QUANTITATIVE ULTRASOUND ASSESSMENT OF THE RAT CERVIX

BY

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THESIS

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LIST OF ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care-International
B-mode	Brightness-mode
BRL	Bioacoustics Research Laboratory
CDC	Centers for Disease Control and Prevention
GAG	Glycosaminoglycans
IL	Interleukin
MMP	Metalloproteinase
NHANES	National Health and Nutrition Examination Survey
NO	Nitric Oxide
PVDF-TrFE	Polyvinylidine Fluoride- Trifluoroethylene
RF	Radio Frequency
ROI	Region of Interest
RR	Relative Risk
TIMP	Tissue Inhibitor of Matrix Protease
TNFα	Tumor Necrosis Factor α
UIC	University of Illinois at Chicago
UIUC	University of Illinois at Urbana-Champaign

SUMMARY

Preterm birth is a significant public health problem in the United States. Presently there is not an instrument available to determine cervical ripening, which precedes preterm labor and birth.

A new ultrasound technology, quantitative ultrasound, which examined the microstructure of tissues, was used to determine cervical ripening in one group of nonpregnant rats and five groups of pregnant rats. There were 13 rats in each group. The results indicated that the ultrasound variables of acoustic concentration of the scatterers, scatterer diameter, and scatterer strength factor were significantly different as pregnancy progressed and were correlated to hydroxyproline in the cervix. The hydroxyproline concentration in the cervix decreased as pregnancy progressed and the hydroxyproline content increased. Quantitative ultrasound was significantly associated with the changes in hydroxyproline concentration and content. Water content of the cervix samples was not significantly different between the groups. Discriminant analysis was used to build a model to predict gestational age from the ultrasound variables.

The results of this study confirmed the following hypotheses of the conceptual framework of this study: (a) the hydroxyproline content (μg hydroxyproline/mg cervix) of the cervix increased as the pregnancy advanced in the rat; (b) the hydroxyproline concentration (% hydroxyproline) decreased as pregnancy advanced; (c) ultrasound acoustic concentration of the scatterers decreased as the pregnancy advanced; and (d) hydroxyproline had significant correlations with rat groups and with ultrasound variables of scatterer diameter, acoustic concentration, and scatterer strength factor.

Compositional analysis of the cervix tissues for water content did not detect a difference between the rat groups.

I. INTRODUCTION

> The Angel that presided oer my birth
> Said Little creature, formd of Joy and Mirth,
> Go love without the help of any King on earth.
> (Blake, 1982, p. 502)

The birth of a healthy baby is the expectation of every mother and family as they anticipate the birth of their new family member. It is the beginning of a new future and a potential of a new human being. As Blake noted in his poem, it has been an expectation that has been anticipated by families throughout history (Blake, 1982). It is not only the expectation of the mother and family, but the birth of a healthy baby at term is also the goal of midwives and obstetric health providers when providing prenatal care (Cunningham, Leveno, MacDonald, Gant, & Gilstrap, 1993). The importance of starting life healthy not only has value for individuals and families but also for societies. Societies need productive members who can contribute to the growth and well-being of the society.

A. Background

1. Incidence of prematurity.

Raising a healthy full-term newborn baby was not the experience of the parents of 480,849 preterm infants (12.1% of all births) born in 2002 (MacDorman, Martin, Mathews, Hoyert, & Ventura, 2005). The incidence of preterm births has actually risen since 1992. In 1992, the preterm birth rate was 10.7% and in 2002 it was 12.1% (J. A. Martin et al., 2003). The incidence of preterm births in Illinois in 1992 was 11.8% and in 2002, 12.6% (J. A. Martin et al., 2003). There remains a racial disparity in the prematurity rates in the United States, with African-American women experiencing a preterm birth rate of 17.3%, White women, 11%, and Latina women, 11.6% (Hamilton,

Martin, & Sutton, 2003). In 2002, 19.2% of African-American pregnant women in the city of Chicago delivered a preterm infant (J. A. Martin et al., 2003).

2. Morbidity and mortality

The morbidity and mortality associated with prematurity is characterized both in the gestational age and the weight of the infant at birth (MacDorman et al., 2005). Preterm birth is defined as newborn infants born less than 37 weeks gestation and very preterm birth as those newborn infants born less than 28 weeks gestation (MacDorman et al., 2005). Infant mortality is the death of a live born infant in the first year of life (Arias, Anderson, Kung, Murphy, & Kochanek, 2004). Figure 1 displays the relative risk of death by gestational age at birth (Kramer et al., 2000). Relative risk is the ratio of the risk of disease in exposed individuals versus the risk of disease in non-exposed individuals (RR = $a/(a+b)/(c/(c+d))$. Infants born before 28 weeks gestation are at extreme risk of death. Preterm infants born after 32 weeks gestation have a 97% incidence of survival (Larroque et al., 2004).

