopsy was taken for histological diagnosis. The biopsy site was chosen based only on clinical examination. All patients were investigated at the Special Laboratory of Laser in Dentistry, University of São Paulo (LELO-USP) and Hospital A C Camargo, São Paulo, after a written informed consent was signed.

A homemade fluorescence spectroscopy system was used in this study. The system is composed of two excitation lasers (408 nm diode laser and 532 nm frequency-doubled Nd:YAG laser), a Y-type probe (Ocean Optics, USA), a USB-spectrometer (USB-4000-Ocean Optics, USA) and a laptop. The Y-type probe with two 600 μm optical fibers is connected on one end to the excitation laser and the other end to the spectrometer, and the investigation tip is enclosed in an aluminum handpiece. The external diameter of the interrogation tip is 2.5 mm.

All subjects answered an anamnestic form with medical information about habits associated with the etiology of cancer (smoking and alcohol), family history, among others. A detailed clinical examination was carried out, resulting in a clinical diagnosis following the classification in normal mucosa and abnormal mucosa (erythroplakia, leukoplakia or erythroleukoplakia) and the definition of the biopsy site.

A mouth washing with saline solution was performed just before optical measurements to minimize possible contaminants in the mucosa, such as food scraps. The subjects had fasted for at least 1 h before the examination to prevent dye intake and alteration of the fluorescence pattern of the investigated mucosa.

Each measurement site was assessed using excitation wavelengths at 408 and 532 nm. For patients with a clinically detectable lesion with a diameter of less than 1 cm, the entire altered area was screened with the needed points to cover the lesion surface. In patients with lesions greater than 1 cm in diameter, and in the case of identification of surface heterogeneity, representative regions were chosen to correlate with different clinical patterns, avoiding any area of necrosis. In each chosen site, at least five spectra, for each excitation wavelength, were taken to check the variability on measurement performance. All the optical measurements were taken by a single operator (ALNF).

Four-mm punch biopsies were taken after the previous dentist/physician decision based on clinical impression, and the correlated fluorescence spectrum identified. The tissue sample was stained for HE analysis and the slides were evaluated by certified pathologists blinded to the clinical impression and fluorescence spectra (figure 1).

Normal volunteers were investigated at distinct oral sites: lateral border of the tongue, dorsum of the tongue, floor of the mouth, lower lip mucosa, buccal mucosa, gingiva and hard palate, with five optical measurements per excitation wavelength for each site. Cytological material was collected from all investigated sites using Oral CDX brush biopsy and fixed with 95% ethanol for the smear slide.

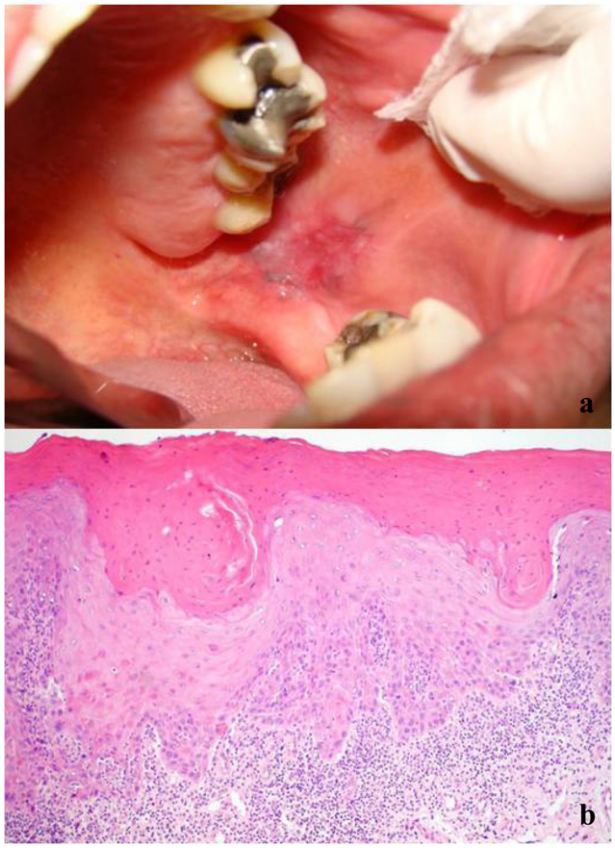

Figure 1. Comparative clinical pathology (a) erythroleukoplakia disorder in the buccal mucosa and (b) histopathological results of moderate dysplasia.

