

Changes in rat cervical collagen during gestation and after antiprogesterone treatment as measured in vivo with light-induced autofluorescence

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OBJECTIVE: This study was conducted to evaluate use of light-induced autofluorescence for measuring changes in collagen of cervical connective tissue during gestation.

STUDY DESIGN: In a conventional laboratory setting light-induced autofluorescence from the cervix of Charles-Dawley timed pregnant rats was measured in vivo. Eight distinct groups at various times of gestation were examined. Measurements were also performed on rats at day 17 of gestation 24 hours after treatment with RU 38486.

RESULTS: The amount of light-induced autofluorescence decreases significantly as pregnancy approaches term (means of the arbitrary total counts per spectrum on day 19 vs day 21 = 2.8×10^5 vs 1.9×10^5 , $p < 0.01$) and reaches its lowest point when the pups are engaged in the cervix (day 19 vs day 22 = 2.8×10^5 vs 0.95×10^5 , $p < 0.001$) and during delivery (day 19 vs delivery = 2.8×10^5 vs 0.83×10^5 , $p < 0.001$). Preterm treatment with RU 38486 also caused a significant decrease in the native fluorescence compared with vehicle-injected controls (day 17 control vs day 17 RU 38486 = 2.8×10^5 vs 1.5×10^5 , $p < 0.001$).

CONCLUSION: Significant decreases in cervical collagen at parturition parallel the results of previous studies that used various indirect methods to analyze collagen content. These data support the use of light-induced autofluorescence for detecting and studying the changes in cervical and uterine connective tissue during gestation in vivo. (AM J OBSTET GYNECOL 1995;173:1550-6.)

Key words: Antiprogesterone, collagen, rat cervix, light-induced autofluorescence, pyridinoline, parturition, pregnancy

Cervical ripening and dilatation at parturition are the results of complex biochemical mechanisms.¹⁻⁴ These changes occur predominantly in the connective tissue, the major tissue constituent of the cervix. The mechani-

cal properties of the cervix are determined by collagen and proteoglycan macromolecules that comprise the extracellular matrix of the connective tissue.⁵ Immediately before labor collagen fibers of the cervix become less densely packed and collagen concentration decreases,^{3, 6, 7} in part because of increased collagenase activity.^{8, 9} In humans the collagen concentration decreases between 30% to 50% compared with nonpregnant controls,¹⁰ and a 20% to 40% reduction occurs in rats.¹¹ Clinical studies correlate a high concentration of cervical collagen with the slow progress of labor.⁶

Collagen produces a characteristic fluorescence spectrum when it is exposed to an excitation light. The native fluorophor in collagen is thought to be pyridinoline, a 3-hydroxypyridinium ring derived from three

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residues of hydroxylysine.¹² There is further evidence that pyridinoline is one of the major cross-links within the primary structure of collagen fibrils.¹³ Cross-linking provides the collagen fibers with high tensile strength and stability.¹⁴ Pyridinoline is considered a more permanent cross-link because it is nonreducible. Pyridinoline is reported to exist in human and rat uterine walls.¹⁵ The specific cross-linking molecule in the rat cervix has not been identified yet, although cervical collagen fibrils do contain structurally cross-linked collagen fibers.¹

Fluorescence spectroscopy is a widely used research tool in the biosciences. Fluorescence spectra reveal important details on the structure and dynamics of macromolecules and concentrations and locations of molecules at the microscopic level. Autofluorescence (or native fluorescence) from intrinsic fluorophors in tissues and cells is a valuable property to exploit when diagnosing certain diseases. Intrinsic fluorophors, functioning as part of unique but complicated biologic systems, change along with the status of cells and tissues. If appropriately used this technique is a minimally invasive yet highly reliable diagnostic tool. Recently, many studies have used light-induced autofluorescence to characterize the physiologic state of tissues and their abnormal conditions.¹⁶⁻²² Fluorescence spectroscopy is used to investigate various aspects of diseases in the human reproductive organs, including the nonpregnant cervix, ovary, and uterus.^{19, 22, 23} In this paper data are presented supporting use of light-induced autofluorescence spectroscopy to examine changes in cervical collagen concentrations of rats during gestation and labor. Changes in the light-induced fluorescence spectra were also observed in rats treated with RU 38486, an antiprogesterone agent that causes premature delivery.²⁴ The techniques used in this study may prove valuable in the application of rapid *in vivo* analysis of cervical collagen changes in humans during gestation and labor.

