Autofluorescence reveals menstrual phase in the endometrium

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Summary

Objective: Tissue contains fluorophores that autofluoresce without additional dye or photosensitizer with the appropriate light excitation. This technique has been widely applied for discrimination between normal and precancerous tissue. The aim of this study was to explore the capability and reliability of autofluorescence phase determination in samples of human endometrium. *Methods:* A total of 70 measurement sites from 31 endometrial tissue samples from hysterectomy were enrolled. Xenon light (330 nm) was directed at the endometrial tissue and the resultant autofluorescence intensity recorded. Spectra were then grouped according to the proliferative and secretory phase, with multivariant analysis, partial least square (PLS) and analysis of variance (ANOVA) used for evaluation of the statistical significance of phase determination. *Results:* Both proliferative and secretory autofluorescence spectra showed a similar characteristic triple-peak curve shape pattern, however, each of the intensities at the three peaks between the two phases varied markedly ($p < 0.01$). PLS analysis confirmed that collagen, NADH and FAD autofluorescence were the principle determinants of endometrial spectrum; the sensitivity and specificity of phase determination by autofluorescent was 100% and 97%, respectively. *Conclusion:* Autofluorescence measurement provides real-time information on endometrial phase status and, based on our results, it appears reasonable to suggest that it may be promising as a clinical tool for prompt phase interpretation.

Key words: Autofluorescence; Menstrual cycle; Endometrium.

Introduction

The menstrual cycle is a physiological reflection of the estrogen effect at the proliferative phase and progestin-dominant effect at the secretory phase on the endometrium. An ultrasound endometrial study is still the most convenient and practical method of determining the menstrual cycle phase [1]. An additional serum hormone level offers an alternative study for women who have irregular menstrual cycles, especially those with polycystic ovarian syndrome [2, 3]. Although the role of endometrial sampling for menstrual cycle dating remains controversial, the method remains the standard, especially in cases involving luteal phase defects [4, 5]. Unfortunately, of these modalities, only sonography can provide instant endometrial phase information in a clinical setting; however, thorough training and experience is required.

Autofluorescence measurement is a distinct diagnostic tool, allowing direct collection of biological data from the tissue using appropriate light excitation without the need for dyes or photosensitizers. This investigative modality has been used to provide instant discrimination between normal and precancerous tissue, including cervical intraepithelial neoplasms [6, 7]. The subtle change of the two kinds of autofluorescence emitted by collagen and reduced-nicotinamide-adenine-dinucleotide (NADH), at 390 and 470 nm wavelengths, respectively, determines if the tissue is normal or precancerous. Minute variations in tissue architecture and metabolic changes are reflected in variations in autofluorescent spectral intensity, also referred to as optical biopsy [8].

The investigative focus of this pilot study was to test the discriminative capability of autofluorescence for physiological endometrial cyclic change, and to determine the corresponding reliability in phase determination.

Material and Methods

Patient selection and sample preparation

In vitro endometrium immediately after hysterectomy from patients without endometrial polyps, malignancy and pre-hysterectomy curettage were obtained from the Department of Gynecology, Taipei Veterans General Hospital from January through April 2004. Once the corpus uterus was detached from a patient, the endometrium was exposed and rinsed using normal saline to remove blood clots and extra mucus. The specimen was then secured on a tissue stand in preparation for subsequent testing. Randomized one to five sites of each hysterectomy endometrium were chosen for the following autofluorescent measurement. To avoid intra-observer error, each measurement was repeated three times within four hours of extraction, to ensure that the tissue fluorophores had not decayed [9]. Additional specimens were obtained at each measuring site and sent for further pathological evaluation.

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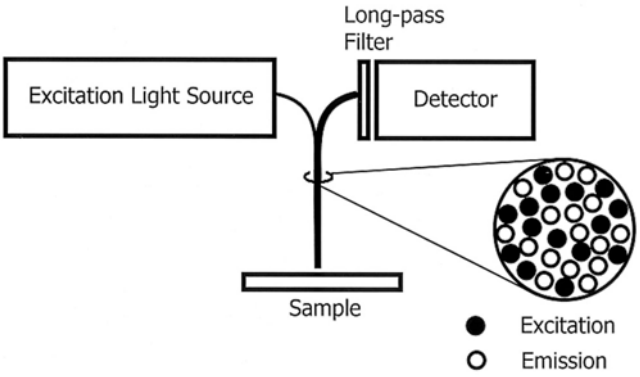

Figure 1. — Schematic of setup for endometrial autofluorescence measurement.

Equipment setup and environmental setting

In our study, a xenon lamp (Jobin-Yvon Optics and Spectroscopy, France) was used as the light source, with a specific wavelength (330 nm) produced using a monochromator (Jobin-Yvon H10 UV, grating 1200 g/mm). The light was output to the surface of the endometrium via a Y-shape optic fiber containing 18 output and 19 input sub-fibers. With the aid of a tissue stand, the terminal end of the Y-shape fiber was placed in vertical contact with the endometrial surface. The input sub-fibers simultaneously transmitted emission autofluorescence through another monochromator (Jobin-Yvon DH10 VIS, grating 1200 g/mm) in the range of 370-540 nm in 2-nm increments, with the discrete outputs amplified by an R928 photo multiplier tube (Hamamatsu, Japan; rise time < 2.2 ns). The resultant data were managed using a program based on LabVIEW6i (Laboratory Virtual Instrument Engineering Workbench, National Instruments, Austria) run on a portable computer (ThinkPad, IBM Inc.).