Low birth weight infants are infants who are born less than 2500g at birth. Very low birth weight infants are those infants born less than 1500g at birth. Figure 2 displays the contribution of birth weight of infant mortality in the United States from 1983 to 2001. Infants weighing over 2500 g at birth contributed the least to infant mortality and infants weighing less than 1500 g. There has been relatively little change in all of the infant mortality rates since 1990.

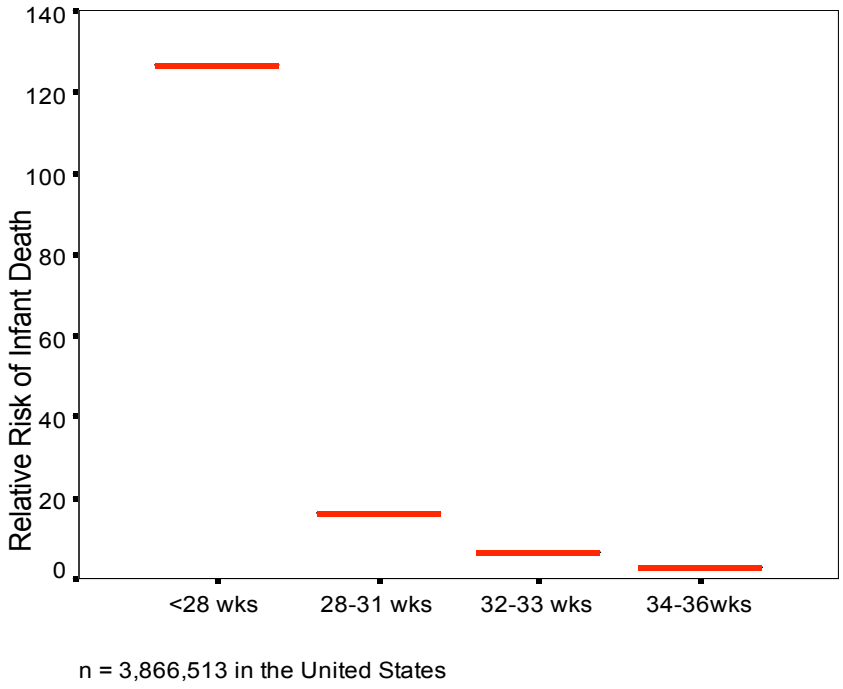

Figure1. Contribution of preterm birth to infant mortality (Kramer et al., 2000).

3. Cost of prematurity

The cost of prematurity to society in dollars and to families in human suffering is enormous. Preterm birth is the most common cause of hospitalization of pregnant women and the second leading cause of infant mortality (Beato, 2003; MacDorman et al., 2005). The leading cause of infant mortality is congenital malformations (R. N. Anderson & Smith, 2005). It is estimated that a 250 g increase in newborn birth weight would reduce medical expenses in the first year of life by $12,000–16,000 and a birth weight of at least 2500 g would save $60,000 (Beato, 2003). The cost of the initial neonatal care for preterm infants is estimated to cost $6

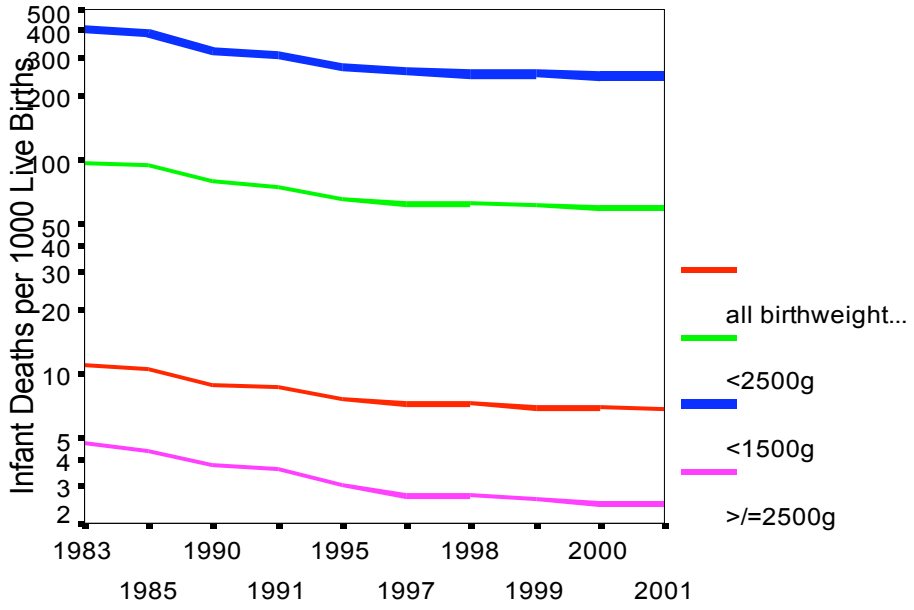

Figure 2. The contribution of birth weight to infant mortality (Mathews, Menacker, & MacDorman, 2003).

billion annually (St. John, Nelson, Cliver, Bishnoi, & Goldenberg, 2000). Almost 20% of preterm infants do not survive the first year of life (Larroque et al., 2004).

