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In vivo Raman spectroscopy for biochemical monitoring of the human cervix throughout pregnancy

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Abstract

BACKGROUND—The cervix must undergo significant biochemical remodeling to allow for successful parturition. This process is not fully understood, especially in instances of spontaneous preterm birth. In vivo Raman spectroscopy is an optical technique that can be used to investigate the biochemical composition of tissue longitudinally and non-invasively in human beings, and has been utilized to measure physiology and disease states in a variety of medical applications.

OBJECTIVE—The purpose of this study is to measure in vivo Raman spectra of the cervix throughout pregnancy in women, and to identify biochemical markers that change with the preparation for delivery and postpartum repair.

STUDY DESIGN—In all, 68 healthy pregnant women were recruited. Raman spectra were measured from the cervix of each patient monthly in the first and second trimesters, weekly in the third trimester, and at the 6-week postpartum visit. Raman spectra were measured using an in vivo Raman system with an optical fiber probe to excite the tissue with 785 nm light. A spectral model was developed to highlight spectral regions that undergo the most changes throughout pregnancy, which were subsequently used for identifying Raman peaks for further analysis. These peaks were analyzed longitudinally to determine if they underwent significant changes over the course of pregnancy ($P < .05$). Finally, 6 individual components that comprise key biochemical constituents of the human cervix were measured to extract their contributions in spectral changes throughout pregnancy using a linear combination method. Patient factors including body mass index and parity were included as variables in these analyses.

RESULTS—Raman peaks indicative of extracellular matrix proteins (1248 and 1254 cm^{-1}) significantly decreased ($P < .05$), while peaks corresponding to blood (1233 and 1563 cm^{-1})

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significantly increased ($P<.0005$) in a linear manner throughout pregnancy. In the postpartum cervix, significant increases in peaks corresponding to actin (1003, 1339, and 1657 cm^{-1}) and cholesterol (1447 cm^{-1}) were observed when compared to late gestation, while signatures from blood significantly decreased. Postpartum actin signals were significantly higher than early pregnancy, whereas extracellular matrix proteins and water signals were significantly lower than early weeks of gestation. Parity had a significant effect on blood and extracellular matrix protein signals, with nulliparous patients having significant increases in blood signals throughout pregnancy, and higher extracellular matrix protein signals in early pregnancy compared to patients with prior pregnancies. Body mass index significantly affected actin signal contribution, with low body mass index patients showing decreasing actin contribution throughout pregnancy and high body mass index patients demonstrating increasing actin signals.

CONCLUSION—Raman spectroscopy was successfully used to biochemically monitor cervical remodeling in pregnant women during pre-natal visits. This foundational study has demonstrated sensitivity to known biochemical dynamics that occur during cervical remodeling, and identified patient variables that have significant effects on Raman spectra throughout pregnancy. Raman spectroscopy has the potential to improve our understanding of cervical maturation, and be used as a noninvasive preterm birth risk assessment tool to reduce the incidence, morbidity, and mortality caused by preterm birth.

Keywords

cervical remodeling; in vivo; optical spectroscopy; postpartum; pregnancy; Raman spectroscopy

Introduction

Over half of all spontaneous preterm birth (sPTB) cases do not fall within any specific high-risk category, making the prediction and management of sPTB difficult.¹ While labor and parturition are coordinated processes involving many systems and organs for proper function, a full understanding of the cascade leading to labor and parturition has not been achieved, particularly in instances of sPTB. An arguably underinvestigated but highly important organ for pregnancy maintenance and proper labor is the cervix, a dense extracellular matrix (ECM) that is infiltrated by cells, blood vessels, and smooth muscle.^{2,3} The normal process of cervical remodeling that facilitates vaginal delivery occurs in 4 overlapping stages (softening, ripening, dilation, and repair) that have unique biochemical features and vary in duration among patients.⁴ Furthermore, these processes are affected by patient factors including parity,^{5–8} prior vaginal delivery,⁹ and body mass index (BMI).^{10–14}

Biochemically, the most abundant component of the ECM is fibrillar collagen, accounting for >70% of the cervix tissue dry weight.⁹ Other important ECM components include elastin, hyaluronan, sulfated glycosaminoglycans, proteoglycans, and water. During the softening phase, hydration and vascularity increase, remodeling the collagen matrix.⁴ The ripening phase involves an accumulation of hydrophilic molecules including glycosaminoglycans such as hyaluronic acid, further increasing cervical tissue hydration. Additionally, collagen becomes increasingly soluble. While the total amount of collagen remains the same, the collagen concentration decreases due to an influx of water and other matrix constituents.¹⁵ Next, cervical dilation occurs rapidly so the cervix can expand and

dilate for delivery. Finally, during cervical repair, the collagen matrix of the cervix rebounds through an inflammatory process similar to wound healing,^{16,17} with an increase in macrophages and neutrophils that orchestrate ECM rebuilding.¹⁸ Collagen remodeling occurs in all phases of cervical maturation, and has been identified as the most important contributor to the mechanical strength in the cervix.^{9,19} A firm relationship has been established between the extent of collagen cross-linking and tensile strength in both the pregnant mouse²⁰ and human cervix tissues.^{21–23}

