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Fluorescence Probe for Cervical Examination during Various Reproductive States

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ABSTRACT

These studies represent further investigations that have been done utilizing the fluorescence from pyridinoline, one of the major crosslinks of type I and III collagen, to evaluate cervical connective tissue changes during various female reproductive periods. Based on our previous studies, a prototype instrument has been constructed. The instrument was specifically designed for the purpose of vaginal examination of cervical connective tissue by measuring light induced fluorescence directly from the surface of the external os of the cervix. The studies were carried out on nonpregnant rats, rats during gestation at different periods, rats at different times during postpartum, and rats during preterm birth after being treated with antiprogesterone drugs. A study has also been done on humans during pregnancy and postpartum. The results parallel previous investigations that have used various invasive methods to analyze cervical extensibility, cervical collagen content and collagenase. In consideration of the important role of the collagen fibers and their turnover in the process of cervical function during pregnancy (softening or ripening at term), this method could be a useful tool for evaluating treatment strategies of the cervix. Moreover, the instrument could be serve as a device for the non-invasive estimation of cervical status in the clinic and the diagnosis of the changes in the cervix during the preparation for labor.

Keywords: collagen, pyridinoline, fluorescence spectroscopy, cervix, dilation, pregnancy, clinic examination

1. INTRODUCTION

Fluorescence spectroscopy has been widely used in bioscience research for nearly half a century. Recently, many study results have introduced the great possibility of using the fluorescence spectra from intrinsic fluorophors in the biological tissue to analyze the tissue's physiologic state. The great attraction is its possibility as a non-invasive medical procedure.

Labor involves regular uterine contractions of high amplitude in short duration and the dilation of the uterine cervix. The softening, dilation and effacement of the uterine cervix progresses as a result of a self-contained mechanism; not as a result of uterine contractions.[1] Abnormality of the uterine cervix during pregnancy is a major concern to obstetricians. Extensive ripening before term may result in abortion or preterm delivery; absence of spontaneous ripening may indicate a prolonged labor or post term pregnancy. At present, palpation is the commonly practiced method for clinical examination of the cervix. Diagnosis of labor remains a challenge to obstetricians.

It is known that collagen degradation plays an important role in the softening and ripening process of the uterine cervix during pregnancy and labor.[2,3] Collagen takes approximately 70% of the cervical tissue[4], nearly 62-80% of which is type I collagen (with the other 20% type III collagen).[5,6] Collagen degradation reduces the cervical toughness and increases its extensibility.[7] Although the mechanisms of spontaneous cervical ripening are still unknown, there is a great deal of evidence suggesting that cervical collagen changes during pregnancy and labor. Changes such as 1) increase of the interior space between collagen fibers, 2)shortening of the length of collagen fibers, and 3)increase of the collagen acidic solubility, progress as the pregnancy approaches term.[8,9,10]

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We first introduced the method of using the light induced autofluorescence to measure the cervical tissue changes during gestation and labor.[11] Collagen gives characterized fluorescence whose maximum is around 390nm (figure. 1). The intrinsic fluorophor is believed to be pyridinoline, which is considered one of the major crosslinks within the primary structure of collagen fibrils.[12] Our previous investigations were done by measuring the serosal surface of the medium bond of the cervix of rats *in vivo*. The results showed a correlated fluorescence intensity decrease in the later gestational days and at parturition. It also showed the measured collagen fluorescence intensity drop from the rats who were treated with antiprogesterone compound RU 38.486.

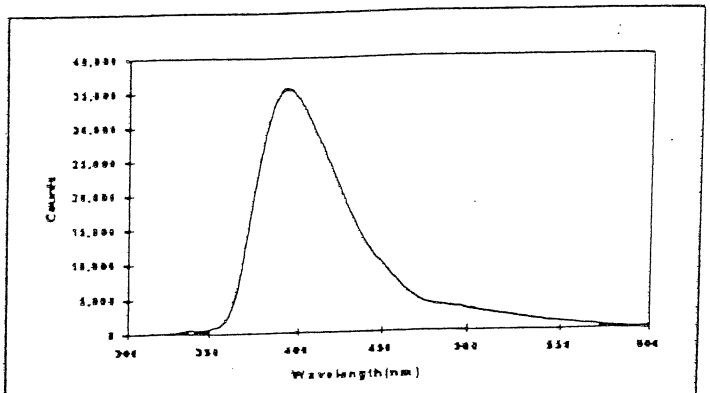

Figure 1. The fluorescence spectrum from the collagen type I (from Sigma).