4. <u>Neurological impairment</u>

More than 90% of all neonatal deaths occur from infants born prematurely and those that do survive are more likely to be neurologically impaired than infants born at term (Mathews, Menacker, & MacDorman, 2002). One fifth to one quarter of all premature infant survivors suffer at least one major disability, such as impaired mental development, cerebral palsy, blindness, or deafness (Lorenz, 2001). Surviving preterm

infants have a 40 times relative risk of developing cerebral palsy compared to a full-term infant (Hack & Faranoff, 1999). Additionally, infant survivors born less than 28 weeks gestation or less than 1000 g continue to display cognitive, behavioral, and educational impairments in childhood (P. Anderson, Doyle, & Victorian Infant Collaborative Study Group, 2003).

5. Impact of multiple births on preterm births

There has been concern that the increase in the number of multiple births has contributed to the preterm birth rate and infant mortality rate. In 2002, the twin birth rate was 31.1 per 1000 live births, which decreased from 38% since 1990 (22/1000 live births) and 65% since 1980 (J. A. Martin et al., 2003). The incidence of triplet and higher order multiples soared from 37/100,000 live births in 1980 to 193.5/100,000 live births in 1998 to the due to the increased usage of assistive reproductive technologies (J. A. Martin et al., 2003). The higher order multiple birth rate decreased in 2001 to 184/100,000 live births (J. A. Martin et al., 2003). The incidence of multiple pregnancies has had an impact upon the preterm birth rate and the infant mortality rates. Table I contrasts the differences in morbidity and mortality between singleton newborns and multiples (MacDorman et al., 2005; J. A. Martin et al., 2003). Infant mortality is defined as the number of infant deaths per 1000 live births in the first year of life (Mathews et al., 2003). In a 1996–1997 study of 42,463 infants conceived by assisted reproductive technologies, 43% were singletons, 43% were twins, 12% were triplets, and 1% were quadruplets or higher order multiples (Schieve et al., 2002). The infants born in the study accounted for only 0.6% (0.2% would have been spontaneously expected) of all of the births to women over the age of 20 years old in the United States, but accounted

TABLE I

CONTRIBUTION OF PLURALITY TO BIRTH WEIGHT, PREMATURITY, AND INFANT MORTALITY[a]

	Singletons	Twins	Triplets/higher order multiples
Preterm (%)	10.4	58.2	93.5
Very preterm (%)	1.6	11.9	58.1
Gestational age at birth			
mean weeks (SD)	38.8 (2.5)	35.3 (3.7)	30.2 (4.17)
Percent low birth weight	6.1	55.4	95.8
Percent very low birth weight	1.1	10.2	59.8
Mean birth weight g, (SD)	3332 (573)	2347 (645)	1367 (620)
Infant deaths	6.1	30.2	67.5

[a] (MacDorman et al., 2005; J. A. Martin et al., 2003).

for 3.5% of the total low birth weight newborns and 4.4% of all very low birth weight newborns in the United States (Schieve et al., 2002). Of interest in their study was the fact that even the singletons had a 2.6 relative risk (95% CI: 2.4–2.7) of low birth weight as compared to the general population (Schieve et al., 2002), which suggests that there were factors associated with the diagnosis of infertility or the procedures with assisted reproductive technologies that may have placed the infants at risk for low birth weight (Schieve et al., 2002).

6. Healthy People 2010

The Centers for Disease Control and Prevention (CDC) cited the reduction of premature births and neonatal deaths as one of their priorities for Maternal and Infant Health in the Healthy People 2010 initiative (Centers for Disease Control and Prevention [CDC], 1999). The goal is to reduce the preterm birth rate to 7% for all live

births and to 9% for African-American women by 2010 (CDC 1999). These goals were set forth to focus on the current health status of a large segment of the American population and predictors of the health for the American population for the next generation (Beato, 2003). In assessing the health care needs of the nation, the initiative noted that maternal child health care disparities still exist with vulnerable populations of the poor and women of color (Beato, 2003). The Healthy People 2010 taskforce is developing strategies via research, public service programs, health care provider education programs, systems changes, and food fortification to make an impact on the improvement of maternal child health (Beato, 2003). Reducing the preterm birth rate is one of the initiatives of the taskforce. Guidelines are being developed to reduce the number of higher order multiple pregnancies in order to reduce the preterm birth rate. The CDC established a Pregnancy Risk Assessment Monitoring System that identifies maternal behaviors and experiences that may place women at risk in pregnancy for preterm birth (Beato, 2003). The Department of Health and Human Services has funded Healthy Start Centers in targeted vulnerable communities to provide support services and home visits to families in an effort to reduce factors leading to preterm birth (Beato, 2003).