Researchers are working to develop new tools to quantitatively and noninvasively evaluate cervical remodeling in vivo to aid both basic understanding of the remodeling process and clinical decision-making. These methods include magnetic resonance, acoustic, mechanical, electrical, and optical techniques.^{24–26} Light-based approaches can enable the study of tissue morphology and biochemistry depending on the light-tissue interaction process used. Two optical methods have been used in vivo to specifically monitor collagen. Fluorescence from collagen can be obtained by exciting tissue at ~340 nm. Previous studies have shown that fluorescence decreases over the course of pregnancy in mice and women.^{27–29} Excitation at ~950 nm can produce an optical phenomenon termed “second harmonic generation” in collagen fibers, and can be used to create collagen-specific images that show well-defined collagen networks and ECM remodeling over the course of pregnancy.^{30,31}

Beyond collagen, near-infrared spectroscopy has been used to monitor other factors, including hydration, hemoglobin, and optical scatterers by measuring diffuse light scattering.^{32,33} Absorption by hemoglobin and optical scattering were shown to increase throughout pregnancy, and absorption by water significantly increased during cervical ripening after exposure to the prostaglandin misoprostol.^{32,33} Ectocervix surface area determined by images from an endoscopic camera revealed increased surface area in pregnant patients throughout pregnancy, and was attributed to increased hydration.³⁴ While fluorescence, second harmonic generation, near-infrared spectroscopy, and white light imaging have been applied in vivo, these methods provide only a partial measure of the biochemical microenvironment. A single method that can probe hydration, proteins, and nuclear content as well as changes in vasculature could evaluate the role of each biomarker as well as the interplay between them during cervical remodeling.

Raman spectroscopy is an inelastic scattering technique that probes the vibrational energy levels of molecules. A Raman spectrum contains spectrally narrow peaks that are created by light interaction with distinct vibrational modes of molecules and therefore provides specific molecular fingerprints that are related to biochemistry and structure. Raman signals corresponding to lipids, proteins, saccharides, nucleic acids, and water offer direct information regarding the biochemical makeup of the tissue, and signal intensity is linearly related to molecule concentration. Care must be taken during the interpretation of spectra in instances of peak overlap, such as the overlap of protein and water at 1657 cm⁻¹. In such cases, all Raman features of a given molecule must be analyzed, and spectral unmixing methods may be employed. Using Raman spectroscopy for direct understanding of molecular processes is difficult in vivo due to the large number of biochemical constituents that contribute to Raman spectra. Thus, in vivo information mainly provides relative abundance of biochemical constituents and in some cases structure that may be used to

inform biology. Due to its high sensitivity and specificity, Raman spectroscopy has been increasingly used in biomedical applications,³⁵ including detecting dysplasia in the human cervix.^{36–42} In recent years, sensitivity to the hormonal status of cervical tissue has been seen in Raman spectra,^{43–45} which was the first step in using Raman spectroscopy to study cervical remodeling in mouse models of pregnancy.^{46,47} Although mouse models allow for observation of pregnancy in a highly controlled manner, not all biology is conserved between mice and women, and clinical implementation introduces its own set of challenges.

The goal of this study is to test the feasibility of measuring Raman spectra of the in vivo cervix in pregnant women, and to characterize Raman signatures throughout pregnancy and postpartum. To this end, 68 pregnant patients were enrolled in the study, and their cervixes were measured throughout pregnancy and postpartum. Statistical analysis was performed longitudinally on the collected spectra, and highly significant changes in the spectra are observed as a function of gestation. In addition, patient factors including BMI and obstetric history had significant effects on Raman spectra. To our knowledge, this is the first report using Raman spectroscopy to study cervical remodeling in pregnant women, demonstrating feasibility of the approach and identifying biochemical features associated with the phases of cervical remodeling.

