Visible-Near Infrared Spectroscopic Assessment of Urogenital Tissue in Premenopausal and Postmenopausal Women

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ABSTRACT:

BACKGROUND: A clinical study was conducted to evaluate the feasibility of using visible and near-infrared (NIR) spectroscopy as a potential noninvasive measure of genital skin health in premenopausal and postmenopausal women.

METHODS: A total of 45 female subjects (aged 21-70 years), all of whom gave fully informed consent to participate, were enrolled in the study and assigned to 1 of 3 groups: 15 premenopausal (Pre-M), 15 postmenopausal receiving hormone replacement therapy (Post-M HRT), and 15 postmenopausal receiving no form of hormone replacement therapy (Post-M non-HRT). Spectral measurements were taken at the vaginal mucosa, and spectral data were evaluated for the erythema index (EI), hemoglobin index (HI), bilirubin/β-carotene, and melanin. The color index (CI; calculated as the ratio of absorbance at 480 nm/540 nm) was also determined. Results were compared with previously published results on biomarkers and physical characteristic of genital tissue measured on the same groups of women.

RESULTS: Spectral measurements from the Post-M Non-HRT subjects indicated a significant reduction in HI compared with the Pre-M group (P= .0003) and to the Post-M HRT group (P= .0001). Similarly, EI was reduced in the Post-M Non-HRT (P< .0001 and P= .0041 for the Pre-M and Post-M HRT groups, respectively). In contrast, the Post-M Non-HRT subjects exhibited a significant increase in β-carotene compared with the Pre-M subjects (P= .0098). Bilirubin and melanin were not significantly affected. The Post-M Non-HRT group exhibited a significant increase in CI, indicating a shift away from the hemoglobin absorption region (510-620 nm wavelength) and toward the bilirubin/β-carotene absorption region (450-490 nm wavelength). This change was significant when compared with both the Pre-M group (P< .0001) and the Post-M HRT group (P= .0048). The changes in spectral measurements were consistent with previously reported changes in physical parameters (vaginal atrophy, increased pH, decreased skin temperature) and with decreased concentrations of the biomarkers histamine and histidine.

CONCLUSIONS: Hemodynamic spectral characteristics differ in postmenopausal vaginal tissue compared with tissue in premenopausal women, with decreased absorbance in the hemoglobin absorption region (510-620 nm wavelength) and an increased absorbance in the bilirubin/β-carotene absorption region (450-490 nm wavelength). A change in absorbance in the visible and NIR wavelengths is a promising, additional measure of genital skin health related to menopause and vulvovaginal atrophy.

KEYWORDS: Near-infrared spectroscopy, urogenital tissue, vulvovaginal atrophy, postmenopausal, hormone therapy, microvasculature indicators, urogenital biomarkers

Introduction

For decades, it has been known that skin can be evaluated spectrophotometrically, and that blood flow and the level of oxygenation of blood can have major influences on the spectral absorption wavelengths of the skin. As early as 1929, Brunsting and Sheard reported that oxygenated blood near the surface of the skin tends to shift the dominant absorption wavelength toward the red end of the spectrum (620–660 nm), whereas venous blood shifts the dominant absorption wavelength toward the blue end of the spectrum (490–500 nm).¹ Understanding this basic principle enabled the development of the first practical, lightweight oximeter in the early 1940s to measure hypoxia in pilots.² The development of pulse oximetry as a noninvasive means to continuously monitor oxygen levels in patients was developed in the 1970s and early 1980s.³

In addition to oxygenation, spectroscopy using absorption bands in the visible and near-infrared (NIR) spectral region can be used in the monitoring and diagnoses of a variety of inflammatory and disease processes, including tissue edema, hydration, and the degree of tissue perfusion.⁴ The technology can be used to evaluate the severity of burn wounds based on the oxygen saturation of the tissue and regional blood flow⁵ or to monitor real-time changes in regional oxygen saturation of cerebral and somatic tissues following hypoxic neurologic injuries.⁶ Near-infrared spectroscopy has a history of successful use...
in evaluating oxygen saturation in the retinal vessels \(^7\) and as a diagnostic tool for periodontal diseases.\(^4,8\text{–}10\)