The standard dye, Rhodamine solution, was used for device calibration at the beginning of measurement. Since autofluorescence is easily overcome by visible light, the procedure was conducted in a dark-room environment.

Data processing and statistical evaluation

Triple-repeat measurement data for each site were first averaged and then used to produce a raw autofluorescence spectrum. The extreme diversity of each spectrum resulted from several factors e.g., excitation-emission efficacy, signal-noise ratio, and fiber-tissue surface interaction. To eliminate such confounding effects, area normalization was performed.

The spectral data were categorized into proliferative (P) or secretory (S) phase group based on the pathological report. For advanced statistical discrimination between the two groups, a multivariate regression analysis algorithm, the partial least square (PLS), [10] which is widely accepted as a standard statistical tool in autofluorescence studies [11, 12] was applied. Through the PLS calculation, each spectrum was simplified as an integer digit, called cross-validation score. The score dispersion yields an index of the reliability of the diagnostic algorithm. The PLS also reveals the location of the significant autofluorescent wavelengths among the spectrum, named principle components. The principle components are the most representative elements of the spectra so that they can establish the statistical comparison between two groups with significance set at $p < 0.05$ by analysis of variance (ANOVA). Diagnostic accuracy was then evaluated by determination of diagnostic sensitivity and specificity using the receiver operating characteristics (ROC) curve.

Results

A total of 31 patients underwent total hysterectomy, with a total of 70 measurement sites enrolled in the study. The average age ± SD of the patients was 42.58 ± 13.40 years. The most common indication for hysterectomy was uterine fibroids (61.29%). There was no statistical significance comparing the two groups (P vs S) for age (42.34 ± 15.60 vs 36.16 ±11.44 years).

All individual endometrial autofluorescence spectra demonstrated the characteristic triple-peak pattern (normalized group average spectra are shown in Figure 2) where the peaks corresponded to collagen, NADH and flavin-adenine-dinucleotide (FAD) autofluorescence. There was reciprocal change in these peaks comparing the two groups.

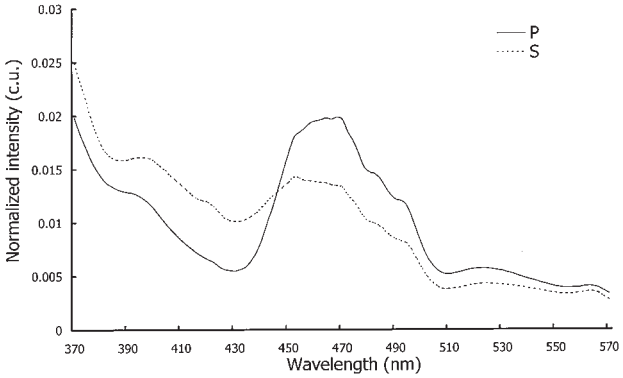

Figure 2. — Endometrial normalized autofluorescent spectra. Note triple-peak spectral pattern with apexes at 395 ± 5, 470 ± 5 and 530 ± 5 nm, corresponding to collagen, NADH and FAD, respectively, in both proliferative (P) and secretory (S) phase spectra.

PLS analysis revealed three principle components affecting the pattern of endometrial autofluorescence at 395 ± 5, 470 ± 5 and 530 ± 5 nm, wavelengths identical to the triple-peak location. There was intensity statistical significance between the two groups on each of the three peaks ($p < 0.01$; Figure 3). All cross-validation scores of

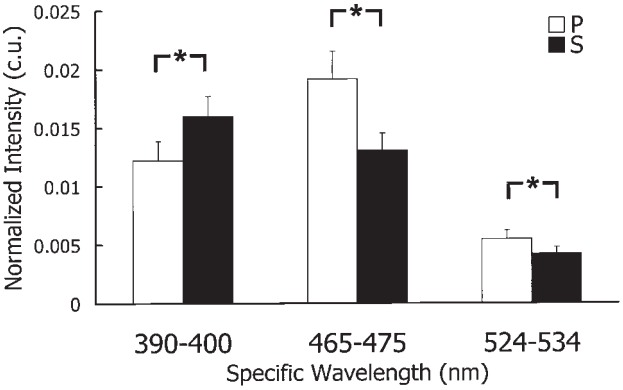

Figure 3. — Comparison of the normalized intensity of the three specific peaks for the endometrial P and S phases. I-bars represent standard deviation. Statistical significance was achieved for each of the three sets (*$p < 0.01$).

the 70 spectra by PLS algorithm are lined up in Figure 4. The sensitivity and specificity of the endometrial phase, as determined by autofluorescence, were 100% and 97%, respectively.