Materials and Methods

Patient recruitment

This study was approved by the Vanderbilt University Medical Center Institutional Review Board (no. 100544) and followed the guidelines of the Declaration of Helsinki. Pregnant patients receiving prenatal care at the Vanderbilt Women's Health Center were recruited using informed, written consent. To be eligible for enrollment, patients had to be at least 18 years old and able to provide legally valid prospective informed consent. Relevant patient information such as obstetric history, medications, age, BMI, and estimated date of delivery were all included in each patient's research chart. At the end of the study, patient outcome, delivery date, and time were also recorded. Patient demographics are listed in the Table. A total of 68 patients were enrolled in this study.

Experimental setup

A portable Raman spectroscopy system was used for data collection (Figure 1, A). The system consisted of a 785 nm diode laser (Innovative Photonics Solutions, Monmouth Junction, NJ), an imaging spectrograph (Kaiser Holospec, Ann Arbor, MI), a deep-depletion thermoelectrically cooled CCD camera (Princeton Instruments, Trenton, NJ), a laptop computer, and a Raman spectroscopy fiber optic probe (EmVision LLC, Loxahatchee, FL). The Raman probe (fiber optic probe approximately 2.1 mm in diameter, plus external stainless-steel casing totaling 6.3 mm in outer diameter) consists of 1 optical fiber that transmits light from the laser to the tissue, surrounded by 7 collection fibers that deliver the backscattered light to the spectrograph. The laser power at the sample was set to 80 mW.

Clinical protocol

After obtaining written, informed consent, the patient underwent a speculum exam performed by a certified women's health nurse practitioner. The cervix was exposed and cleaned with a cotton swab soaked in sterile saline before the Raman probe was gently placed in contact with the cervix to obtain a Raman spectrum (Figure 1, B). The overhead room lights were turned off, and 3–5 measurements were taken from separate locations around the ectocervix, taking care to ensure that the probe was placed on the squamous epithelium. Each measurement used an integration time of 3 seconds, and the entire process took <10 minutes per visit. The Raman probe was disinfected after each patient in accordance with standard disinfection procedures. For each patient, spectra were acquired monthly in the first and second trimester, weekly in the third trimester, and at 6 weeks postpartum.

Data processing

Each day, the Raman spectroscopy system was wavelength calibrated using a neon argon lamp. The exact laser excitation wavelength and Raman shift were calculated using acetaminophen and naphthalene standards, which have well-characterized Raman shifts. The spectral response of the system was first determined using a National Institute of Standards and Technology–calibrated tungsten lamp, and then calibrated daily using a piece of green glass with a similar broadband spectrum. The resultant calibration factor was applied to each spectrum based on the spectral throughput measured each day. Raman spectra were processed to remove ambient background light, noise smoothed using a Savitzky-Golay filter, and fluorescence subtracted using the modified polynomial method.⁴⁸

Data analysis

To determine how the average Raman spectrum changes over the course of pregnancy and postpartum, a restricted cubic spline function was used for Raman shift (cm^{-1}) with k degrees of freedom or $k-1$ knots, where k was set to 65. A total of 65 knots were chosen to minimize the number of variables in the model without sacrificing spectral information. Additional patient variables, including prior vaginal delivery and BMI, were incorporated as continuous variables in this spectral model, resulting in a series of Raman spectra representative of various stages of pregnancy and postpartum.

Spectral regions that appeared to change throughout pregnancy based on observations from the spectral model were used for calculating changes in Raman peaks and Raman peak ratios over pregnancy. Peak ratio calculations provide data normalization and thus allow improved comparison across data sets with varying signal-to-noise ratios. Using this approach, the following peaks were identified for further analysis: 1006, 1055, 1125, 1157, 1248, 1254, 1304, 1339, 1451, 1563, and 1657 cm^{-1} . To determine whether peak or peak ratio intensities changed as the cervix prepared for delivery, generalized estimating equations (GEEs)^{49,50} (a type of linear model that clusters data obtained from each patient over time to account for the inherent correlation between measurements from the same patient) were developed for longitudinal monitoring. The change in each peak or peak ratio over time was modeled and plotted with its mean and its 95% confidence interval based on the GEE output, and analysis of variance was performed on the model output to determine statistical significance.

(*indicates $P < .05$ over the course of pregnancy). GEE output plots enable the analysis of cervical remodeling as a continuous variable, such that not only the spectral peak levels can be observed in each trimester, but also the rate of change within, between, and throughout all trimesters. Next, major biochemical contributors to the Raman spectra were tracked throughout pregnancy and postpartum using a biochemical model termed “nonnegative least squares” (NNLS) analysis. Pure components known to be present in the cervix were measured and the tissue spectrum was recreated using a linear combination of the pure components. The components used for the model were measured in their pure form and include actin, adenosine triphosphate, cholesterol, collagen type I, deoxyribonucleic acid, glucose, glycogen, phosphatidylcholine, phosphatidylethanolamine, progesterone, prostaglandin E_2 (Sigma, St Louis, MO), glucose-6-phosphate (Roche Diagnostics GmbH, Mannheim, Germany), hyaluronic acid (Abcam, Cambridge, MA), blood (excess human blood from use in institutional review board-approved protocol no. 151532), and deionized water. All measurements were acquired using the same integration time and are displayed in Supplemental Figure 1.