This study investigated the possibilities of measuring the collagen fluorescence alteration through the surface of the external os of the cervix. With an improved instrument setting and a scope style probe which contains optical fibers, we were able to measure the signal from the surface of the external os by approaching it through the vagina. The study was performed on nonpregnant rats, middle to later pregnant rats, rats in parturition, rats during postpartum and rats during preterm birth after treated with antiprogesterone drugs. A study has also been conducted using human subjects during pregnancy and postpartum. The results further demonstrate the potential of this method as a convenient, non-invasive tool for evaluation of states of the cervix during the reproductive period.

2. MATERIAL AND METHODS

2.1 Optical Instrument

This first prototype unit was assembled by Instrument S.A., Inc. under the specifications given by investigators, based upon previous studies. The instrument (Figure 2) includes four main portions. The first portion includes a non-ozone xenon lamp (250 W) and a selective filter system which filters out the UV light with wavelength centered at 339nm±3nm. The second portion includes a monochromater and CCD spectroscopic analysis system. They both are placed in an enclosed unit which links to the third portion for data acquisition and a notebook computer as a monitor and controller. The fourth portion includes a collascope probe with an optical fiber cable which connects to the main unit (which includes the first and second portions). It delivers the

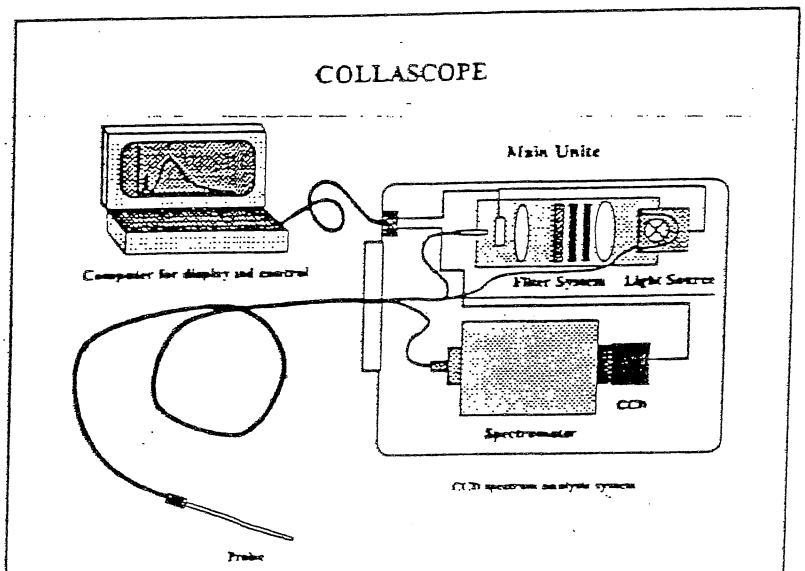

Figure 2. Diagram of the instruments for cervical measurement.

selected excitation light (339nm) from the first portion to the probe and also carries the fluorescence signal from the measurement site back to the second portion for data recording and analysis. An additional optical fiber has also linked the light source, probe and CCD detector in order to obtain a reference signal which represents the intensity of the excitation light.

The Collascope probe, which is in contact with the measuring site, is formed with two parts: fiber-optical probe and sheath. The fiber optical probe is a stainless steel rod with optical fiber bundle in the center. The sheath, used for isolating the optical fiber probe from the measuring site, is hollow stainless steel with a fixed sapphire window on one end. In the application, the optical fiber probe is inserted into the sheath, and fixed with a thumb screw. The probe is then inserted into the vagina with the sapphire window in contact with the surface of the external os of the cervix for measurement.