The specific objective of this study was to evaluate the feasibility of using visible and NIR spectroscopy as a potential noninvasive measure of genital skin health, vaginal inflammation, and other skin conditions related to vulvovaginal atrophy and menopause-related genitourinary symptoms. We conducted a study of biomolecular and physical measures in the urogenital skin of 3 groups of women in different life stages: premenopausal, postmenopausal with benefit of hormone replacement therapy (HRT), and postmenopausal taking no HRT. Recently, we reported that skin temperature was lower in both postmenopausal groups indicating reduced blood perfusion.\(^11\) Histamine and histidine levels were also reduced significantly. This current report presents results of the visible and NIR spectroscopic evaluation conducted during this study.

### Materials and Methods

Testing was conducted in compliance with the Good Clinical Practice Regulations (21 Code of Federal Regulations [CFR] 50) and in accordance with the Declaration of Helsinki.\(^12\) Test protocols were approved by the test facility’s Institutional Review Board (Liberty IRB, Deland, FL, USA). Details of the study inclusion/exclusion criteria and demographic information for the subjects were detailed in an earlier publication.\(^11\)

Briefly, 45 female subjects, aged 21–70 years, who fully gave their informed consent to participate and met all entrance criteria, were enrolled. A summary of the test group demographics is presented in Table 1. The groups consisted of 15 premenopausal women (Pre-M), 15 postmenopausal women receiving HRT for at least 12 consecutive months (Post-M HRT), and 15 postmenopausal women who were not receiving any type of HRT (Post-M Non-HRT). A number of end points were measured in this study including physical measures (such as vaginal atrophy, skin surface pH, and temperature), histamine/histidine levels, cytokines, and natural moisturizing factor. The collection and analyses of these data were described previously.\(^11\)

For this phase of the study, spectral measurements were obtained from 4 different body sites: vagina, introitus, labia minora, and labia majora. The specifics of the instrument used to measure reflectance spectra have been described previously by Liu and colleagues.\(^3\) Spectra were collected using a portable PDA512-ISA spectrograph (Control Development Inc., South Bend, IN, USA) interfaced to a customized bifurcated fiber optic probe designed for use in the oral cavity (Fiberguide Industries, Stirling, NJ, USA). The probe consisted of 7 central fiber optic bundles to deliver light to the measured site and an outer ring of 22 fiber optic bundles arranged in a circular pattern to collect light emitted from the tissue. Relative absorbance was recorded.

### Table 1. Summary of demographics and baseline vaginal atrophy scores.

| PARAMETER                      | PREMENOPAUSAL GROUP (PRE-M) N=15 | POSTMENOPAUSAL WITH HRT GROUP (POST-M HRT) N=15 | POSTMENOPAUSAL NON-HRT GROUP (POST-M NON-HRT) N=15 |
|--------------------------------|----------------------------------|--------------------------------------------------|---------------------------------------------------|
| Age, y                         | 33.0 ± 6.4                       | 60.5 ± 3.6                                       | 60.7 ± 3.6                                       |
| Height, inch                   | 64.7 ± 3.7                       | 63.9 ± 2.5                                       | 63.5 ± 2.8                                       |
| Weight, lb                     | 156.0 ± 22.8                     | 147.8 ± 23.7                                     | 149.2 ± 27.0                                     |
| BMI, %                         | 26.2 ± 3.3                       | 25.5 ± 4.3                                       | 25.9 ± 3.6                                       |
| Years since last period        | NA                               | 14.2 ± 8.2                                       | 15.8 ± 9.3                                       |
| Average time on HRT            | NA                               | 5 y, 2 mo                                        | NA                                               |
| Ethnicity                      |                                  |                                                  |                                                  |
| African-American               | 6 (40)                           | 0 (0)                                            | 2 (13)                                           |
| White                          | 8 (53)                           | 15 (100)                                         | 13 (87)                                          |
| Other                          | 1 (7)                            | 0 (0)                                            | 0 (0)                                            |
| Vaginal atrophy scores         | 0.29 ± 0.12                      | 0.71 ± 0.22                                      | 6.98 ± 0.44\(^a\)                                |
| Vaginal pH                      | 4.72 ± 0.12                      | 4.36 ± 0.20                                      | 6.77 ± 0.25\(^a\)                               |

Abbreviations: BMI, body mass index; HRT, hormone replacement therapy; NA, not applicable.