NNLS analysis is most accurate when noncorrelated spectral components are used in the model; therefore, the correlation coefficient between each biochemical signal was calculated (Supplemental Figure 2). If any 2 biochemical components had a Spearman correlation coefficient > 0.75 , 1 of the 2 components was chosen to be input for the model and the other was not included. The model was computed using MATLAB (MathWorks, Natick, MA). The pure component spectra were loaded into a matrix with each column containing a pure component spectrum. Patient spectra were input to the model and the best fit coefficients from each component were calculated. In addition, the residual between the model fit and the tissue spectrum was calculated to ensure all regions of the Raman spectra were adequately represented.

To longitudinally monitor how each biochemical molecule changed throughout pregnancy, the biochemical coefficients were analyzed using GEEs as explained above. In addition, patient variables including obstetric history (parity) and BMI were incorporated into the model as continuous variables to determine their effects on Raman spectra throughout pregnancy. Studies have shown that BMI and parity have an influence on the ability of Raman spectroscopy to correctly characterize cervical spectra,⁵¹ and are factors that contribute to the timing of parturition.^{10,11,14}

Results

Spectral changes throughout pregnancy

Raman spectra collected throughout pregnancy changed significantly as the cervix prepared for delivery. Representative spectra from 1 patient measured throughout her pregnancy and postpartum are shown in Figure 1, C. Multiple regions show considerable variation including 1030–1100 cm^{-1} , 1118–1200 cm^{-1} , 1230–1260 cm^{-1} , 1270–1330 cm^{-1} , 1440–1460 cm^{-1} , 1550–1600 cm^{-1} , and 1630–1670 cm^{-1} . The postpartum spectrum is particularly distinct from the prenatal measurements, and most closely resembles the earliest measurement obtained at the patient's 8-week prenatal visit. However, significant increases in the 1118–

1200, 1300–1350, and 1630–1670 cm^{-1} regions are observed, unique to the postpartum period.

The spectral model, which was developed to determine the average Raman spectra over the course of pregnancy for the entire patient population studied, is shown in Figure 2. Several significant changes were observed over pregnancy and postpartum, which are highlighted by the gray shaded regions in Figure 2 (*significant change over the course of pregnancy with $P < .05$). The 1530–1600 cm^{-1} region attributed to peaks observed in blood increases with advancing gestation, whereas bands characteristic of ECM proteins (as observed in the collagen spectrum) (1030–1100 cm^{-1} , 1230–1290 cm^{-1} , 1440–1460 cm^{-1} , and 1630–1670 cm^{-1}) and carotenoids (1130–1200 cm^{-1}) decrease throughout pregnancy. In addition, the left shoulder of the 1657 cm^{-1} peak appears to increase (Figure 2). Postpartum, bands observed in ECM proteins return to early pregnancy levels and there is significantly higher intensity in the 1270–1330 cm^{-1} range. Although Figure 2 depicts a computational representation of the data, the majority of the spectral changes that occurred throughout gestation are seen in the example patient spectra in Figure 1, C, demonstrating the utility and accuracy of the model.

Several peaks undergo significant changes over the course of pregnancy (* $P < .05$), including peaks at 1157, 1248, and 1254 cm^{-1} , which significantly decrease throughout pregnancy, and the 1563 cm^{-1} peak, which significantly increases as a function of gestation week (Supplemental Figure 3). Postpartum measurements are significantly higher ($P < .05$) in the 1304 and 1339 cm^{-1} peaks compared to early pregnancy (<22 weeks of gestation), and significantly lower in the 1006 and 1657 cm^{-1} peaks. A similar comparison of postpartum spectra to those taken at the end of gestation (>37 weeks) reveals significantly higher intensities postpartum at 1248, 1254, and 1339 cm^{-1} , and significantly lower intensities in 1006, 1563, and 1657 cm^{-1} . Raman peak ratio calculations reveal many features that change significantly throughout pregnancy and postpartum (Figure 3), including the 1254/1304 cm^{-1} ratio (ECM protein to actin and glycogen ratio), which decreases as term approaches ($P < .0001$) and is significantly lower postpartum compared to early pregnancy (Figure 3, A). In addition, the 1563/1248 cm^{-1} peak ratio (blood to ECM protein ratio) steadily increases over gestation ($P < .0001$), and postpartum measurements are significantly lower than late pregnancy (Figure 3, B). The 1157/1339 cm^{-1} peak ratio (carotenoid to ECM protein ratio) significantly decreases throughout gestation until term ($P < .005$), and postpartum measurements are significantly lower than early pregnancy (Figure 3, C).