Material and methods

Optical apparatus. Fluorescence spectral measurements of rat cervixes were done with an optical setup using a xenon lamp as an excitation light source (150W-GS, Hamamatsu Photonics, Bridgewater, N.J.). A lens was placed in front of the lighthouse with a crown glass window to collect the light. The excitation wavelength was then obtained with a narrow band optical filter at a centering wavelength of 340 nm (Oriol, Stratford, Conn.). The 340 nm wavelength was used for excitation because shorter wavelengths are known to penetrate the tissue less. The crown glass window and the 340 nm narrow band filter shaped the excitation light spectrum as a narrow peak centered at 348 nm with band width of 5 nm. The filtered light was focused through a fused

silica lens ($f = 2.5$ cm, Esco Products, Oak Ridge, N.J.) at one end of an optical fiber (Ensign-Bickford Optics, Avon, Conn.) for delivery of the excitation light directly on the tissue surface. Detection of the fluorescence emission light was accomplished by collecting it with a second optical fiber. One end of the second optical fiber was mounted in the same plane 15 degrees from the excitation fiber and the other end located at the focal point of a BK7 glass lens. Collected signals were sent through a long-path color glass filter (0-51) (Esco Products) with a cutoff wavelength at 360 nm (1% transition at this wavelength). The signal was then focused by an achromatic lens (PAC052, Newport, Chino, Calif.) into a grating monochromator (1200 G/mm, Instruments SA, Metuchen, N.J.). Both fiber tips (total diameter 600 μ m) were positioned 1 mm from the surface of the cervix. The spectra signals were then directed to an optical multichannel analyzer (EG & E Princeton Applied Research, Bedford, Mass.). A complete spectrum measurement was obtained in seconds (100 msec accumulation \times 100 times).

Animals and *in vivo* measurement. Timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were kept in the animal care facility until used. All animals were given free access to food and water. Day 1 of pregnancy was designated as the day a sperm plug was observed. Normally the rats are delivered on the morning or early afternoon of day 22. The autofluorescence measurements on the cervix of rats were performed *in vivo*. Rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine (15 mg:2 mg). The abdominal cavity was opened and the optical fiber tips placed directly on the cervix. Multiple fluorescence spectral measurements (two to four) were performed on the serosal surface of the medial band located proximal (1 to 2 mm) to the vaginal-cervical junction. Some fluorescent measurements were also made on the external os of the cervix after the vagina was surgically opened. The rats were killed after the measurements were taken.

A total of 37 rats at various stages of gestation and at two distinct laboring states were used. The rats were separated into nine groups. The groups were first divided according to the days of pregnancy: 15, 17, 19, 20, and 21. At term, on day 22 of pregnancy, there were three groups: nondelivery (all measured in the morning), pup engaged in the cervical canal, and at delivery with two to three pups delivered. Also, a group of rats was injected with RU 38486 (subcutaneous, 10 mg per rat) in a castor oil-benzoyl benzoate vehicle at 16 days of pregnancy and measured 24 hours later. A control group injected with vehicle only was measured concurrently (day 17). The typical changes seen with anti-progestin treatment were clearly observed after dissec-

