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ORIGINAL PAPER

Fluorescence Spectroscopy for Diagnostic Differentiation in Uteri's Cervix Biopsies with Cervical/Vaginal Atypical Cytology

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Abstract This work aims the diagnostic differentiation of chronic inflammation (CC), low-grade Intraepithelial squamous lesions (LGSIL) and high-grade intraepithelial squamous lesions (HGSIL) in biopsies of cervix of uterus from patients with atypias (ASC-US and ASC-H) and lesions (LGSIL and HGSIL), traced in the cervical/vaginal cytology by using Laser-Induced Fluorescence Spectroscopy (LIFS), with 488 nm excitation wavelength. Ninety seven biopsies from 32 patients with atypical cervical/vaginal cytology were collected. The biopsies were guided by colposcopy and taken at the squamous-columnar junction. Fluorescence emission spectra of each biopsy were collected by means of an optical fiber cable coupled to an argon laser at 488 nm as excitation source and addressed to a spectrograph and CCD camera/controller. Spectra were separated into three groups, CC, LGSIL and HGSIL, based on the cytopathology. It was detected similar mean spectra profiles for CC and LGSIL, and differences for HGSIL. An algorithm was developed for tissue classification based on the intensity of the multiplication of each spectrum by the mean spectrum of each group, searching for a discriminator that would address this spectral difference. The sensitivity and specificity of HGSIL identification, compared to CC and LGSIL was 89% and 100%, respectively. The LIFS using excitation wavelength of 488 nm could be used to differentiate HGSIL lesions from LGSIL and CC inflammation, and could help a precocious and less invasive diagnosis of cervix lesions.

Keywords Fluorescence spectroscopy · Diagnostic · Uteri's cervix · Cervical/vaginal cytology

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Introduction

Cancer has become an important health problem around the world. It has actually ranked as the second death issue in the developed countries and is in between the three major causes of death in the developing ones [1]. The world's cancer incidence has increased significantly in the past decade. It is known that 70% of all cancer deaths occur in countries where the population has middle to a low income rate, very little or no education, limitation from cancer prevention, diagnosis and treatment [2]. Statistics has demonstrated that in USA the cancer is the second major death cause preceded only by circulatory diseases [3].

It is estimated that the uterine cervix cancer is the women's third most common malignant neoplasia, with about 19,260 new cases yearly, being surpassed only by non-melanoma skin cancer and breast cancer. It is also foreseen that the uterine cervix cancer is the fourth cause of deaths in women [4]. Nevertheless, among all neoplasias, the uterine cervix cancer actually outlines the highest

potentials for prevention and cure, reaching near to 100% when there is an early diagnosis [4].

The incidence peak of uterine cervix cancer occurs between the age of 40 to 60 year. However, this age has been dropping due to the increased incidence of Human Papilomavirus (HPV), especially in youngsters under age of 20 years [5]. The uterine cervix cancer has been etiology linked to the HPV in about 90% of cases [6].

The main strategy for an early detection of the uterine cervix cancer applied in Brazil is the screening survey which means performing cervical colpocytology examination (Papanicolaou) in women without any symptom of the disease. The goal is to identify those who may have the illness in the very initial stage, where the treatment can turn out to be effective [4].

In the Brazilian public health, the diagnostic differentiation of the Squamous Cell Atypia (ASC) is not done. This differentiation could be performed from the colposcopy and/or from the uterine cervix biopsy [7].

Optical methods for detecting human diseases have been gaining evidence in the last decades. Light could be used for tissue characterization without need for biopsies. These techniques could be inserted into the clinics for fast, cost effective and reliable diagnostics. The Laser-Induced Fluorescence Spectroscopy (LIFS) has becoming a promising tool in the early diagnosis of a great variety of neoplasias in esophagus, lungs, bladder and uterine cervix, showing good results in the differentiation among normal tissues and several types of cancers. It is based on the excitation of the sample with a short wavelength radiation (ultraviolet and visible) and the observation of the sample autofluorescence in long wavelengths (visible and near-infrared) through optical fiber cables [8, 9].

Tissue identification is based upon the determination of spectral differences between the chromo-fluorophores in the normal tissues and the dysplastic ones, caused by changes in the biochemical composition that occur following the neoplasic alterations in the uterine cervix tissue, as long as those tissues progress to the Squamous Intraepithelial Lesions [10, 11].