Raman spectra of purified biochemical components

Six biochemical components were used to construct the least squares model (actin, blood, cholesterol, collagen I, glycogen, and water), and their Raman spectra are depicted in Figure 4, A–F. These spectra showcase the specificity of Raman spectroscopy to different types of biomolecules. An example of an in vivo spectrum obtained from cervical tissue, overlaid with its least squares fit and resulting residual, are displayed in Figure 4, G. The residual is small and fluctuates above and below zero, with an R^2 value of 0.922, indicative of a good fit.

The contributions of 2 biochemical components change significantly as a function of gestation (*indicates $P < .05$) (Figure 5). The contribution from blood significantly increases in a linear manner ($P < .0005$), whereas the contribution from collagen decreases over the course of pregnancy ($P < .0001$). Comparing postpartum measurements to early pregnancy (<22 weeks of gestation) reveals significantly higher ($P < .05$) postpartum levels of actin, and significantly lower contributions from collagen type I and water. Additionally, comparison of postpartum to the end of gestation (>37 weeks of gestation) reveals significantly higher actin and cholesterol contribution, and significantly lower blood contribution in the postpartum fits.

Effects of patient variables

While several patient variables were considered, parity and BMI were found to have an effect on Raman spectra during pregnancy and postpartum (Figure 6). From NNLS analysis blood/vascularity signals have an inverse relationship with parity, with nulliparous patients showing significant increases in blood/vascularity signals as the cervix remodels, compared with a relatively stable blood contribution in parous patients (Figure 6, A, and Supplemental Figure 4, A) ($P < .05$). Individual peak analysis reveals that the 1125 cm^{-1} peak increases steadily in nulliparous patients, but does not increase until about 30 weeks of gestation in parous patients (Supplemental Figure 4, B). Additionally, the 1254 cm^{-1} peak is significantly higher at the beginning of pregnancy and significantly lower near term in nulliparous patients, although parous patients do show decreases in this peak throughout pregnancy (Supplemental Figure 4, C). Postpartum, parous patients had significantly higher contributions from cholesterol (Supplemental Figure 4, D), while nulliparous patients had significantly higher intensities at 1006 (Supplemental Figure 4, E) and 1055 cm^{-1} (possibly caused by ECM proteins or actin) (Supplemental Figure 4, F) than parous patients.

Evaluation of the effects of BMI shows that low BMI patients (demonstrated by spectral model with BMI = 25) have decreasing actin contributions as the cervix prepares for delivery ($P < .05$) that levels out as BMI increases (Supplemental Figure 5, A). In addition, low BMI patients start with higher signal from 1248 cm^{-1} , a peak characteristic of ECM proteins, but decreases to a greater extent than high BMI patients at term (Supplemental Figure 5, B). Postpartum spectra from low BMI patients have higher actin contribution and higher 1003 cm^{-1} peak intensity compared to high BMI patients (demonstrated by model with BMI = 40 in Figure 6 and in Supplemental Figure 5, A and C). In addition, high BMI patients have higher contribution from collagen than low BMI patients in the postpartum period (Supplemental Figure 4, D).

Comment

Monitoring the cervix during pregnancy has many purposes; these include improved understanding of normal and abnormal physiology of cervical remodeling, estimating delivery time, and determining patient readiness for labor. This last clinical task is currently conducted using the Bishop score and in some cases, ultrasound; however, these methods provide limited information, mainly pertaining to cervical structure. In this study, Raman spectroscopy was employed to noninvasively measure the biochemistry of the cervix over

the course of pregnancy in vivo in women for the first time. This approach successfully measured known biochemical changes observed in multiple stages of pregnancy and postpartum repair, and established patient variables that affect the remodeling process, demonstrating the utility of Raman spectroscopy for cervical evaluation during pregnancy.