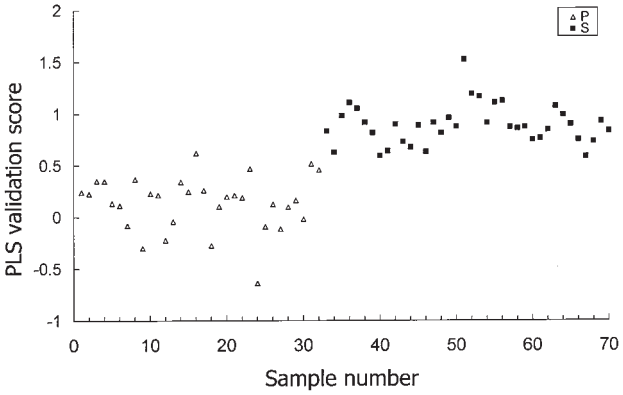

Figure 4. — Scatter plots of total cross-validation scores the using partial least square (PLS) method. Samples 1-32 and 33-70 on the horizontal axis are proliferative and secretory phases, respectively.

Discussion

First reported in 1924, tissue autofluorescence is a universal physical finding for different organ types [13]. The sources of autofluorescence are collagen NADH, FAD and other minor components [9]. Among the known fluorophores, collagen is the major supportive component of tissue, and NADH, FAD are the key coenzymes in the redox metabolic pathway. It has been demonstrated that autofluorescence studies provide an investigative alternative for discrimination between normal and abnormal cervical tissue, with comparable sensitivity and outstanding specificity relative to colposcopy performance [7, 14]. To our knowledge, however, endometrial autofluorescence performance is still the frontier to be explored. In this study, we found that *in vitro* endometrial tissue can also unexceptionally yield autofluorescence and the peak pattern is identical to other tissue [8, 12].

Both the proliferative and secretory endometrial autofluorescence spectrum show the triple-peak pattern, however, statistical comparison revealed significant differences between them. The satisfactory high sensitivity and specificity indicate the reliability of autofluorescence on phase discrimination. We attribute this advantageous discrimination to the entirely different dynamic, either structural or biochemical change of the endometrium which provides a favorable condition for the autofluorescent measurement approach. Cyclic endometrial change is the result of an ovarian hormonal synergistic effect. Collagen is the main supportive component of tissue architecture, however, the reported extent of endometrial collagen change during the menstrual cycle remains controversial, varying from 2% to 6.6% [15-17]. A descriptive transvaginal sonography study has demonstrated that endometrial thickness increases from the day of menstruation to ovulation, with a transition in crescent echogenicity revealing the change in thickness increase [18]. After the ovulation event the endometrial echogenicity becomes enhanced. It is postulated that the hyperechogenicity arises from the lengthening and coiling of the endometrial glands [19]. This indicates that the endometrium is more compact in the secretory phase relative to the proliferative analog. Condensation accounts for the increased collagen autofluorescence intensity in the secretory phase demonstrated in our study.

As is known, the endometrium recovers soon after the menstrual flow and proliferous cells pile up from the stratum basalis. Cell proliferation is related to the elevation of serum estrogen levels. It has been observed that direct estrogen administration to the endometrial cells evokes increasingly cellular nucleotide uptake and marked RNA synthesis in 15-30 minutes [20]. The subsequent glycogen, phospholipid and fluid increases contribute to protein synthesis, promoting cell growth in the next two hours [21]. The latest evidence supports the proposition that estrogen-mediated cell proliferation is accomplished via activation of the mitochondria [22]. NADH and FAD are the key coenzymes in the cellular redox pathway of respiratory chain reaction and they are the markers of mitochondrial bioactivity [23]. This expectably dramatic redox activity is compatible with the statistically significant increase in NADH and FAD autofluorescence in the proliferative phase shown in this study.

In comparison to the gold standard of pathology, an outstanding phase correlation is achieved for specimens categorized with autofluorescence. By contrast, Forrest et al., reported an overall accuracy for sonographic endometrial phase interpretation of 93% [24]. Ultrasound generates information with respect to tissue texture via sonic waves, which are received by a transducer and then transformed into dynamic images, so this is a physical diagnostic tool. Autofluorescence uses the fluorophores photo emission to portray the pathological or physiological status of tissue, which is also a kind of physical approach. PLS assay in this study has demonstrated the reliability of phase discrimination determined by autofluorescence. Such excellent performance is likely because autofluorescence reflects not only tissue architectural changes but also bioactivity events at the time of measurement. The combined information promotes the autofluorescent diagnostic efficacy and leads to the high accuracy.

Conclusion

This pilot study has generated autofluorescence data from *in vitro* endometria. Autofluorescence is a physical phenomenon that occurs when tissue fluorophores luminesce in light in a dark environment. The endometrial cavity is a naturally enclosed compartment in the *corpus uterus* that provides an ideal field for autofluorescence measurement. Herein, we have demonstrated that autofluorescence is an alternative measurement tool that can instantly and quite precisely reveal two major endometrial phases. Given these preliminary results, it appears reasonable to suggest that its potential capability will be confirmed by more detailed analyses of phase discrimination in the near future.

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