B. Statement of the Problem

Normal labor and birth requires collaboration between the corpus of the uterus and the cervix via a complex series of mechanisms. The corpus of the uterus and the cervix represent two histologically and functionally different structures. Without a softened or ripe cervix, the rhythmic contractions of the corpus of the uterus will not allow the progression of labor. Correct timing of cervical ripening is essential to maintain

the pregnancy, as too early ripening can lead to premature birth (J. D. Iams, 2003). Thus, an understanding of how cervical softening occurs is a clinically significant and public health problem, in order to improve the ability to treat cervical ripening and ultimately to reduce the number of premature births.

The underlying causes of preterm labor leading to preterm birth are heterogeneous, making the assessment of the time of delivery difficult. Some investigators have postulated that it is a complex series of events that results in the delivery of the preterm infant (Romero et al., 1994). This may be why a simplistic approach of arresting contractions has been ineffective in delaying the birth of the preterm infant. The preterm birth rate has not changed since the introduction of medications to stop contractions (J. D. Iams, 1996). In fact, once there is advanced cervical change there may be little that can be done except to delay birth for 48 hours with tocolytics (medications to halt contractions) to administer steroids to stimulate fetal lung maturation (de Vaciana, Porto, Major, & Barke, 1995; J. D. Iams, 1996). The prolonged use of tocolytics has been questioned due to their ineffectiveness and the seriousness of the side effects to the mother and fetus (A. Anderson, 1981; Keelan, Coleman, & Mitchell, 1997; Salokorpi, Eronen, & von Wendt, 1996).

C. Factors Associated with Preterm Labor

1. Socioeconomic factors

Socioeconomic factors have been implicated in women with preterm labor. These include psychosocial stress leading to corticotropin releasing hormone increases, sexual behaviors leading to infections, substance abuse, low folate levels, and genetic polymorphisms. Genetic causal pathways may also influence how a woman or a fetus

may respond to environmental toxins or stress or develop uteroplacental lesions (X. Wang et al., 2001; Wildschut, Lumey, & Lunt, 1991).

2. Infection

Infection and inflammation with resulting cytokine release has been postulated as a factor involved in the onset of cervical ripening and preterm labor (Romero et al., 1994). Women with preterm labor have been found to have positive amniotic fluid cultures; however, the incidence in one study varied from 0% to 30% (Gibbs, Romero, Hillier, Eschenbach, & Sweet, 1992). Other markers indicating a biochemical mechanism for preterm labor in the setting of infection, including prostaglandins, tumor necrosis factor, and pro-inflammatory cytokines (interleukin-6 and interleukin-8), have been identified (Baumann et al., 1993; Gibbs et al., 1992; Rizzo, Capponi, Angelini, & Romanini, 1998). There is strong evidence for inflammation and infection as having a role in preterm labor (Romero et al., 1994; Romero et al., 1989). However, treating infection once labor has begun has had mixed results in preventing preterm birth. Both American and British randomized controlled trials of antibiotics have found that antimicrobial therapy did not prevent preterm birth in women admitted in preterm labor with intact membranes (Cox, Bohman, Sherman, & Leveno, 1996; Kenyon, Taylor, Tarnow-Mordi, & ORACLE Collaborative Group, 2001b). However, in women admitted with preterm rupture of the membranes, the ORACLE study from the United Kingdom found a prolongation of pregnancy and a decrease in neonatal morbidity with erythromycin and an increase in neonatal necrotizing enterocolitis with broad-spectrum antibiotics (Kenyon, Taylor, Tarnow-Mordi, & ORACLE Collaborative Group, 2001a; Kenyon et al., 2001b). The American randomized trial of antibiotics given

to women with preterm rupture of the membranes found significantly less infection in the antibiotic group and less neonatal morbidity (Mercer et al., 1997). Other infections such as syphilis have resulted in prematurity and fetal death (McFarlin & Bottoms, 1996; McFarlin, Bottoms, Dock, & Isada, 1994).

The efficacy of treating other vaginal infections and cervical infections to prevent preterm labor has been less clear. In a large multicenter randomized-controlled trial in Finland, treating bacterial vaginosis between 10 and 17 weeks with vaginal clindamycin was not effective in decreasing the incidence of preterm birth. However, women who remained free of bacterial vaginosis had a significantly lower incidence of preterm birth (Kekki et al., 2001).