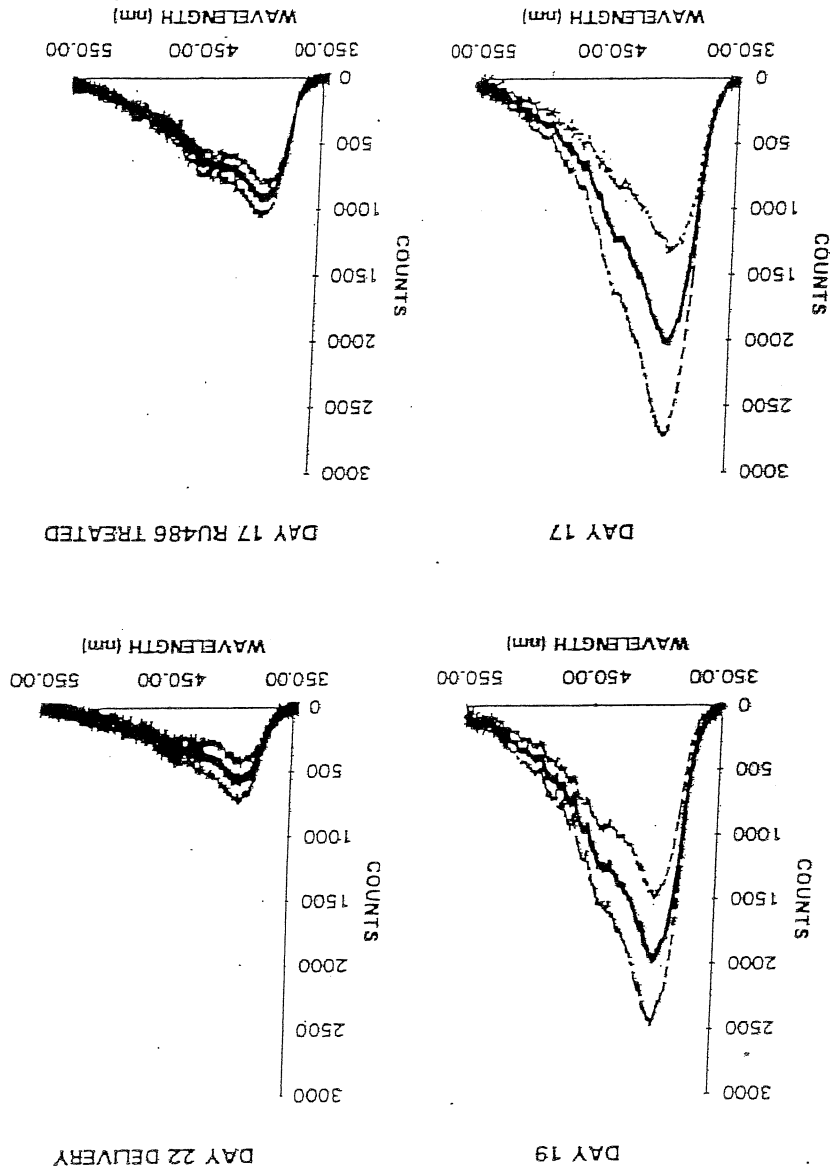


Fig. 1. Average uncorrected spectra and 95% confidence intervals as detected from serosal surface of the cervix of rats at different days of pregnancy (day 19 and day 22 delivery) and after RU 38486 treatment (day 17 control and day 17 RU 38486 treated).

shoulder around 440 nm. The average spectra and their 95% confidence margins measured on the external surface of the cervix on gestational day 19 and day 22 (delivery) are shown in Fig. 1. Similarly, the average spectra and the confidence margin from rats at day 17 and on day 17 after treatment with RU 38486 are also shown in Fig. 1. Spectral intensities and features observed vary because of inherent differences between rats and differences between measurement sites within the same rat. Variance may also arise because of slight changes in the optical setup, although measurements of a control dye were completed to attempt to normalize daily variations of the optical apparatus. The results indicate that the peak intensity at delivery (day 22) is

Statistical methods. The results are presented with means \pm SEM. The spectra are presented with 95% confidence intervals. Analysis of variance was used to indicate significant differences by group comparison. Differences between mean values of groups were statistically analyzed by the Fisher's least-significant difference test. A value of $p < 0.05$ was considered significant. Spectral profiles obtained from the cervix showed a major fluorescence peak at 395 nm with an overlapping