LIFS has been shown effective in the differentiation of the pre-neoplasic and neoplasic lesions in squamous intraepithelial alterations of uterine cervix. This technique has been investigated using several excitation wavelengths in vitro and in vivo, with algorithms based on spectral differences among diseased and health tissues in order to verify which one present more diagnostic differentiation capacity. For in vitro samples of cervix tissues, Lohmann et al. [18] used wavelength of 365 nm, Ramanujan et al. [19] used 340, 380 and 460 nm, Glassman et al. [20] used 340, 440 and 460 nm and Mahadevan et al. [21] used 330 and 385 nm. Other researchers have been detecting cervix neoplasia in vivo, such as Ramanujan et al. [12] using excitation wavelength of 337 nm, Ramanujam et al. [13] using 337, 380 and 460 nm, Agrawal et al. [14] using 337 nm, Nordstrom et al. [15] using 355 nm, Weingandt et al. [16] using 375 and 440 nm, Georgakoudi et al. [17] using between 337 and 610 nm and Rivoire et al. [10] using between 300 and 470 nm. Results of all works indicate that LIFS has a potential for such a differentiation, depending on the wavelength and algorithm used.

The main interest in this research is due to the fact that ASC (ASC-US-undetermined significance atypical squamous cell and ASC-H—atypical squamous cell, that could not exclude the high grade lesion) and SIL (squamous intraepithelial lesions, low grade—LGSIL or high grade—HGSIL) alterations are a problem in gynecological practice because there is no consensus about the most adequate conduct for women with such a cytologic diagnostic. For instance, there may be presented a more serious tissue alteration being treated as inflammatory alteration (reactive). With a proper diagnose, LIFS could contribute to a more conservative and less invasive practice, using only the cytological follow-up.

The importance of the present work is the possible use of LIFS as a screening for the Bethesda Colpocytology Classification System, that has been introduced because orientates to a more adequate therapeutics and which just recently has become routinely used in the medical community. So, the objective of this work is to develop a diagnostic algorithm using LIFS with excitation wavelength at 488 nm (argon ion laser) applied to the pre-neoplasic and neoplasic uterine cervix tissue, using the nomenclature adopted by the Bethesda System [22] for colpocytology screening, to obtain a fast, reliable and non-invasive diagnostic of precocious lesions in atypical cytology, thus avoiding unnecessary procedures (colposcopy and biopsies) due to the unspecificity of the standard approach and reducing the interval between diagnostic and therapeutics, diminishing suffering of patients.

Material and methods

Subjects and ethics The research was submitted to the Ethics Committee at the UNIVAP's Institute of Research and Development and approved by protocol number L208/2005/CEP. It was selected 32 patients from the Health Basic Unit Facility, Family Health Departments and the Center for Infect-Contagious and Parasite Diseases in the city of Fernandópolis, São Paulo, Brazil. Those patients were females ranging from 18 to 65 years of age (32.8 years mean age and 12.2 years standard deviation), with an active sexual life. Three patients were in the menopause and 29 in the menacme (reproductive period), being the biopsies done in such patients at the 5th day of menstruation [23]. Those

patients were chosen due to the uterine cervix colpocytology diagnosed as atypias as follows: ASC (ASC-US, ASC-H) and SIL (LGSIL and HGSIL). The patients were oriented about research goals and methods.

Samples The cervix tissue samples used in this work were obtained from 97 biopsies of 32 patients that underwent colposcopy evaluation as well as uterine cervix biopsy. Biopsies were collected on the particular Headmaster's office after patient's Term of Consent. For so, which is a gynecological routine, it was used a video-colposcopy, (model M 900M, DF Vasconcelos, Brazil) with binocular and cold light focus, with magnification of 3/4/7/13/17 to locate the ideal region for the procedure, in this case, in the transformation zone, at the squamous columnar junction (SCJ). Medina's pliers were used for the biopsies. After withdrawn, biopsy fragments were placed in polyethylene containers and wrapped up with aluminum foils before cryogenic conservation, by immersion into liquid nitrogen in an storage and transportation container (Cryometal, Brazil, model DS 18). The cryogenic preservation is recognized and used since 1972 as a preservation method of the physic-chemical properties of tissues from biopsies, as from in vitro fertilization samples [24], although some changes in the fluorescence profile could occur in emission wavelengths of 500 to 700 nm due to sample degradation [25].