\(^a\)Post-M Non-HRT group significantly different from Pre-M and Post-M HRT groups (\(P<.0001\)).
for the spectral range of 420 to 700 nm wavelengths. Table 2 shows the effectiveness of data acquisition. Measurements taken at the vaginal mucosa were successful in all study participants. As the anatomic site of the measurement moved from mucosal to stratified squamous epithelium, there was a higher fraction of rejected spectra, likely due to the attenuation by melanin. Because data acquisition was most reliable at the vaginal site, this was the focus of subsequent data evaluation.

Previously generated reference spectra for oxygenated and deoxygenated hemoglobin (HbO₂ and Hb, respectively) were used for comparison, and relative absorption was in the 510 to 620 nm wavelength region. Absorbance of bilirubin was in approximately the 450 to 490 nm wavelength region. Absorbance of β-carotene was also in the 400 to 500 nm range. Melanin absorbs over a broad range of wavelengths with absorption increasing linearly in the range of 620 to 720 nm and exponentially toward shorter wavelengths (300-600 nm).

Equations used to calculate the erythema index (EI) and hemoglobin index (HI) were adapted from Feather and colleagues. The EI was determined by establishing an arbitrary baseline joining points 510 and 610 nm in the spectrum of oxyhemoglobin and calculating the area under the curve above this baseline. It was determined by Feather and colleagues that this approach reduces the effect of the tissue matrix in the measurement. The EI is calculated from the following expression:

$$EI = 100\left( 1.5 \times OD_{545} + OD_{575} - 2 \times OD_{510} + OD_{560} \right)$$

The HI was based on measurements at wavelengths in the absorbance spectra at which absorbance is independent of the state of oxygenation, ie, the isosbestic points. Differences between absorbances at the isosbestic points at wavelengths 527.5, 544, and 573 nm define the HI, calculated from the following expression:

$$HI = 100\left( \frac{OD_{544} - OD_{527.5}}{16.5} - \left( \frac{OD_{573} - OD_{544}}{29} \right) \right)$$

Absorbance of β-carotene was also in the 400 to 500 nm range.

Table 2. Spectral data acquisition.

| GROUP                | SUCCESSFUL DATA COLLECTION |
|----------------------|----------------------------|
|                      | VAGINA | INTROITUS | LABIA MINORA | LABIA MAJORA |
| Pre-M (N=15)         | 15     | 8         | 9            | 6            |
| Post-M HRT (N=15)    | 15     | 12        | 15           | 7            |
| Post-M Non-HRT (N=15)| 15     | 10        | 11           | 6            |
| All combined (%)     | 45 (100)| 30 (67)   | 35 (78)      | 19 (42)      |

Abbreviation: HRT, hormone replacement therapy.
lowest. The Post-M HRT group showed an intermediate relative absorption in this wavelength range. In contrast, absorption in the 450 to 490 nm region was lowest in the Pre-M group, intermediate in the Post-M HRT group, and highest in the Post-M Non-HRT group. This wavelength region reflects absorption from bilirubin and β-carotene.

Using a fiber optic probe designed for use in the oral cavity as described in the “Materials and Methods” section, overall optical spectra were measured in the vaginal region for 3 different test groups: premenopausal women (Pre-M), postmenopausal women receiving HRT (Post-M HRT), and postmenopausal women receiving no form of HRT (Post-M Non-HRT). Relative absorbance was recorded for the spectral range of 420 to 700 nm wavelengths.

Based on absorption at selected wavelengths, end points of interest were determined as described in the previous section. Results are presented in Table 3. For the HI, the mean absorptions for the Pre-M and Post-M HRT groups were not significantly different (P = .06). However, the Post-M Non-HRT group had a mean absorption that was significantly lower than the Pre-M group (P < .0001) and the Post-M HRT group (P < .0001 vs. Pre-M, and P = .0041 vs. Post-M HRT).