Once the probe is in position, the operation of the data collection process is operated by the notebook computer terminal with a programmed process. The used maximum accumulation measurements is 2 seconds for rats and 8 seconds for humans. In the presented spectra, all the data are normalized on the peak of the reference light. Ratio between fluorescence signal from cervix to reference signal is calculated.

2.2 Rats in Normal Pregnancy and Measurement

Rats are the commonly used model for the study of the reproductive science. Sprague-Dawley rats (timed-pregnancy, first pregnancy) were purchased from Charles River Laboratories (Wilmington, Mass.) and kept in the UTMB animal care facility. All animals were given free access to food and water. Day 1 of gestation is defined as the day a sperm plug is observed. Normally the rats start to deliver on early afternoon of day 22 of gestation.

The rats were anesthetized before the measurement with an intraperitioneal injection of ketamine hydrochloride mixed with xylazine. The fluorescence spectra were obtained from the external os of the cervix. The measurements were performed *in vivo* by using a nasal speculum to help open the rat vagina, cleaning away the discharges with a cotton application Q tip, then inserting the probe in to reach the surface of the external os. After the measurement, the rats were put back into the cage to recover from the anesthetics and to await further scheduled measurements. The rats were sacrificed at the end of the experiment with CO_2 inhalation.

The rats were measured at their day 13, 14, 15, 17, 20 gestation. Day 22 non-delivery data were measured in the early morning of day 22 gestation, before delivery. The day 22 delivery data were obtained in the afternoon, after rats delivered (two to five) pups. The rats with postpartum days 1, 2, 3, 5, 10, 12, 17. Each group had a minimum of 5 rats. A group of six non-pregnant rats were also measured, as a control group.

The ratio R between the peak value of the fluorescence (in the range of 370nm to 430nm) and the peak value of the reference peak (in range 330nm to 345nm) were calculated after each measurement.

2.3 Preterm Labor Rats treated with Antiprogesterone

The same kind of Sprague-Dawley Rats (from Harlan Sprague Dawley Inc., Houston, TX) were treated with 10mg per rat of the onapristone (ZK98 299), an antiprogesterone agent, at their day seventeen of gestation. Measurements of the cervical fluorescence were conducted 12, 16, 20, and 24 hours after the administration of the onapristone. Each individual group had 4 rats. A same set of control rats were again measured for comparison. The measurement technique was the same as described in section 2.2. The preterm parturition of the pups occured between eighteen and twenty hours after the zk98.299 administration.

2.4 Human Subjects

Pregnant and postpartum volunteer human subjects were recruited for the study. The measurements were similar, in nature, to a pap smear examination. The major difference was the use of an optical scope as part of the examination and results were obtained within a minute. The first step of the human study

was to establish a longitudinal profile of the distribution according to the weeks of the pregnancy and postpartum. The cervical external os were gently cleaned of residuals with rayon-tipped proctoscopic swabs before the measurement. The measuring sites were selected at 12 clock position and away from any observable lesion site. The presented data includes a patient population representative of various conditions (The population includes patients from primipara to multipara with several children, patients who had previously miscarried, patients from perfect pregnancy to pregnancy with conditions, postpartum patients who were and were not breast feeding, and some nonpregnant patients were also using birth control).