In cases of bleeding Albocresil© solution was used for chemical cauterization, which is often used as a routine for little discomfort. An asepsis with 0.9% saline solution was done and there was no application of acetic acid. According to Agrawal et al. [14], the acetic acid application improves the fluorescence sensitivity because of the evidenced white-acetic lesions, so it allows better lesion identification for in vivo examinations. Nevertheless, Ramanujan et al. [12] recommend additional studies to assess an eventual change in the tissue fluorescence by the acetic acid. The Schiller test was not used due to the known fluorescence properties of iodine. Two biopsies were performed, in the lower lip and in the upper lip of the uterine cervix, specifically in the cervical cryptal region, that is, in the surroundings of the functional and actual SCJ [26].

Fluorescence spectroscopy Prior fluorescence spectroscopy, biopsy fragments were brought to room temperature and moisturized with saline. In the laboratory, each sample was excited in two points by the fluorescence spectrometer described as follows (Fig. 1). An argon laser (model Stabilite 2017, Spectra Physics, USA) was used as an excitation source, with a power of about 2 mW at 488 nm wavelength. This monochromatic beam was coupled to an excitation proximal end of an optical fiber cable (2 mm external diameter) especially designed for biological use [27]. The light, after exciting the tissue through the central fiber of the distal end and interacting with the tissue, was collected by the optical fiber catheter distal end. The light from the proximal end of the cable has passed through a low-pass filter with a cutoff frequency of 500 nm for blocking the laser light and was coupled to the spectrograph (model IS3100 Chromex, USA) that disperses the light in the region of 500 to 700 nm. Fluorescence was then detected by a CCD detector which converts the light into electric signal for being stored by the computer. After spectroscopy, the biopsy fragments were fixed in 10% formaldehyde, labeled and submitted to standard histological examination.

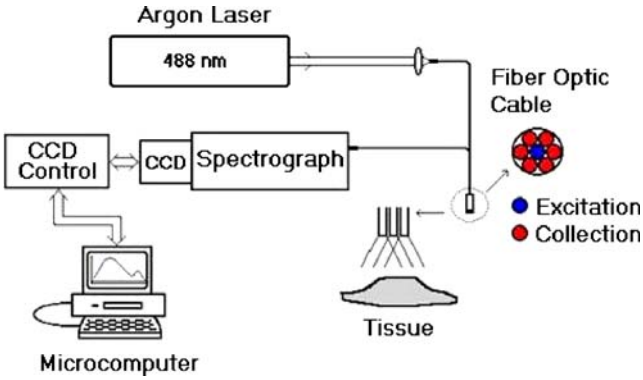

Fig. 1 Schematic diagram of the spectrofluorimeter using an argon laser at 488 nm as an excitation source for autofluorescence collected from cervix biopsy samples

Spectra handling and processing The spectra were intensity and wavelength calibrated using intensity standard (Oriel tungsten lamp) and wavelength standard (Oriel HgAr pen lamp) by a routine using the Matlab software and then plotted using Excel software. After histopathological examinations, the spectra were separated into three groups according to the type of lesion: CC, LGSIL and HGSIL and the mean spectrum of each histopathological group was calculated and plotted to identify differences in the intensity and profile of the observed emission spectra that could be used for tissue separation.

In order to differentiate each type of alterations through differences observed in the fluorescence spectra and develop an algorithm for tissue classification, spectra were normalized at the wavelength of 600 nm, this is the wavelength where mean have same intensity. Then, the mean spectrum of each tissue type was obtained and a calculation was performed, as follows: each single spectrum was multiplied by the mean spectrum, resulting in an intensity that represents the similarity of each spectrum to the mean.

To determine if this intensity would be able to differentiate lesions, a Kolmogorov and Smirnov test for normality was done and a parametric Student-*t* or non parametric Kruskal–Wallis analysis was applied to the data, to assess the significance level between the intensity of CC com-

pared to LGSIL and HGSIL samples. The InStat program (GraphPad Software) was used for such statistics.

The resulted intensity was then plotted and a separation line for each histopathological group was calculated, based on the mean Euclidean distance. Euclidean distance was chosen because there is only one variable accounting for the differences, and a more specific discriminator, such as Mahalanobis distance, that takes into account the covariance of the data, would not be significantly better than the Euclidean one if just one discriminator is used.

Results

Histopathological analysis Table 1 shows the main alterations found in the cervical colpocytology and the histopathology results obtained for each biopsy. For 97 biopsies, 54% were classified as chronic cervicitis, and were treated as normal mucosa in this study. Those uterine cervix fragments also demonstrate a discrete hyperplasia with atypia of the basal layer, presence of discrete cell nuclei hypercromasia, light edema and chronic inflammatory infiltrated in the adjacent corium. There was no sign of malignancy.