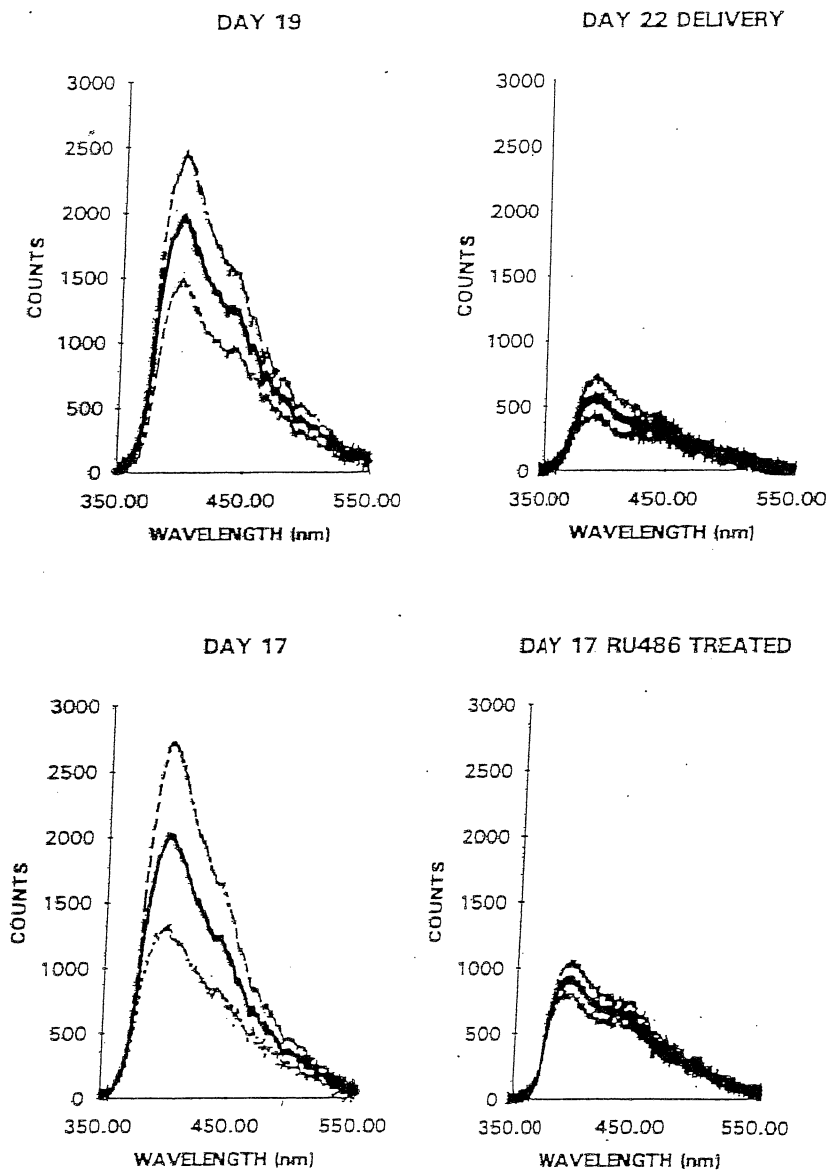


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tion (i.e., placental disruption, vaginal bleeding), but the pups were not yet engaged in the cervix and none had been delivered.

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Results

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Day of Gestation	d15	d17	d19	d20	d21	d22nd	d22eng	d22del	d17RU
d15 (n=4)					*	**	***	***	**
d17 (n=3)					*	**	***	***	***
d19 (n=4)					**	**	***	***	***
d20 (n=4)							***	***	*
d21 (n=6)							***	**	
d22nd (n=4)							*	*	
d22eng (n=4)									
d22del (n=3)									*
d17RU (n=5)									

Fig. 3. Results of statistical comparisons of spectral values from different groups at various times of pregnancy. Least-significant difference test was used to estimate differences between mean values. Asterisk, $p < 0.05$; two asterisks, $p < 0.01$; three asterisks, $p < 0.0001$. Abbreviations as in Fig. 2.