3. EXPERIMENTAL RESULTS

3.1 Rats in normal pregnancy and postpartum

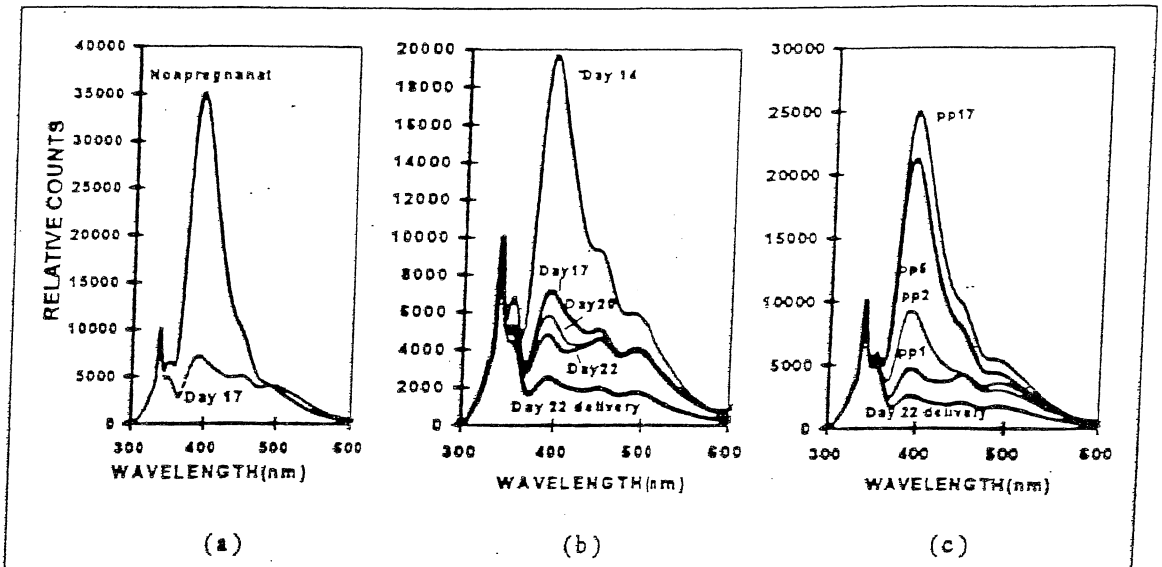

Figure 3. Averaged fluorescence spectra from rat cervical external os *in vivo* (a) nonpregnant rats and day 17 gestational rats, (b) rats during pregnancy, (c) rats during postpartum.

Figure 3 shows the averaged fluorescence spectra profiles from external os of the rats which are a) non-pregnant and day 17 gestation, b) during gestation and in delivery, and also c) in the postpartum period. In the spectra, the sharp peak with center around 339nm is the reference peak of the excitation light. The peak centered around 393nm is recognized as the fluorescence from collagen, or more exactly, from pyridinoline which is a main crosslink molecular in the collagen.[12]

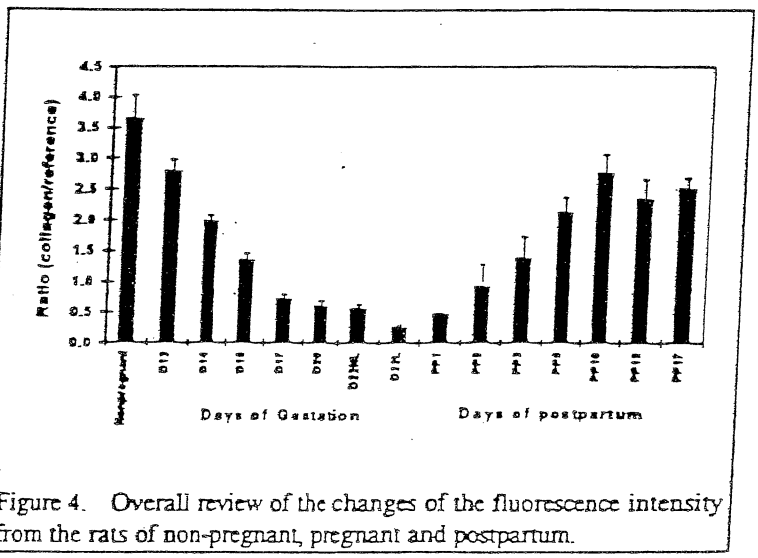

Figure 4. Overall review of the changes of the fluorescence intensity from the rats of non-pregnant, pregnant and postpartum.

The small lump occuring at around 460nm is more likely from NADH in the cells. The lift of the spectra at the tail around 495nm is more likely due to the sensitivity lift of the CCD detector. All of the presented spectra were normalized at excitation light reference peak (peak at 339nm) making peak counts to 10,000. Figure 4 shows all the obtained average ratio R and their SEM from non-pregnant rats, through out the pregnancy to postpartum day seventeen. The variances might be caused by differences between rats. They might also be caused by how well the probe was engaged with rat cervical external os, or in another words the position of the probe.