About 33% of all biopsies were hiatopathology classified as low grade intra-epithelial lesion, with the ectocervix showing grade 1 cervical intra-epithelial neoplasia (NIC I-SIL), with discrete atypical basal hyperplasia of about one third deep into epithelium, with evidence of dispolarity (or alteration of nuclei levels) and a few cells with sketch of coilocytosis. At the adjacent corium it is seen discrete edema and mononuclear inflammatory infiltrated.

High degree intra-epithelial lesion was found in 13% of fragments, with the ectocervix showing grade III intra-epithelial neoplasia (NIC III), atypia and despolarization all over the epithelium thickness, adjacent corium with discrete inflammatory infiltrated, predominantly mononuclear.

Table 1 Alterations found in the pap smear colpocytology (screening) and respective histopathologic results

Results of pap smear colpocytology	Number of patients	Total of biopsies	Histopathologic results		
			CC	LGSIL	HGSIL
ASC-US	22	65[a]	45	19	01
ASC-H	01	03	00	00	03
LGSIL	05	15	06	09	00
HGSIL	03	11	01	03	07[b]
Invasive CA	01	3	00	01	02
Total	32	97	52	32	13

[a] A biopsy disregarded of ASC-US colpocytology patient
[b] In a HGSIL colpocytology patient, it was performed two biopsies more, besides the three pre-established ones

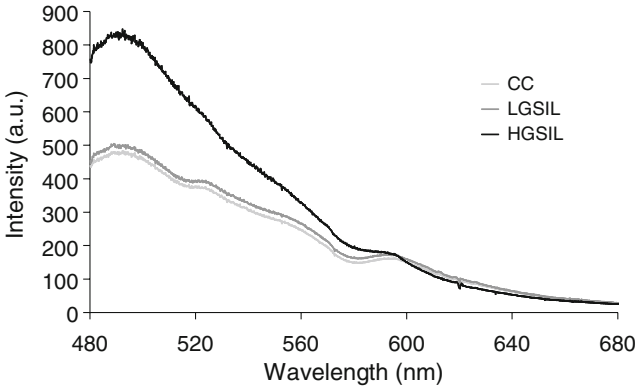

Fig. 2 Autofluorescence mean spectra of uterine cervix biopsies obtained from CC, LGSIL and HGSIL tissues (laser power: 2 mW, excitation wavelength at 488 nm)

Spectral analysis of biopsy fragments A total of 194 fluorescence spectra in the region of 500 to 700 nm were obtained from all 97 biopsies. Ten spectra were eliminated from the analysis due to low signal to noise ratio (<10). The obtained fluorescence spectra were separated into three groups depending on the histopathology: 95 chronic cervicitis (CC), 61 low grade intra-epithelial lesion (LGSIL) and 28 high grade lesion (HGSIL) spectra.

Figure 2 plots the average fluorescence spectra of CC, LGSIL and HGSIL obtained using excitation wavelength of 488 nm. It can be observed that both tissues, CC and LGSIL, although significant differences in individual spectral intensity among all samples, showed mean spectra with similar profile in the spectral region of 500 to 700 nm. At 600 nm the spectra had equal intensities. The mean spectra for the HGSIL showed a considerable variation when compared to ones obtained from CC and LGSIL. The spectra of all tissues showed a valley in the wavelength of 580 nm, that is attributed to hemoglobin absorption of blood (absorption Q band). CC and LGSIL showed an absorption band at 510 nm, also attributed to blood Q band [28].

Diagnostic algorithm Since spectral differences among CC, LGSIL and HGSIL tissues appear as change in the fluorescence intensity profile instead of presence of prominent peaks, it was proposed a diagnostic method based on the intensity of each spectrum compared to the mean spectrum. For so, each spectrum was multiplied by the mean spectrum, and the resulted intensity was plotted. Figure 3 shows the barplot of the resulted intensity and the standard deviation (SD) for the cervix tissue CC, LGSIL and HGSIL. The mean ratio of CC and LGSIL were closer to each other (values 4,605±1,644 and 4,080±1,396, respectively) and the mean ratio for HGSIL was of 8,092±1,717, or 75% above the value of CC.