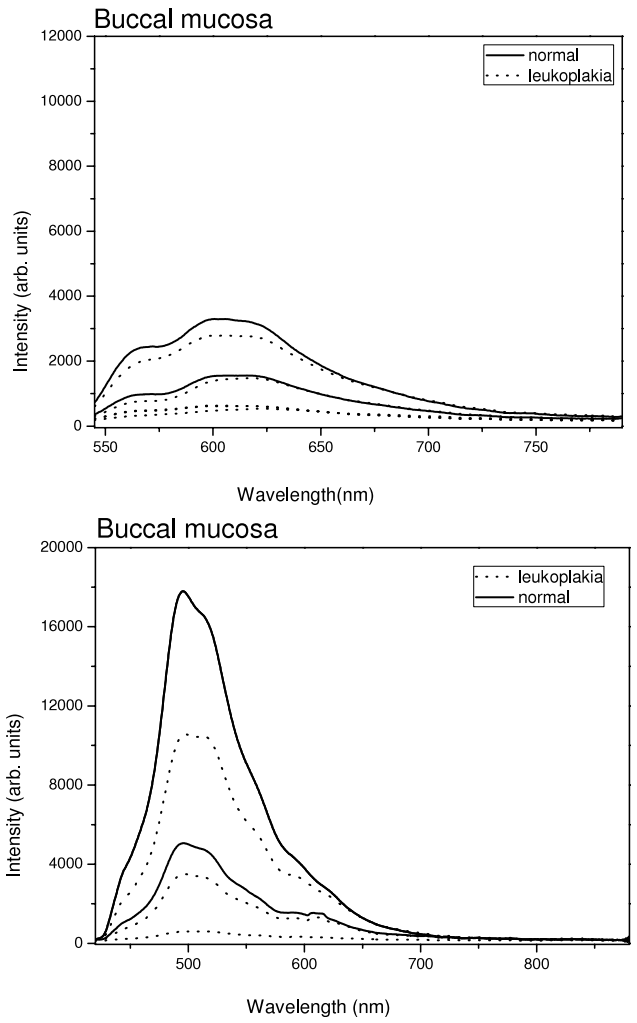

Figure 2. Comparison in the buccal mucosa of spectra in sites of normal mucosa and leukoplakia, at wavelengths of 532 nm and 406 nm, respectively.

Fluorescence spectra were classified using histopathology, the gold standard for diagnostics, in normal epithelium and epithelial dysplasia. For normal volunteers, clinical impressions and cytological results were used for classification. Principal component analysis (PCA) was used for evaluation of the diagnostic resolution of the training set.

3. Results

Thirty patients, 15 female and 15 male, with potentially malignant disorders were investigated in the period of May of 2009 and June of 2010. Table 1 summarizes the clinical features and pathology diagnostics of the study population. The most prevalent disorder type concerning clinical diagnosis was leukoplakia, and the oral sites were tongue and buccal mucosa. The most frequent histopathological classifications were mild and moderate dysplasia. For erythroplakia (4) and erythroleukoplakia (1) lesion dysplasia was graded as moderate or severe. With a minimum follow-up of two years, 20% of cases progressed to cancer, four *in situ* and two moderately differentiated oral squamous cell carcinoma.

Fluorescence spectra showed distinct behaviors considering oral site, clinical diagnosis, and pathological findings. Oral mucosa at different oral sites present distinct fluorescence spectra for each excitation wavelength; in this sense, the discrimination was better achieved when the comparison between normal and altered mucosa was performed individually for each site. At the same time, due to the small sample number, statistical analysis could not be performed for each site, but only in general for the oral cavity.

Leukoplakia and erythroplakia showed different autofluorescence patterns even for the same histological result. Figure 2 shows the fluorescence spectra for both excitation wavelengths in the buccal mucosa. As can be noted for leukoplakia, tissue discrimination is not obtained for the used excitation wavelengths. On the other hand, for erythroplakia, fluorescence spectra under both excitations provided a good discrimination for the suspect lesions (figure 3).