3. Prevention programs

Programs to prevent preterm birth have had mixed results and differences in costs (Mozurkewich, Naglie, Krahn, & Hayashi, 2000). Fetal fibronectin testing is a test designed to predict preterm birth. Fetal fibronectin is a large molecular weight glycoprotein expressed in the cervical and vaginal secretions of women with disruption of the choriodecidual junction either by labor or inflammation. It has been found to have strong negative predictive value and to be reassuring to women who may be having preterm labor symptoms (Andersen, 2000). Fetal fibronectin testing has a strong negative predictive value of predicting delivery within 7–14 days and is strongest when used at 24 to 28 weeks gestation (Peaceman et al., 1995). The fetal fibronectin test has had a significant impact in reducing interventions in women who are having preterm labor symptoms and have a negative fetal fibronectin test.

4. Uterine contractions

Contractions have been the hallmark for diagnosing preterm labor (a retrospective diagnosis). However, the diagnosis of true preterm labor is difficult before significant cervical effacement and dilatation of the cervix occurs, and as many as 50% of women in apparent preterm labor will continue their pregnancies (A. Anderson, 1981; Rinehart et al., 2001). Investigators have noted that the contraction patterns in women leading to preterm labor are different than term labor (Vercoustre, 1997). Clinical experience and pilot research with women in apparent preterm labor supports the difficulty of the diagnosis of the signs and symptoms of preterm labor when evaluating with contractions alone. There have been reports in the literature of the lack of fetal breathing movements being predictive of preterm delivery (Castle & Turnbull, 1983). A study of all of the ultrasound variables in the biophysical profile score (fetal tone, fetal breathing, fetal movement, and amniotic fluid volume) evaluated if it were possible to improve the prediction of preterm delivery (McFarlin, Baumann, Sampson, Kruger, & Cotton, 1994; McFarlin, Brancazio, Baumann, & Kruger, 1999). When women were having significant contractions and cervical dilatation and the fetus was active, then women were unlikely to deliver within 48 hours. Conversely, if the fetus was quiet on ultrasound (lack of movement and poor tone), almost all of the fetuses delivered within 48 hours (McFarlin, Baumann et al., 1994; McFarlin et al., 1999) .

5. Cervical ripening

For many women, the changes that lead to cervical ripening occur without any noticeable contractions. Ultrasound has also been used extensively for cervical length measurements in an effort to predict preterm labor. A shortened transvaginal

cervical length measurement at 22–24 weeks of gestation is associated with preterm birth (Iams et al., 1998; Iams et al., 1995; Johnston, Williams, Hogue, & Mattison, 2001). Although short cervical length measurements have been found to be associated with preterm birth, the sensitivity and specificity demonstrate that the magnitude of the effect may make it of limited use as a screening test in the second trimester in a low risk population (McFarlin, Brancazio, & Dorinzi, 2000b, 2000c; Owen, Yost, Berghella, et al., 2001). Women with a shortened cervix in the second trimester of pregnancy have a significantly higher probability of delivering prematurely. However, not all women who deliver a premature infant have a shortened cervix in the second trimester of pregnancy. Testing the theory of preterm labor as a continuum, the cervix as well as the lower uterine segment was measured transvaginally in the second trimester of pregnancy and indicated no association with preterm birth (McFarlin, Brancazio, & Dorinzi, 2000a).

The initiation of labor entails a complex series of events, which occur in the maternal-fetal compartment and are still not entirely understood. Cervical ripening, which is the physiologic process of softening, distensibility, and effacement of the cervix, happens before labor begins (J. E. Norman, Thomson, & Greer, 1998). This process of cervical softening is related to increased water content in the cervix and a rearrangement in the collagen content, making it possible for the cervix to efface and dilate. The cervix consists of only 10–15% smooth muscle cell, which undergo cell death, playing a role in cervical softening (Leppert, 1995). The rest of the cervix is comprised of collagen elastin, extracellular matrix proteins, and glycosaminoglycans (Leppert, 1995). Disorganization and rearrangement of collagen fibrils is probably under the influence of enzymatic activity and decorin-collagen interaction (Leppert, Kokenyesi,

Klemenich, & Fisher, 2000). It is thought that the collagen fibrils become loosely disorganized, although maybe not destroyed by enzymes as the cervix repairs itself very rapidly postpartum. During this process of collagen disorganization, cervical softening and the water content of the cervix is greater. Quantifying the microstructure changes that are taking place before labor is what we would propose to detect with our new ultrasound technology.