To test the normality of the intensity values of CC, LG and HG it was applied the Kolmogorov–Smirnov test, that

showed normal distribution (Gaussian; $p<0.05$). Then the parametric Student-t test was applied to verify the hypothesis of similarity between the ratio values of the three groups, and it showed a high level of significance for CC and LGSIL, that is, they came from similar samples ($p=0.2$). Comparing the mean ratio of CC and LGSIL with the HGSIL tissues, it was found a very low level of significance ($p<0.001$), showing that the spectra came from different samples. Chronic cervicitis is a chronic inflammatory alteration of uterine cervix, and is not classified as ASC or SIL. The Student-t test showed high significance among the CC and LGSIL ratios, having no significant spectroscopic differences.

Figure 4 plots the CC, LGSIL and HGSIL intensities. Due to the relative high standard deviation of CC and LGSIL, it was not possible separate those tissues, as verified by the statistical t-test. Since it was found significant differences between intensities of CC/LGSIL from HGSIL, it was drawn a separation line based on the mean Euclidean distance which limits the intensity of CC and LGSIL from the HGSIL, providing a way to separate the low grade disease from the high grade one. Therefore, all HGSIL intensities scatter above the diagnostic line. Thus, the sensitivity and specificity of the HGSIL intensity compared to the mean ratio of CC and LGSIL was 89% and 100%, respectively [29].

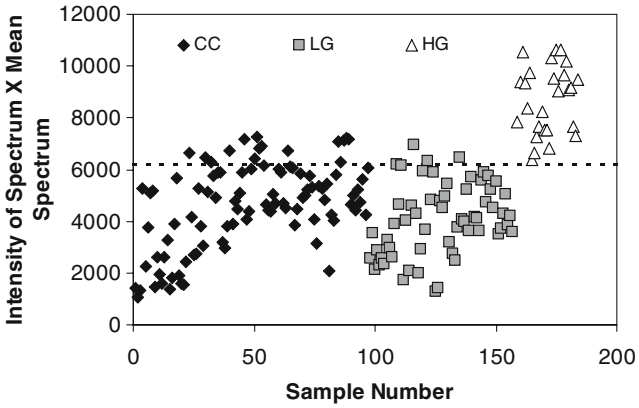

Fig. 4 Scatter plot of the mean intensity after multiplying each spectrum by the mean spectra. A diagnostic (*dotted*) line based on Euclidean distance was found to be 6,217, separating CC and LGSIL from HGSIL

Discussion

A total of 97 biopsies were analyzed from patients that underwent cervical colpocytology with diagnostic of atypical and squamous intraepithelial lesions of uterine cervix. In such biopsies were found about 54% of CC, 33% of LGSIL and 13% of HGSIL. The loss of the regular stratification, proportional to the worsening of the intraepithelial lesion, with increase of the cytoplasm nucleus proportion and variation of shape and size, besides the increased mitotic activity, indicates evolution of the disease. The compromising of the cervical epithelium layer is being another evolution parameter of the lesions, as long as it evolves from CC to LGSIL and to HGSIL.

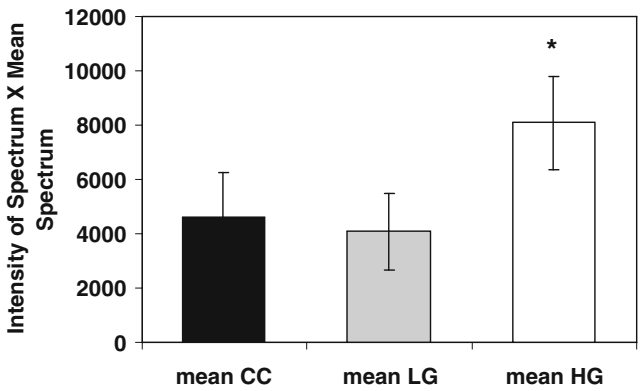

Fig. 3 *Barplot* of the mean intensity resulted after multiplying each spectrum by the mean spectra and the respective SD. *The mean ratio of HGSIL has significance level $p<0.001$ when compared to CC and LGSIL

The average age of patients was of 32.8 years, showing that the age of cancer incidence would be decreasing and thus committing even more young women [5]. This issue could be explained by the increased incidence of Human Papillomavirus in the female population [6]. The cervical pre-cancer and cancer can, however, be found in any age, including above 65 years of age [30]. This justifies the standard deviation of 12.4 years in relation to the patient's age found in this work.