In vivo Raman measurements of the cervix acquired over the course of pregnancy revealed characteristic biochemical changes that occur in preparation for delivery. Decreases in peaks indicative of ECM proteins such as collagen (1055, 1248, and 1657 cm^{-1}) and increases in peaks corresponding to blood (1233 and 1563 cm^{-1}) are observed in the example patient spectra (Figure 1, C), the spectral model determined from 68 patients (Figure 2), and the NNLS analysis (Figure 5). An increase in blood signatures is attributed to the increase in vascularization that occurs as the cervix prepares for delivery⁴ (Figure 5), a phenomenon that has also been observed in pregnant women using near infrared spectroscopy.³³ Decreases in ECM peaks are explained by the process of ECM remodeling where collagen becomes increasingly soluble due to reduced cross-links and dispersed at a lower concentration.^{4,15} Trivalent collagen cross-links have been observed at 1660 cm^{-1} , and reductions in these cross-links (which have been previously reported in women²⁷ and mice^{27,52}) likely contribute to the observed decrease in this peak during pregnancy.⁵³ Furthermore, the 1657 cm^{-1} peak (also known as the amide I region) broadened in preparation for delivery, a phenomenon that is known to be caused by structural disorder such as collagen fiber dispersion.⁵⁴ This broadening may also be attributed to increased blood signals, which have a peak at 1619 cm^{-1} . In pregnant women, collagen solubility has been measured in vivo using laser-induced fluorescence where fluorescent intensity corresponding to collagen cross-links was shown to decrease throughout pregnancy.²⁸ Fiber dispersion has been measured during pregnancy ex vivo in mice and women using second harmonic generation⁵⁵ and optical coherence tomography.⁵⁶ An increase in collagen fiber dispersion was seen using both techniques. In the postpartum cervix, Raman spectral signatures were significantly different from measurements obtained during pregnancy, with increases in spectral features corresponding to actin and cholesterol, and decreases in features corresponding to blood, collagen type I, and water as observed in Figure 5. There are few studies focused on understanding postpartum repair, however, these studies suggest that the repair process is governed by immune cells, which infiltrate the cervix and create a proinflammatory environment that resembles wound healing.^{18,57,58} Microarray studies have found that in addition to inflammatory genes, matrix synthesis and collagen strengthening genes are up-regulated in the postpartum cervix.^{16,17} Based on this evidence, it is possible that the spectral increases of actin and cholesterol are caused by an influx of immune cells and fibroblasts. Although postpartum contributions from glycogen did not reach statistical significance due to variability in measurement time point (2–12 weeks postpartum), a possible explanation for the observed heightened glycogen levels is intensive anabolic activity driving the repair process. Furthermore, the peak at 1160 cm^{-1} is found in many carotenoid antioxidants,^{59–61} which could be elevated postpartum to combat high levels of reactive oxygen species related to macrophages and neutrophil infiltration.

Two patient variables, parity and BMI, affected spectral changes that were observed throughout pregnancy and postpartum (Figure 6). Nulliparous patients started pregnancy with higher spectral contributions from collagen (based on the NNLS model) compared to

parous (Figure 6, A). In fact, this observation is supported by an ex vivo study in which collagen concentration (determined via hydroxyproline assay) was measured from cervical biopsies taken from parous and nulliparous women, and revealed decreasing collagen levels as parity increased.⁸ These findings were corroborated with ex vivo biomechanical tests of nonpregnant tissues from patients with and without prior vaginal deliveries that revealed stiffer, less compliant tissue in patients without prior vaginal deliveries,⁹ and confirmed decreased collagen extractability, as well as increased sulfated glycosaminoglycans and hydration in patients with a history of vaginal delivery. It is likely that a permanent vascular network is established during the first pregnancy similar to permanent changes in mechanical properties,⁹ which would explain why patients with prior pregnancies had significantly higher baseline blood/vascularity levels compared to nulliparous patients. Taken together, these results are unsurprising, as multiparous patients generally have faster labors,⁵⁻⁷ indicating that some level of permanent cervical remodeling occurs after completing all 4 phases of cervical remodeling during a prior pregnancy.

Patients with low BMI have higher spectral signals corresponding to ECM proteins (1248 cm^{-1} peak) at the beginning of pregnancy (Figure 6, B), but this intensity decreases significantly more than high BMI patients by term, indicating that high BMI patients experience a lesser level of ECM remodeling. One possible explanation for this observation is an increase in leptin, an adipokine that is elevated in pregnant patients with high BMI, which has been shown to disrupt matrix metalloproteinase (MMP) action on collagen in myometrial tissues and synthesize myometrial collagen in late pregnancy.^{14,62,63} It is possible that this phenomenon also occurs in the cervix, reducing the extent of cervical remodeling achieved, and contributing to extended labor times and increased cesarean delivery rates observed in high BMI (>30) patients.¹⁰⁻¹⁴ In addition, actin signals decreased throughout pregnancy in low BMI patients, possibly caused by cervical cell apoptosis that occurs prior to labor.¹⁷ However, actin signals did not decrease in high BMI patients, which may also be attributed to high leptin levels that have been shown to prevent cell apoptosis in vitro.^{14,64}

Nine of the patients enrolled in the present study underwent preterm birth, ranging from 35 + 3/7–36 + 6/7 weeks' gestation when they delivered. Analysis was performed to determine if any differences were observed in this data set compared to patients who delivered at term, but none reached statistical significance. This result is not surprising, as these patients had different obstetric histories and BMIs, and all delivered relatively close to term. For a powered analysis comparing patients with and without preterm birth, additional studies that specifically enroll a large number of high-risk patients will be required.