There were no significant differences in the mean absorption when the 3 groups were compared for bilirubin. However,
the absorption reflecting the β-carotene was significantly higher for the Post-M Non-HRT group when compared with the Pre-M group \((P = .0098)\). The absorption reflecting melamin was not significantly different when the groups were compared.

The CI was defined as the ratio of absorbance at 480 nm (ie, in the bilirubin/β-carotene region) to 540 nm (ie, in the hemoglobin region) using equation 3. When the CI was calculated for the 3 groups (Table 3), there was no significant difference between the means for the Pre-M and Post-M HRT groups \((P = .11)\). However, the mean for the Post-M Non-HRT was significantly higher compared with both the Pre-M group \((P < .0001)\) and the Post-M HRT group \((P = .0048)\) indicating a higher absorption at the 480 mm region and a lower absorption at the 540 nm region (Figure 2). The means for each wavelength are shown on the primary vertical axis. The mean ratios of these 2 values are shown in the secondary vertical axis.

A summary of the NIR measures is provided in Figure 3 to illustrate the relative absorbance for the 3 test groups. With the exception of the absorption indicative of bilirubin, all other end points of interest demonstrated a significant difference between the Post-M Non-HRT group and the Pre-M group (ie, means for β-carotene, CI, HI, and EI). In addition, the Post-M Non-HRT group differed significantly from the Post-M HRT group in the means for CI, HI, and EI.

The calculated values for each variable determined in the study are summarized. To allow the data to be presented on a single graph, each variable from Table 3 was subjected to a multiplication factor: 100 × bilirubin, 10 × β-carotene, 1 × CI, 10 × HI, and 0.05 × EI.

Results of the spectral measurements were compared with physical parameters evaluated on the same subjects. The change in CI in the 3 test groups (Figure 4A) was consistent with increased scores for vaginal atrophy (Figure 4B) with significant differences in the mean when the Pre-M and Post-M HRT groups were compared with the Post-M Non-HRT group. A similar pattern was observed with vaginal pH (Figure 4C), with the Post-M Non-HRT group demonstrating a significantly higher mean than either the Pre-M group or the Post-M HRT group. In contrast, the pH measures of skin at the introitus resulted in a significant increase in both the Post-M HRT \((P = .022)\) and the Post-M Non-HRT \((P = .0033)\) groups (Figure 4C).

Indicators of blood perfusion (HI and EI) calculated from spectroscopy results (Table 3) were compared with skin surface temperatures (Figure 5). As discussed earlier, both the HI and EI decreased significantly in the Post-M Non-HRT group compared with the Pre-M and the Post-M HRT groups (Table 3 and illustrated in Figure 5A). Skin temperatures at the labia minora and labia majora sites also decreased significantly in the Post-M Non-HRT group compared with the Pre-M group \((P = .0087\) at the labia minora and \(P = .0025\) at the labia majora; Figure 5B). The Post-M HRT group showed an intermediate skin temperature at the labia minora and a skin temperature similar to the Post-M Non-HRT group at the labia majora. At the introitus, the Pre-M group exhibited the highest skin
temperature, but the difference was not significant compared with either Post-M group.

The calculated HI and EI absorbance measures were also compared with histamine and histidine levels isolated via tape stripping (Figure 6). When compared with the Pre-M group, concentrations of histamine were significantly lower in both the Post-M Non-HRT group (P = .0003 at the introitus and P = .0001 at the labia minora) and the Post-M HRT group (P = .041 at the introitus and P = .003 at the labia minora) (Figure 6B). For histidine, concentrations were directionally lower in the Post-M group (Figure 6C). The differences achieved significance at the labia minora (P = .045 for Pre-M to Post-M Non-HRT and P = .01 for Pre-M to Post-M HRT). The ratio of histidine to histamine was highly variable; however, it was lower in the Pre-M group compared with both Post-M groups.
This difference was significant for the Post-M Non-HRT group at the introitus ($P = .030$) and the labia minora ($P = .017$).

**Discussion**

The vulva and vagina undergo characteristic age-related changes in morphology and physiology, including loss of subcutaneous fat, vaginal mucosal atrophy, and a rise in pH. Aging of the skin is associated with a regression and disorganization of capillaries and small vessels resulting in an overall reduction in blood vessel density. This program was designed to determine whether aspects of female genital health could be evaluated noninvasively using visible and NIR spectroscopy conducted in 3 groups of women in different life stages: premenopausal, postmenopausal with benefit of HRT, and postmenopausal taking no HRT. Results were compared with previously reported biomolecular and physical measures in the urogenital skin conducted among the same test subjects.