Figures 3, 4 and 5 also show some examples of the discrimination obtained for erythroplakia in the buccal mucosa, leukoplakia on the floor of the mouth, and erythroplakia on the border of the tongue, respectively. Other oral sites as palate, gingival, dorsum of the tongue, and inner mucosa of the lip also presented some lesions, but the analysis is difficult due to the low number of cases. Changes on emission intensity and on spectra shape could be noted at

Table 1. Clinical features and histopathology diagnosis of the study population. (Note: one of the erythroplakia was *in situ* carcinoma. BOT = border of the tongue, DOT = dorsum of the tongue, FOM = floor of the mouth, BM = buccal mucosa.)

								Pathology diagnosis			
	Oral sites								Epithelial dysplasia		
Clinical impression	BOT	DOT	FOM	Gingiva	Palate	Lip mucosa	BM	Epithelial hyperplasia	Mild	Moderate	Severe
Leukoplakia	9	1	3	4	0	2	8	5	13	8	1
Erythroplakia	0	0	1	0	1	0	3	0	0	2	2
Leukoerythroplakia	1	0	0	0	0	0	0	0	0	0	1

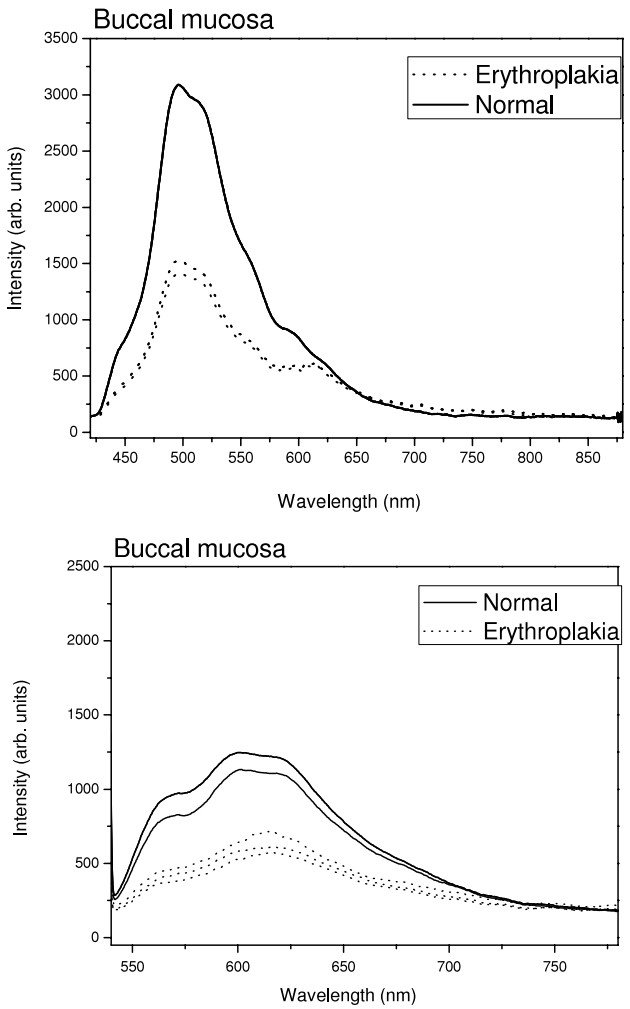

Figure 3. Comparison in the buccal mucosa of spectra in sites of normal mucosa and erythroplakia, at wavelengths of 406 nm and 532 nm, respectively.