D. Conceptual Framework

1. Physiology and morphology of cervical ripening

The physiological and morphological changes of the microstructure of the ripening of the cervix and ultrasound backscatter theory provide the theoretical framework for this research. The uterus has conceptually been viewed as one organ. However, functionally and histologically the cervix and the body of the uterus, the corpus, are different structures (Danforth, 1947). Without a ripe cervix, uterine contractions alone cannot accomplish the work of labor and birth (Danforth, 1947). During the last 40 years many diagnostic and treatment modalities have been developed for preterm contractions (A. Anderson, 1981; Castle & Turnbull, 1983; de Vaciana et al., 1995; Goepfert & Goldenberg, 1996; Kenyon et al., 2001a; Kiss, Ahner, Hohlagschwandtner, Leitich, & Husslein, 2000; Kramer et al., 2001; Mattison, Damus, Fiore, Petrini, & Alter, 2001; Romero et al., 1994). However, none of these interventions have reduced the preterm birth rate. Until recently, much of the focus of controlling preterm labor has been placed on controlling uterine contractions. Very little effort has been placed on controlling the cervical ripening process that precedes preterm labor and birth by weeks or months.

The cervix forms a rigid, connective tissue-rich sphincter at the distal end of the uterus. The corpus of the uterus is composed of primarily smooth muscle, whereas the cervix, a rigid structure, has less than 10% smooth muscle. There is more smooth muscle in the cervix at levels closer to the internal os than the external os. The cervix consists mainly of connective tissue of which collagen is the major component (Danforth, 1947). During the course of pregnancy, the cervix gradually softens as the collagenous stroma is remodeled. Although it is known that the process of cervical ripening is under the control of hormones, cytokines, and gestational age, it is unknown what actually triggers this process. The connective tissue stroma of the cervix has a cellular component and an extracellular matrix. The major cellular components of the cervix are fibroblasts and to a lesser extent smooth muscle cells (5–10%). Fibroblasts are cells of mesodermal origin that have the ability to give rise to other cells of mesodermal origin (fat, bone, cartilage, smooth muscle), collagen, elastin fibers, various glycoproteins and ground substance. Apoptosis, or programmed cell death, of smooth muscle cells in the cervix of rats is associated with cervical ripening by stimulating cytokines and enzymes that break down collagen (Leppert, 1995). Almost 50% of the dry weight of the cervix and 80% of the protein content consists of collagen (Leppert, 1995). Approximately 70% of the collagen in the cervix is Type I, 30% is Type II, and a very small amount in the basement membranes is Type IV (Thomson, Norman, & Greer, 1997). The cervical ripening model that guided this research (McFarlin, 2003) is displayed in Figure 3.

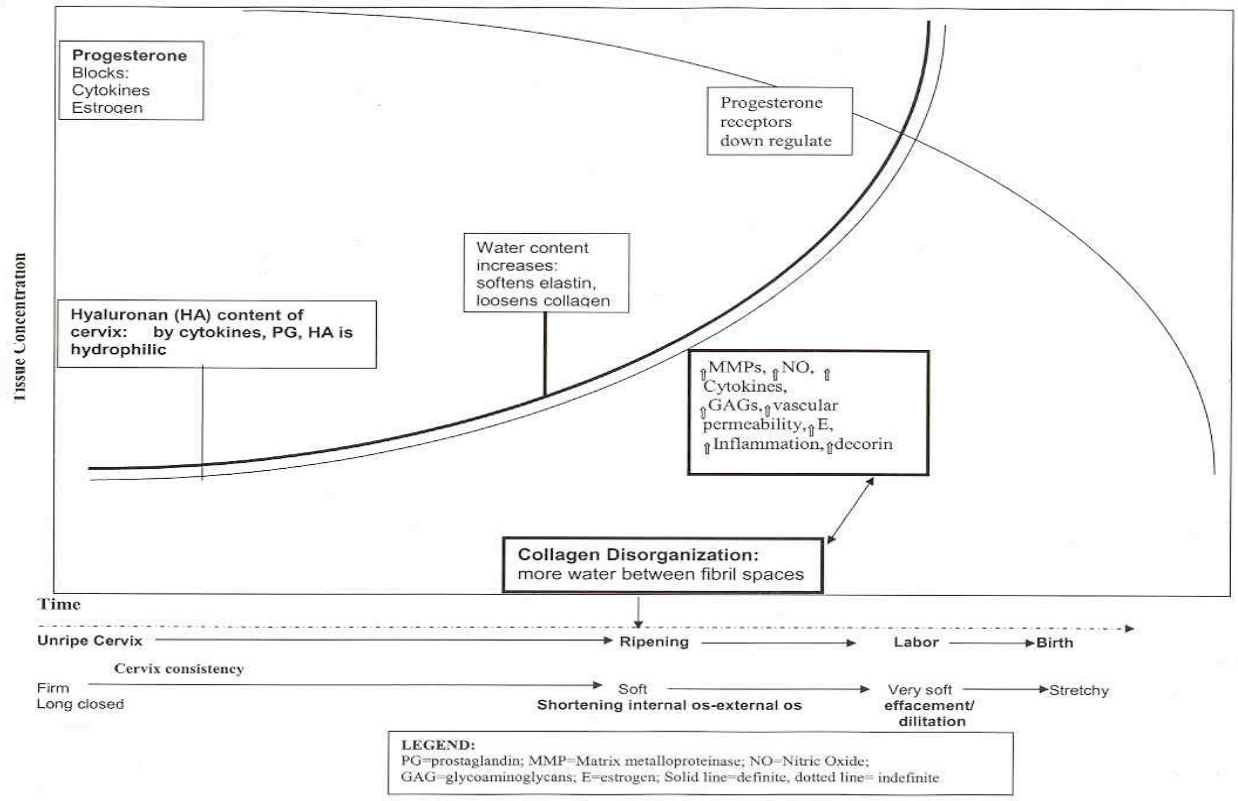

Figure 3. Conceptual model of cervical ripening that guided this research.