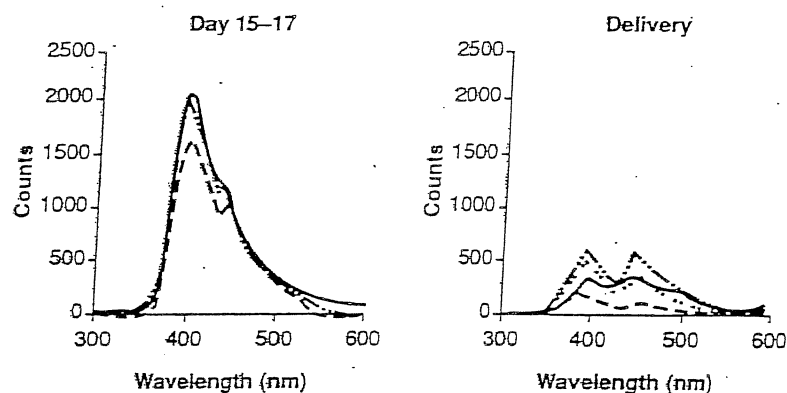


Fig. 4. Spectra obtained from rats at days 15 to 17 of pregnancy (left, $n = 3$) and during delivery (right, $n = 4$) obtained from external os of cervix. Traces of spectra from different rats are superimposed.

gestation are probably signals primarily from pyridinoline cross-linked collagen, although small amounts of elastin and reduced nicotinamide adenine dinucleotide may overlap the collagen spectrum.²⁵ Overlaps probably caused the shoulder at around 440 nm in some spectra collected. Generally, collagen fluorescence produces a relatively sharp fluorescence peak at 395 nm, which corresponds to the peak fluorescence for pure pyridinoline. Elastin has a wide fluorescence spectrum band peaking at 420 nm when the excitation wavelength is close to 350 nm. Free reduced nicotinamide adenine dinucleotide yields a fluorescence band with a peak at 460 nm that is blue-shifted to 450 nm when reduced nicotinamide adenine dinucleotide binds to proteins. Occasionally reabsorption of light by blood causes a small dip in the spectra at wavelengths between 415 to 420 nm, where the blood absorption spectra reaches maximum. However, we do not believe that blood contributes much to the decrease in the spectra as observed in this study, although there may be changes in vascularization of the cervix, because there was no decrease in fluorescence spectra at 415 to 420 nm as was found when the probe was deliberately placed on a blood vessel.

Studies examining cross-linking in uterine collagen

show that the formation of pyridinoline parallels that of collagen.^{27, 28} In fact, pyridinoline maintains an exact proportion to collagen of 0.28 mol/mol in the rat uterus from day 13 through post partum. A rapid increase of pyridinoline in the uterus is thought to provide the necessary mechanical strength to the tissue during labor. Tests of cervical biomechanical properties immediately after delivery demonstrate that the strength of the cervix decreases twelvefold compared with biopsy specimens from nonpregnant women.²⁹ The decline of pyridinoline cross-linked collagen observed in the cervix would favor cervical dilatation and an increased extensibility.

Antiprogesterone treatment during gestation increases cervical ripening by an unknown mechanism(s)³⁰ and initiates preterm birth.²⁴ In the guinea pig treatment with onapristone results in rearrangement of collagen fibers. These changes are similar to those in control animals just before term. Decreased collagen content is also observed in antiprogesterone-treated animals, indicative of increased collagenolysis.³¹ After antiprogesterone treatment the light-induced autofluorescence decreased significantly compared with controls but not quite so dramatic as that observed in the term delivered

rats. However, the RU 38486-treated rats demonstrated vaginal bleeding, but they were not yet delivered. In fact, the pyridinoline cross-linked collagen in the treated animals is most similar to the day 22 nondelivery animals. This provides further evidence that mechanical distention caused by engagement is an important step for complete cervical ripening and dilatation.

Cervical changes associated with dilatation and effacement of the cervix are monitored during pregnancy according to several systems, of which the Bishop score is the most common.³² This method is subjective and often does not adequately predict initiation of labor. Of the five parameters measured in the Bishop score, predication of labor is most highly correlated with the degree of cervical dilatation, followed by cervical consistency.³³ Methods to objectively measure changes in cervical consistency are not widely used.³⁴⁻³⁶ An instrument or procedure to measure cervical changes associated with labor would be invaluable for monitoring the progress of parturition. The described method may also be particularly helpful in predicting premature labor. The measurements could be extended also to include estimates of fetal membranes and to diagnose premature rupture of membranes. Furthermore, this tool might prove useful in diagnosing gynecologic problems associated with structure dysfunction, such as vaginal tissue failure, that is postulated to lead to urinary incontinence.³⁷

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