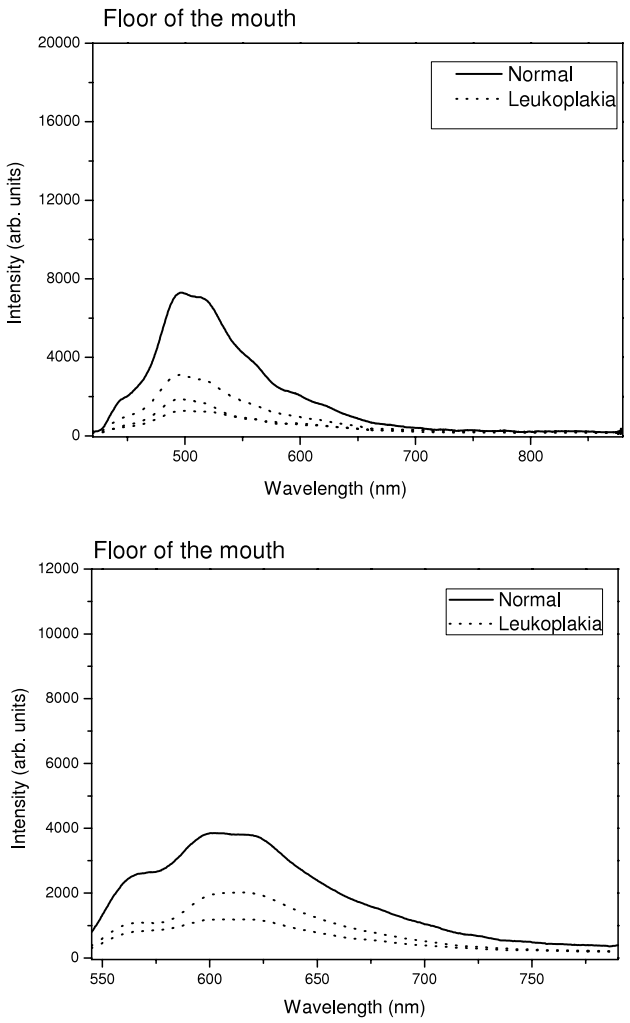

Figure 4. Comparison in the floor of the mouth of spectra in sites of normal mucosa and leukoplakia, at wavelengths of 406 nm and 532 nm, respectively.

some lesion regions, but in other areas the fluorescence spectra were quite similar to the clinically normal contralateral area. The fluorescence spectra varied according to the anatomical site, even for the same clinical characteristics of the lesion, as well as for distinct histopathological diagnosis. Another observation that should be pointed out is that, for the subjects in this study, the normal mucosa of the patients showed distinct patterns when compared to the oral mucosa of the normal volunteers. This behavior should be further investigated, also in comparison with clinically non-malignant mucosa of oral cancer patients. In PCA analysis of the sensitivity and specificity weighted were 0.874 and 0.776, respectively.

4. Discussion

The prognosis of patients with squamous cell carcinoma of the oral cavity involves several demographic, clinical, pathological and therapeutic variables. The most significant prognostic factors are the tumor site and size and the variables related to regional metastases. These factors significantly influence the probability of disease locoregional control and survival rates. Primary and secondary prevention seems to be the best chance to improve long-term survival results and there is a need for the development of diagnosis tools aiming to aid in the diagnosis of potentially malignant disorders and early stage cancers [3, 19].

Point spectroscopy is a potential diagnostic tool that can investigate only superficial lesions, since its response is dependent on the excitation light penetration into the tissue and on the collection of the fluorescence exiting from the tissue surface. The volume of the investigated tissue and the origin of the emitted fluorescence are mainly dependent on excitation wavelength and tissue composition. Also, the amount of keratinized layer or vascularization influences the collected fluorescence. Keratinization causes a higher light scattering, and vascularization a higher absorption, modifying the fluorescence evaluated [20, 21].

The variation in tissue architecture alters the pattern of the fluorescence spectrum collected, since the coupling of excitation light and the optical path of photons at different wavelengths is influenced by the organization