It should be noted that we attempted to collect spectral measurements from several anatomic sites, including the vagina, the introitus, the labia minora, and the labia majora. We found that as the anatomic sites changed from mucosal to stratified squamous epithelium, a substantial proportion of the spectra were rejected. This was likely due to the increasing presence of melanin. For the previously reported biomolecular and physical measures, material was collected for analysis of biomarkers from anatomic sites via tape stripping, which could not be performed in the vagina. This limited evaluation of biomarkers to the introitus and external genitalia (labia minora and labia majora). Furthermore, the postmenopausal subjects in this feasibility study were predominately white. The applicability of the method to women of color will require further investigation.

For most of our observations, the Post-M HRT group exhibited values intermediate between the Pre-M and Post-M Non-HRT groups. Hormone replacement therapy is known to relieve many of the physical and psychological changes commonly associated with menopause, including vasomotor symptoms (hot flushes), sleep disorders, decreased sexual response, genitourinary factors, and mood changes. Based on our observations, HRT appears to reduce the magnitude of the change in the CI in postmenopausal women (Figure 3).

As we reported in an earlier publication, the levels of both histamine and histidine were decreased in postmenopausal women (Figure 6B and C). The ratio of histidine to histamine showed an increase (Figure 6D). Histamine is
derived from the decarboxylation of the amino acid histidine, and an altered ratio of histamine to histidine may indicate a change in the induction of histidine decarboxylase or a shift in the equilibrium between these 2 materials. Histamine has a variety of functions in the body including effects on smooth muscle and blood vessels critical to physiological sexual arousal.32,33 In our earlier publication, we proposed that the reduced level of histamine in the genital area might be related to sexual and lubrication difficulties in postmenopausal women, making histamine an important potential biomarker for genital tissue health.

**Conclusions**

Vaginal atrophy and an increase in skin pH are known to accompany menopause. A change in absorbance in the visible and NIR wavelengths is a promising, additional measure of...
genital skin health related to menopause and vulvovaginal atrophy. Hemodynamic spectral characteristics differ in postmenopausal vaginal tissue compared with tissue in premenopausal women, with decreased absorbance in the hemoglobin absorption region (510–620 nm wavelength) and an increased absorbance in the bilirubin/β-carotene absorption region (450–490 nm wavelength).

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Author Contributions
MAF was the clinical and project innovation leader. TC did Spectroscopy data analysis. K-ZL collected the Spectroscopic measures.