Although not yet ready for the launch of a clinical tool, it is important to think about how such a tool would be used and incorporated into the current workflow. The long-term goals include the ability to measure premature cervical change at the patient bedside, as well as determine the etiology of the changes measured. The authors envision that Raman measurements could be made from the cervix early in pregnancy such as at the first appointment to obtain a baseline measurement. The frequency with which measurements would need to be made has not yet been established, and will likely require studies that evaluate the cost-benefit analysis of a variety of frequencies. Short term, Raman

measurements could be introduced as an additional test for assessment of premature cervical change and ideally provide clues pointing toward the etiology. It is also unclear whether data will need to be normalized to a previous measurement taken from the patient, or whether a single measurement could provide enough information to determine premature cervical change. Advanced predictive algorithms will need to be developed, first indicating abnormal spectral profiles, and then hopefully indicating abnormal changes with information regarding etiology. Based on results in this study, variables including parity and BMI will need to be incorporated into the algorithms. In addition to use as a preterm birth risk assessment tool, Raman spectroscopy could be used to aid our biological understanding of problems such as cervical insufficiency, ascending infection, and monitoring efficacy of new treatment strategies for preterm labor.

Limitations of this study include the need for validation of the Raman spectral findings. Investigation in mouse pregnancy is underway,^{46,47} which has the ability to validate in vivo findings from Raman spectra, and future study of patients undergoing hysterectomy will provide additional opportunity to compare in vivo Raman spectra to ex vivo biochemical assays. In addition, interpretation of Raman spectra can be difficult due to the overlap of many spectral features in biological samples and changes in structure rather than concentration. Although Raman spectral intensity and vibrational mode concentration are linear, multiple biochemical components may include the same vibrational mode such as carbon-hydrogen (C–H) stretch found in organic molecules. Observing the full spectrum can aid in these cases as distinguishing features likely exist between different biomolecules. When using Raman spectroscopy as a biological tool, it is important to remember that Raman spectra yield a relative concentration of biochemical species comprising tissue at the time of measurement. This information can help in understanding of the current microenvironment, but its use for determining molecular function or understanding mechanisms of molecular processes is challenging in vivo due to the nature of high overlap from thousands of biomolecules. Lastly, in this study, the Raman spectra are not depth-resolved, and therefore comment cannot be made regarding whether the biochemical signals originated from the cervical epithelium or stroma. For example, it is currently not possible to determine whether actin signals originated from epithelial cells or fibroblasts and smooth muscle cells deep in the cervical stroma. Raman probes can be developed that allow this capability and may be employed in future studies. The main strength of this study is in the ability to monitor multiple dynamic biochemical changes in the cervix as it remodels to deliver the fetus. Although other optical modalities are sensitive to certain biochemical components such as collagen or blood, Raman spectroscopy provides simultaneous monitoring of ECM proteins, lipids, blood, saccharides, antioxidants, and metabolic enzymes without the addition of exogenous labels or removal of tissue. This technique has high potential for better understanding the etiologies and progression of sPTB, and may also help to unveil the healing process that occurs during postpartum repair. Translation of Raman spectroscopy to clinical settings is becoming more realistic each year; real-time data processing has already been achieved,^{65,66} and fully characterized disease and/or physiology profiles allow for automation of data analysis and tissue classification at the bedside. Hardware advances are consistently moving forward, which promise to reduce complexity of the system.³⁵ These studies also bring us closer to identifying a spectral signature that

defines cervical changes in normal pregnancy, increasing the potential to utilize Raman spectroscopy for predicting the timing of delivery and therefore early risk assessment for preterm birth.