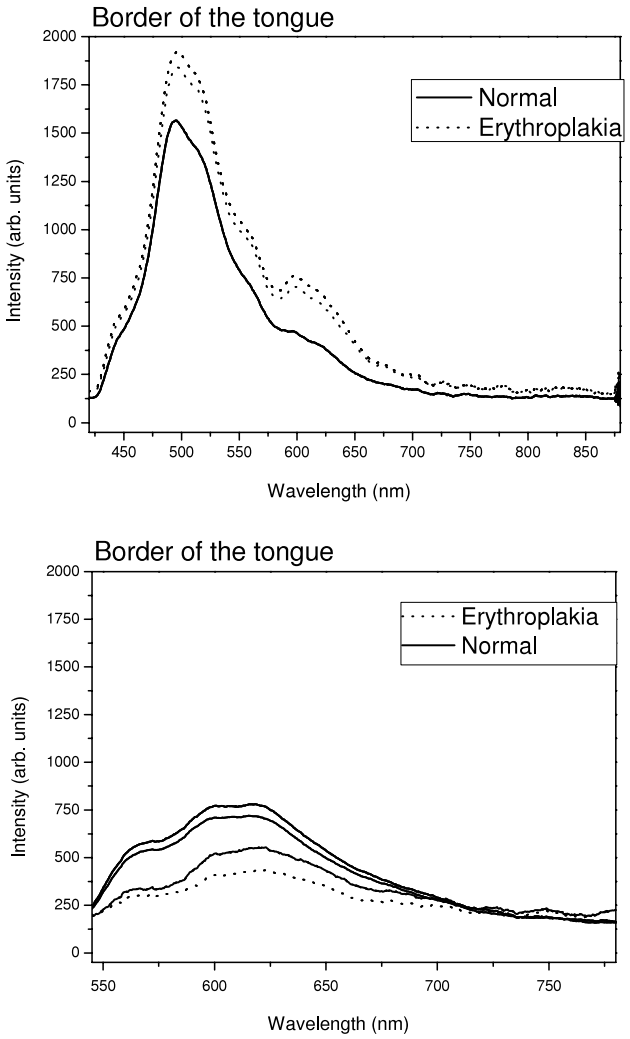

Figure 5. Comparison in the border of the tongue of spectra in sites of normal mucosa and erythroplakia, at wavelengths of 406 nm and 532 nm, respectively.

of biomolecules. The biochemical composition, especially the concentration of absorbers, scatterers and fluorescent biomolecules defines the final emission collected by the system. Monochromatic light is used because it provides a more selective excitation of fluorophores, scatterers or absorbers, improving the efficacy of the method [22]. The analysis of each spectrum was performed according to the histopathological diagnosis without taking other tissue features into account. This clearly reduces diagnostic resolution, since distinct spectral patterns can be found in cases with the same histological phenotype.

Normal mucosa at distinct oral sites shows a distinct histological architecture, composition and fluorescence spectrum. Ramanujam and Chu *et al* have previously discussed this spectral difference at oral sites [23, 24]. Schwarz *et al* also observed spectral differences between oral sites, especially when considering keratinized and non-keratinized oral mucosa. Veld *et al* reported differences between the autofluorescence spectra of normal oral mucosa in a group of 96 volunteers. The most important factor raised by the authors was ethnicity. Based on our results, oral site must be considered when investigating normal versus altered mucosa at different oral sites [25, 26].

Westra and Sidransky have shown that the observed correlation of pathologic features and some genetic alterations indicate that fluorescence visualization is superior to clinical judgment alone in gauging the delimitation of the cancer field. Indeed, the field of optical changes seemed to be more accurate in the diagnosis of the field of genetic changes than traditional light microscopy [27].

Currently, fluorescence techniques have been used as a diagnostic tool in a number of clinical situations and in different medical specialties, aiming not only at the identification of early stage lesions, but also at fluorescence guided biopsy and even fluorescence guided local cancer therapy [28, 29]. Optical diagnosis of the head and neck is a rapidly developing area of clinical research that can be translated to patient treatment [30].

The main problem using point spectroscopy analyses is that only a small tissue volume interacts with the laser. The depth of laser penetration in the region between green and violet is restricted to surface layers of tissue and the intensity of fluorescence emitted is very low. This represents a limitation of this technique, especially when considering heterogeneous lesions, such as most of the potentially malignant disorders of the oral cavity. It was found that fluorescence spectra collected in the same tissue show different patterns depending on the excitation used. This variation occurs because the penetration depth and the fluorophores excited at each wavelength are distinct. Laser excitation at 532 nm shows a higher penetration depth compared to excitation at 408 nm [20], so the contribution of fluorophores of deeper layers may be more important at the collected fluorescence. Furthermore, some of the characteristics of the tissue in which the endogenous fluorophores are present have a great influence. Hemoglobin that is present in vascular structures absorbs a portion of the emitted fluorescence, particularly visible in the excitation at 408 nm. Epithelial fluorophores such as NADH also play a major role in the characteristics of the spectra of shallow depths and stromal fluorophores because collagen contributes to the measured signal from deeper regions [16, 31, 32].

Schwarz *et al* suggest that the short wavelengths may be more sensitive for the detection of initial changes in the epithelium, such as nuclear size and nucleus/cytoplasm ratio, and in the superficial stroma region where the early tissue changes during malignant transformation occur. In non-keratinized tissue, diagnostic performance was achieved using optical spectra only at superficial and medium depths. The discrimination of the spectra of normal and abnormal sites is somewhat better when used with shorter excitation wavelengths, which are heavily constrained to the epithelial layer and minimize the effects of absorption of hemoglobin [32].