REFERENCES
1. Brunsting LA, Sheard C. The color of the skin as analyzed by spectrophotometric methods. III. The role of superficial blood. J Clin Invest. 1929;7:593–613.
2. Millikan GA. The oximeter, an instrument for measuring continuously the oxygen saturation of arterial blood in man. Rev Sci Instrum. 1942;13:434–444.
3. Severinghaus JW, Honda Y. History of blood gas analysis. VII. Pulse oximetry. J Clin Monit. 1987;3:135–138.
4. Liu KZ, Xiang XM, Man A, et al. In vivo determination of multiple indices of periodontal inflammation by optical spectroscopy. J Periodontal Res. 2009;44:117–124.
5. Seki T, Fujisaka M, Fukushima H, et al. Regional tissue oxygen saturation measured by near-infrared spectroscopy to assess the depth of burn injuries. Int J Burns Trauma. 2014;4:40–44.
6. Drayna PC, Abramjo TJ, Estrada C. Near-infrared spectroscopy in the critical setting. Pediatr Emerg Care. 2011;27:432–439; quiz 440.
7. Beach J. Pathway to retinal oximetry. Trans Vis Sci Technol. 2014;3:2.
8. Xiang X, Sowa MG, Iacopino AM, et al. An update on novel non-invasive approaches for periodontal diagnosis. J Periodontal Res. 2010;45:186–198.
9. Zhang C, Xiang X, Xu M, Fan C, Sowa MG, Liu KZ. Assessment of tissue oxygenation of periodontal inflammation in patients with coronary artery diseases using optical spectroscopy. BMC Oral Health. 2014;14:25.
10. Duarte PM, Sowa MG, Xiang XM, et al. Assessment of the hemodynamic properties of the capillaries in the cervix of diabetic patients with periodontitis by optical spectroscopy. J Periodontal Res. 2015;50:594–601.
11. Farage MA, Wehneyer K, Fadlay G, et al. Urogenital biomolecular and physical measures in pre- and post-menopausal women. J Clin Gynecol Obstet. 2015;6:237–250.
12. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013;310:2191–2194.
13. McEwen M, Reynolds KJ. Noninvasive detection of bilirubin in discrete vessels. Paper presented at: Proceedings of the World Congress on Engineering; July 24-26, 2014; London, England.
14. Babin SM, Sowa RM. Preliminary development of a fiber optic sensor for measuring bilirubin. Anal Chem Insights. 2014;9:59–65.
15. Doumas BT, Wu TW, Jendrzejczak B. Delta bilirubin: absorption spectra, molar absorptivity, and reactivity in the dioxane reaction. Clin Chem. 1987;33:769–774.
16. Drayna PC, Abramo TJ, Estrada C. Near-infrared spectroscopy in the critical setting. Burns Trauma. 2004;122:492–496.
17. Ou-Yang H, Stamatas G, Kollias N. Spectral responses of melanin to ultraviolet irradiation. J Invest Dermatol. 2004;122:492–496.
18. Feather JW, Hajizadeh-Saffar M, Leslie G, Dawson JB. A portable scanning reflectance spectrophotometer using visible wavelengths for the rapid measurement of skin pigments. Phys Med Biol. 1989;34:807–820.
19. Anderson RR, Parrish JA. The optics of human skin. J Invest Dermatol. 1981;77:13–19.
20. Hannemann RE, DeWitt DP, Wiechel JF. Neonatal serum bilirubin from skin reflectance. Pediatr Res. 1978;12:207–210.
21. Prince MR, Frisoli JK. Beta-carotene accumulation in serum and skin. Am J Clin Nutr. 1993;57:175–181.
22. Sayre RM, Black HS. Beta-carotene does not act as an optical filter in skin. J Photochem Photobiol B. 1992;12:83–90.
23. Zonios G, Bykowski J, Kollais N. Skin melanin, hemoglobin, and light scattering properties can be quantitatively assessed in vivo using diffusion reflectance spectroscopy. J Invest Dermatol. 1997;118:518–524.
24. Haaland DM, Easterling RG. Improved sensitivity of infrared spectroscopy by the application of least squares methods. Appl Spectrosc. 1980;34:539–548.
25. Farage MA, Maibach HI. Morphology and physiological changes of genital skin and mucosa. In: Surber C, Elsner P, Farage MA, eds. Topical Applications and the Skin. Basel, Switzerland: Karger; 2011:9–19.
26. Castelo-Branco C, Cancelo MJ, Villero J, Nohales F, Julia MD. Management of post-menopausal vaginal atrophy and atrophic vaginitis. Maturitas. 2005;52:46–52.
27. Farage MA, Miller KW, Elsner P, Maibach HI. Functional and physiological characteristics of the aging skin. Aging Clin Exp Res. 2008;20:195–200.
28. de Rigal J, Des Mazis I, Dissolbou S, et al. The effect of age on skin color and color heterogeneity in four ethnic groups. Skin Res Technol. 2010;16:168–178.
29. Trojahon C, Dobos G, Lichterfeld A, Blume-Peytavi U, Kotter J. Characterizing facial skin aging in humans: disentangling extrinsic from intrinsic biological phenomena. Biom Res Int. 2015;2015:318586.
30. Freedman MA. Quality of life and menopause: the role of estrogen. J Women Health (Larchmt). 2002;11:703–711.
31. Meston CM, Frohlich PF. The neurobiology of sexual function. Arch Gen Psychiatry. 2006;63:10:12–30.
32. Adakan PG, Kairin SM. Male sexual dysfunction during treatment with cimetidine. Br Med J. 1979;1:1282–1283.