In summary, this study highlights Raman spectroscopy as a tool to track meaningful chemical signals in cervical tissue throughout pregnancy and during postpartum repair. This is the first study to demonstrate the utility of Raman spectroscopy as a basic research tool in obstetrics via its sensitivity to known dynamic biochemical changes that occur during cervical remodeling, and marking an important step toward development of a clinical tool. With continued advancement, Raman spectroscopy has the potential to improve patient care by arming providers with information on the biochemical state of the cervix, close gaps in our understanding of cervical maturation, and identify women at risk for preterm birth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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AJOG at a Glance**Research Question: Why was this study conducted?**

Improved understanding of the biochemical changes that occur during cervical remodeling in pregnancy is needed. Raman spectroscopy allows in vivo, biochemical, and longitudinal monitoring of the cervix throughout pregnancy and postpartum repair.

Key Findings

Spectral analysis revealed significant decreases in contributions from extracellular matrix proteins and significant increases in vascularity throughout the course of pregnancy. Parity and body mass index had significant effects on Raman spectra, and may help our understanding of adverse outcomes in delivery.

What does this add to what is known?

This is the first report of Raman spectra measured from the cervix in pregnant women and the first study to noninvasively measure differences in the cervical biochemical environment based on patient factors. This work has high potential for translation and may aid understanding of premature cervical change in populations at high risk for preterm birth.

FIGURE 1. In vivo Raman spectroscopy of the cervix during pregnancy

In vivo Raman spectroscopy of the cervix during pregnancy. **A**, Portable Raman spectroscopy system. **B**, Raman probe measurement from cervix in vivo. **C**, Raman spectra from 1 patient over course of her pregnancy and postpartum (PP).

FIGURE 2. Computational model of Raman spectra during and after pregnancy

Computational model of Raman spectra changing throughout pregnancy and postpartum (PP). Spectral bands that exhibited significant changes (shaded regions) throughout pregnancy: * $P < .05$ over course of pregnancy, # $P < .05$ when comparing early pregnancy (<20 weeks) and PP, and ‡ $P < .05$ when comparing late pregnancy (>37 weeks) to PP measurements ($P < .05$). Spectral regions associated with specific molecular peaks are labeled.

a, actin; *ATP*, adenosine triphosphate; *b*, blood; *car*, carotenoids; *chol*, cholesterol; *col*, collagen type I; *g*, glucose; *PP*, postpartum; *w*, water.

FIGURE 3. In vivo peak ratios modeled over the course of pregnancy

In vivo peak ratios modeled over the course of pregnancy. * $P < .005$ over the course of pregnancy, # $P < .05$ when comparing early pregnancy (<20 weeks) and postpartum (PP), and ‡ $P < .05$ when comparing late pregnancy (>37 weeks) to PP measurements.

FIGURE 4. Nonnegative least squares model of in vivo Raman spectra

Nonnegative least squares (NNLS) biochemical model of in vivo Raman spectra. Raman spectra from pure biochemical components (**A**, actin; **B**, blood; **C**, collagen type I; **D**, cholesterol; **E**, glycogen; and **F**, water) and **G**, representative cervix tissue spectrum (blue) with its model fit (pink) and residual (green).

FIGURE 5. Biochemical contributions over the course of pregnancy and postpartum

Biochemical contributions over the course of pregnancy. Fitted component coefficient (for **A**, actin; **B**, blood; **C**, cholesterol; **D**, collagen type I; **E**, glycogen; and **F**, water) plotted against gestation week (mean and 95% confidence interval). * $P < .005$ over course of pregnancy, # $P < .05$ when comparing early pregnancy (<20 weeks) and postpartum (PP), and ‡ $P < .05$ when comparing late pregnancy (>37 weeks) to PP measurements.

FIGURE 6. Computational model comparing Raman spectral changes observed from patient factors

Computational model comparing Raman spectral changes in **A**, nulliparous ($n = 29$) vs parous ($n = 37$) patients. **B**, Patients with normal (model set to body mass index [BMI] = 25, $n = 51$) and high (model set to BMI = 40, $n = 15$) BMI. Gray bars highlight areas that underwent significant change over pregnancy or PP, and patient variable had significant effect on spectral region *over the course of pregnancy and #PP repair, respectively.

TABLE

Patient demographics and outcome

Variable	
Total patients, n	68
Obstetric history	
None, n	28
Pregnancies, n	40
Vaginal deliveries, n	30
Prepregnancy BMI ^a	
Mean ($\pm$ SEM)	29.3 (7.6)
Underweight/normal BMI <25, n	19
Overweight BMI 25–30, n	25
Obese BMI 30–35, n	8
Severely obese BMI 35–40, n	7
Morbidly obese BMI >40, n	8
Obstetric outcome ^b	
Delivered vaginally at term, n	32
Delivered vaginally preterm, n	4
Cesarean delivery at term, n	25
Cesarean delivery preterm, n	5

BMI, body mass index.

^aNot available for 1 patient;

^bNot available for 2 patients.