High-resolution fluorescence and confocal microscopy have elucidated variable oral tissue autofluorescence and the dispersion characteristics of the layers of the epithelium and stroma under conditions of normal tissues, benign diseases and dysplasia. Pavlova *et al* suggest that

the oral epithelium can be divided into three layers with different optical properties. The oral epithelium is composed of a superficial layer of keratin, which varies in thickness depending on the anatomical location. The main fluorophore of the superficial epithelium is keratin, which is above the non-keratinized epithelium occupied by intermediate and basal cells. The fluorescence from the non-keratinized epithelium is associated with metabolic fluorophores NADH and FAD, which increase in samples of oral dysplasias [16, 33].

The carcinogenic process involves a biochemical signaling between the epithelium and the extracellular matrix, with greater alterations in the optical properties in the superficial stroma than those in the deep stroma by disease progression. The fluorescence related to collagen, elastin and angiogenesis is significantly reduced in oral dysplastic lesions and inflammatory lesions, especially in the stromal layer underneath the dysplastic epithelium. The progression of dysplasia in oral mucosa results in a decrease in the volume fraction of collagen, decreased dispersion of the stroma, and angiogenesis that may also be more prominent in the superficial stroma [10, 33].

The presence of inflammation may be another complicating factor for spectroscopic diagnosis of oral lesions. Autofluorescence of inflammatory conditions and cancer may be difficult to distinguish, since both are correlated to reduced emission. As inflammation mainly affects the stroma with concomitant changes in the dysplastic epithelium, depth-sensitive spectral data, in particular the data derived from the more superficial layers, may furnish more useful information for the discrimination of benign lesions from malignant or dysplastic lesions [32].

Erythematous lesions show lower fluorescence intensity when compared to leukoplakia lesions, likely due to a higher concentration of hemoglobin, the most important endogenous absorber, which absorbs excitation light in both spectral regions of violet and green as well as the emitted fluorescence in the tissue. Leukoplakic lesions behave differently in re-emission of light because they are rich in keratin, which has a prominent role in the phenomena of scattering, absorption and fluorescence intensity re-emitted [18].

We also observed distinct spectral features at the fluorescence emission between 500 and 560 nm. This emission region has been correlated with the endogenous fluorophores NADH and FAD, both present at the epithelial cells and relevant for metabolic evaluation. Our optical setup does not discriminate at what tissue layer the emission originates. The collected fluorescence spectrum is a result of the emission from the epithelium and superficial stroma. Possibly an excitation/collection setup as described by Schwarz *et al* would improve the diagnostic resolution [32].

Pavlova *et al* studied the fluorescence emission under UV excitation from four different locations in the tongue of a single patient. One of the four sites was clinically and histologically normal; the other three sites were oral lesions that were histologically diagnosed as inflammation, dysplasia and cancer, respectively. The normal oral tissue was characterized by strong epithelial and stromal autofluorescence. In contrast, the lesion diagnosed with severe inflammation showed a relevant decrease in the fluorescence of both epithelium and stroma. Moreover, while the fluorescence of normal tissue stroma originated from collagen fibers, the changes observed at the inflammatory condition was due to the inflammatory cells in the stroma. The oral lesion diagnosed with dysplasia was characterized by increased epithelial thickness with fluorescent cells throughout the epithelium and a decrease in fluorescence of the superficial stroma. A diagnostic technique with a fast response can provide important information to clinicians, helping in the classification of the lesion, the scanning of large areas, border delineation of the lesion, and also the choice of biopsy site [17, 18, 10, 19, 20, 33].

Distinguishing clinical features were observed in different behaviors of the fluorescence spectra. The violet excitation showed better discrimination of normal versus malignant potential in comparison with excitation at 532 nm. The results show the potential use of fluorescence spectroscopy on the discrimination of oral lesions. When there is a progression from the normal state to an altered state, this is reflected in the spectral characteristics of fluorescence of tissues, which may be correlated with the histopathological examination of tissues. Our results demonstrate the potential of fluorescence spectroscopy to diagnose objectively and noninvasively distinguishing sites of dysplastic oral mucosa. Moreover, these results support the use of depth-sensitive optical spectroscopy to improve performance in the diagnosis.

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