In the nonpregnant cervix, water content comprises about 75% of the extracellular matrix. Under the influence of hyaluronan, the water content increases to about 86% as the cervix ripens. The increase in water content is important for the cervix to soften, the collagen fibrils to become loosened, the elastin to soften, and the cytokines to disperse throughout the matrix of the cervix (Golichkowski, King, & Mascaro, 1980). Without hydration, elastin would not stretch and collagen fibers would remain firmly packed, making the cervix hard. Hyaluronan content in the cervix increases 17–fold by term in the rat cervix (Golichkowski et al., 1980) and has a high capacity to attract and bind water molecules.

Collagen content in the rat cervix increases during pregnancy, peaking just before labor. However its concentration actually decreases during pregnancy, probably due to the increased water content of the cervix (Golichkowski et al., 1980). Decorin, a proteoglycan (GAG), regulates the intrafibrillar distance of collagen by organizing and ordering the collagen fibrils, thus allowing collagen to become disorganized and loose during cervical ripening (Leppert et al., 2000). Collagen rapidly becomes organized after birth, remodeling the cervix again to a firm state. The role of inflammatory factors such as cytokines and leukocytes in cervical ripening is unclear. As their concentrations increase toward labor, they stimulate prostaglandin production and are thought to have a role in tissue remodeling (Young et al., 2002).

Progesterone has long been thought of as the hormone responsible for maintaining pregnancy. Progesterone catabolism late in pregnancy in mice is influenced by an enzyme 5 α-reductase type 1. Mice lacking this enzyme have a high level of progesterone in the cervix at term and lack cervical ripening (Mahendroo, Porter, Russell, & Word, 1999).

Exactly what triggers the changes associated with cervical ripening is unknown. The dotted line in the model (Figure 3) represents time or the gestational age of pregnancy. What is unknown is the influence of these interdependent factors on timing of ripening in pregnancy. It is known that these factors are involved in cervical ripening, but it is not clear which trigger(s) come first. For the purposes of this research this model focuses on the importance of the water content of the cervix in altering the cellular and extracellular matrix (Figure 3). It was theorized that the number and

concentration of acoustic scatterers in the cervix tissues as measured by ultrasound would be related to the amount of cervical ripening.

2. Backscatter theory

Standard B-mode (brightness-mode) ultrasound images of living tissues created with a clinical ultrasound system are processed from radio frequency (RF) echo signals. The RF echoes are created by reflections from interfaces between acoustically different regions and by incoherent scattering from tissue microstructures. The RF echoes contain frequency-dependent information about the smaller-scale structures (less than the wavelength of sound) in the tissues. Conventional B-mode ultrasound processing removes the frequency-dependent information. Clinical B-mode ultrasound images are good at displaying information about larger scale structures (those that are larger than the wavelength), but in order to resolve and quantify smaller scale structures in tissues, the frequency dependent information must be utilized.

Scattering occurs when a sound wave (acoustic wave) moves across tissues or fluid of different impedances (Oelze et al., 2002). Backscatter theory assumes that tissues can be modeled as complex fluids (Insana, 1995). The size of the scatterer is approximately equal to the acoustic wavelength and the size and shape of the scatterer reflect the frequency that the sound will be scattered (Oelze et al., 2002). The scatterer estimate can be made by comparing the sample backscattering spectrum from each region of interest (ROI) in the tissues being evaluated, to a theoretical backscatter power spectrum. The formula for the theoretical backscatter spectrum is as follows (Insana et al., 1991):

$$W_{theor}(f) = C(a_{eff},n_z)f^4F(f,a_{eff})$$

where *f* is the frequency in megahertz; C is a constant that depends on the average effective radius, $a_{eff,}$ of the scatterers in millimeters; n_z is the average concentration of acoustic scatterers in cubic millimeters; and *F* is the form factor of which the Gaussian form factor has been found to work the best in rat mammary tumors (Oelze et al., 2002). The Gaussian form factor assumes that the scatterers are spherical and randomly distributed (Oelze, Zachary, & O'Brien, 2002a). Compensation for tissue specific insertion loss of acoustic energy is made in the calculation of the backscatter equation. Insertion loss is the loss of acoustic energy that results when inserting the sample (in this case the cervix sample) into the sound beam. Figure 4 displays a representation of scattering of ultrasound in tissues.