The obtained results shows remarkable decreases in the collagen (pyridinoline) fluorescence intensity when one compares the day 17 gestation rats to non-pregnant rat (Figure 3(a)). The R value changes from 3.7±0.34 to 0.72±0.08.

During the pregnancy, there is a gradual decrease of the collagen fluorescence intensity as the gestation days increase. At middle of the pregnancy, the R value decreases relatively quickly from the ratio value R of 2.8±0.12 at day 13 gestation, to 0.72±0.08 on day 17 gestation. The R value decreased relative slowly from day 17 to day 22 non-delivery period At the time of the delivery, the R value is dropped to 0.26±0.04, comparing to the R value (0.57±0.07) of the day 22 non-delivery rats (Figure 3(b)).

During the postpartum period, the collagen fluorescence intensity gradually increased in the first a few days from day 22 delivery R value to 2.1±0.3 on postpartum day 5. It seems the R value then floats around between 2.2 to 2.7 from the postpartum day 5 on to postpartum day 17. It seems that the R value can never recover to the value of non-pregnant rats (figure 3(c)).

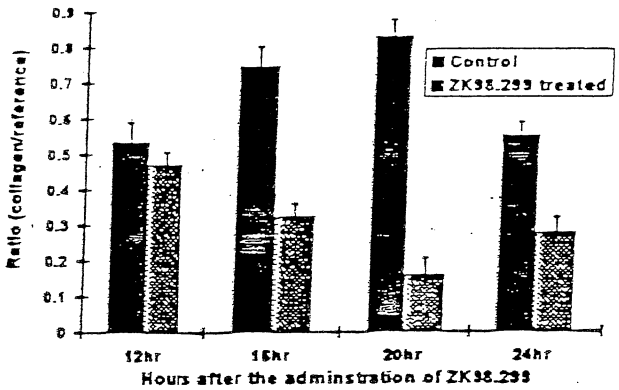

Figure 5. Fluorescence intensity changes from the rats treated with Onapristone in comparison with the controlled rats.

3.2 Pregnant rats treated with antiprogesterone

The onapristone treated rats (10mg/rat) were giving preterm delivery at 18 to 20 hours after the treatment. Figure 5 shows the results from the cervical fluorescence measurement of the treated rats in comparison with the control rats. It clearly shows a gradual decrease in the fluorescence intensity of the onapristone treated rat while they were approaching the preterm delivery.

3.3 Human subject in pregnancy and postpartum

Human subject data are presented in figure 6. The R value is calculated as the tissue fluorescence counts at 393nm divided by the counts at 339nm where the peak of the reference for excitation light is. So far, a total of twenty-four patients (including 8 non-pregnant patients) have been involved with the study. Several of the

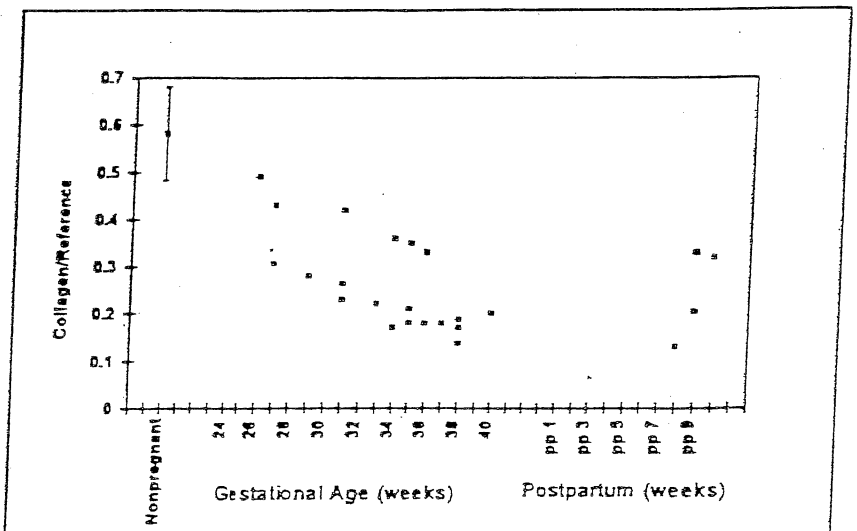

Figure 6. Human cervical fluorescence intensity changes in vivo during pregnancy and postpartum.