It is known that the cervical cytology (cervical–vaginal smear) is a tracking examination that needs the histopathology for the final, conclusive diagnostic. In the cervical colpocytology the most frequent and prevalent alteration is the ASC-US. In this work, 68.7% of cervical smear showed ASC-US alterations, and from those 69.2% were diagnosed as CC, 29.2% as LGSIL and 1.6% as HGSIL. The usual conduct in such case is the follow-up, but a more serious lesion can be present, (HGSIL), that eventually could being treated with less seriousness. The results for the cytology alterations of LGSIL showed a histopathology correspondence of 40% of CC, 60% of LGSIL and 0% of HGSIL. In the HGSIL cytology, the histopathological correspondence was 9.1% for CC, 27.3% LGSIL and 63.6% HGSIL.

These discrepancies observed between cytology and the histopathology in the alterations and atypias of cervix tissue could be diminished with the development of a technique based on fluorescence spectroscopy as an immediate diagnostic method that is fast, safe, reliable and costless, decreasing the number of invasive procedures and providing a more adequate follow-up of suspicious lesions.

By analyzing the fluorescence profile of the samples, it was observed that the mean spectra of CC and LGSIL were similar (Fig. 2). Then, a spectroscopic differentiation between CC and LGSIL was not possible. Ramanujan et al. found that the intensity of the cervix squamous epithelium fluorescence in the 460 nm wavelength is high, while the columnar tissue (glandular) is low [13]. Weingandtat, et al. demonstrated that 94% of all pre-neoplasic and neoplasic cervix lesions are located in the transformation zone, in the SCJ, that is the more adequate region to collect biopsies [16]. However, the autofluorescence intensity could have variation, depending if it is made inside or outside the transformation zone [31]. In this work, the fluorescence spectra of LGSIL and columnar epithelium were greatly alike, with high degree of variation of the fluorescence profile including overlapping of spectra.

The differences in the intensity profile in the wavelength between 500 and 600 nm has been verified by a number of works that testify the ability of fluorescence spectroscopy to differentiate intraepithelial lesions (mainly HGSIL lesions) in the uterine cervix, since the fluorescence emission is a function of some fluorophores with metabolic activity. Carrying molecules $NADH_2$ e $FADH_2$, presented at mitochondria, donate energy (electrons) to the organelle membrane to form ATP from ADP. Then the produced reduced NAD and the oxidized FAD, when excited by ultraviolet/blue light, behave as biochemical markers and the main fluorophores [17, 32, 33, 34, 35, 36]. In the transformation from normal to atypical cells, as much intense the atypia more the energetic needs, one expects higher fluorescence in such circumstances.

In this work it was observed a difference in the HGSIL mean spectra when compared to LGSIL and CC in the wavelength region of 500 to 600 nm (Fig. 3). The separation line can differentiate the intensity of CC and LGSIL from the HGSIL (Fig. 4). The diagnostic of HGSIL based on the ratio values had high sensitivity and specificity.

In this work it was possible to differentiate the high degree intraepithelial lesion from chronic cervicitis and the low grade intraepithelial lesion. However, it was not possible to differentiate chronic cervicitis from low grade intraepithelial lesion. In Gynecology the differentiation of CC/LG from HG is important, once the CC patient have to be submitted to the cervix-vaginal smear tracking each year.

The LGSIL has 90% of its incidence depending on the Human Papillomavirus infection. The suggested conduct is the follow-up with cervix colpocytology every six months until the lesion is subdued, that normally occurs within 60 to 70% of all cases for Mattos [37] and in 47% of all cases after 24 months for Melkinov et al. [38]. However, the HGSIL conduct demands immediate complementary procedures, mostly surgical, once the alteration can propitiate a NIC II, NIC III or even a carcinoma in situ, which require quick conducts for the cure. The fluorescence spectroscopy could address this screening and follow-up, becoming a fast and effective examination that will contribute to the early diagnostics, avoiding unnecessary and costly procedures.

The use of spectrofluorimeter with multiple excitation wavelengths lower than 488 nm will enable a better understanding of biological tissue emission of different fluorophores. Wavelengths at 400, 440, 460 and 515 nm are proposed as adequate for the collagen, NADH and FAD emission, respectively [39]. A multiple line argon laser, with proper filtering and fiber optic coupling, could address this issue.

Conclusion

The Laser-Induced Fluorescence Spectroscopy using excitation wavelength of 488 nm applied to the diagnostic of alterations in the cervix intraepithelial tissues demonstrated significant differences of the spectral intensity profile for the HGSIL compared to CC and LGSIL. A method for identification of the CC/LGSIL from the HGSIL tissue type based on the intensity resulted from the multiplication of each spectrum by the mean spectrum was proposed, with high sensitivity and specificity. However, the differentiation between CC and LGSIL could only be made through histopathologic analysis, not by the fluorescence spectra.

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