subjects have been measured two to three times during their pregnancy and postpartum period. There have then been a total of thirty two measurements have. The results show a gradual decrease of the fluorescence as the pregnancy approaches term, and recovery during the postpartum period. Two early dilated patients have shown lower R value at week of 31, 33 and 34 of gestation. More patients are needed for us to statistically analyze the differences between various patients' conditions.

4. DISCUSSION

Stiffness and tensile strength of the collagen are highly dependent on the cross-link bonds between collagen fibers and other matrix proteins.[13] Pyridinoline, a mature collagen crosslink, is known to exist in uterus[14] and may very well exist in the cervix[15]. Decrease or deformation of collagen crosslink could contribute to rearrangement, loosening and shorenting of collagen fibers. The concentration decreases of pyridinoline will directly effect its measured fluorescence intensity.

It is possible, as shown in the results of these studies, to measure the collagen fluorescence and its alternation along with the gestation from the surface of the external os of the rat cervix. The results suggest that there is a small amount of change in the collagen during early pregnancy (non-pregnancy to day 13), and then quick restructuring during day 13 to day 17, and then a slowing of the process during the later stages of pregnancy. There is then a quick drop from day 22 non-delivery to day 22 delivery time, indicating a further collagen structure change for the success of the parturition. These two changes may correlate with the two phases of the cervix change – softening and dilatation. This is agree with the study done with light and electron microscope [13] which showed the fibrous collagen fibers were well organized and densely packed in the cervix of non-pregnant rats and early pregnant rats (less then 10 days gestation). In the softening cervix (18-21) the structure of the collagen fibers changed to more fragmented fibrous networks with increased intrafibrous spaces (loosly packed). At term, the ripened cervix was filled with collagen microfibers, with much fewer of the long fibers. The softening provides a certain readiness for cervical dilatation.

Furthermore, the fluorescence measurement displayed a gradually increasing signal level during postpartum, which represents the recovery of the cervical collagen structure. It is interesting to see that the level of the signal does not return to the level of non-pregnancy time. This may indicate that there is some kind of permanent change after first time parturition.

The pattern of the fluorescence decreases measured from the surface of cervical external os is slightly different than the measurement from the serosal surface of the medial band.[11] This might be attributed to the tissue structural difference and time difference on the physiological change throughout the cervical tissue, as suggested by P.C. Leppet.[2] It is clear that the collagen degradation can be observed throughout the cervix, but may be different in time.

There is not yet a clear picture of how human cervix change progresses during pregnancy and postpartum. This is because *in vitro* biopsy study is the only method available in current research, and this compunded by difficulties in obtaining the *in vitro* samples for such studies. Some human studies have offered results suggesting that prolonged labor is related to the higher collagen content in the cervix; and that cervical collagen fiber breaks down after patients were treated with the antiprogestrone, RU486. The non-invasiveness of this method provides a good tool for the study of changes in the human cervix. Our initial human data has shown that samilar spectra and changes exist in the human cervix *in vivo*. A great deal more human research is needed for establishing a good understanding of the way cervical change progresses during human pregnancy.

The significance of this method is its non-invasiveness, easiness, and instantaneousness. It will be a more practical method for examinig the maturity of the cervix during the gestation and parturition. In comparison to the current clinic palpation practice, it is certainly a more regulated method, which overcome the probelm of differences between individual examiners. The information is related the microstructure molecular, and likely directly related to the collagen crosslink -pyridinoline. It could be used for the prediction of the pre-term labor clinical settings. It is also useful to monitor the progress of

induced labor, help diagnose the reasons for prolonged labor, and to monitor the recovery progress during the postpartum period.

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