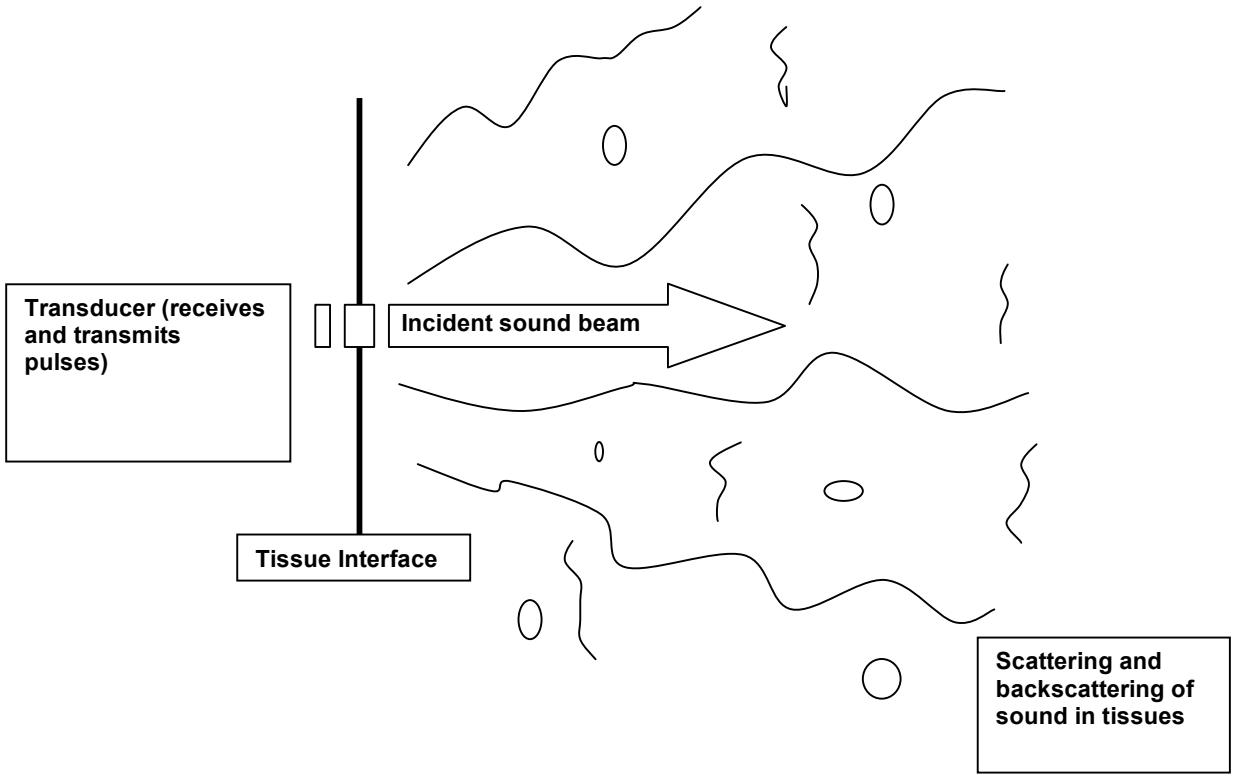

Figure 4. Model of the scattering of ultrasound (Shung & Thieme, 1993).

Typically three measurements are measured from the backscatter tissue volumes: scatterer diameter, acoustic concentration, and scatterer strength factor. Scatterer diameter is the correlational length of the scatterer measured in μm. Acoustic concentration is the number of scatterers/mm^3 × (average impedance of scatterers)^2. Impedance is the density of a media × the sound propagation speed (Kremkau, 1998). If impedances are the same in a media there will be no reflection of sound. Scatterer strength factor is acoustic concentration × (average radius of scatterers)^6.

Backscatter measurements are taken from a specific tissue volume ROI that consists of many parallel ultrasound A-lines, which are averaged. A backscatter power spectrum is then calculated for each ROI, which typically is 1 × 1 mm (Oelze & O'Brien, 2002). Each ROI can be converted into a color pixel that represents the average scatterer diameter. An example of a parametric image from a rat cervix is displayed in Figure 5. The scatterer diameter in this cervix sample ranged from 12–19 μm.

Studies by Pohlhammer and O'Brien (1981) found that the amount of scattering in biologic tissues can be mathematically equated to the amount of collagen in tissues (Pohlhammer & O'Brien, 1981). Based upon previous work done in this laboratory and by others, this technology has the potential to be very useful in quantifying cervical collagen.

Ultrasound backscatter technology has been commercially used successfully in ophthalmology with a device called ultrasound biomicroscopy. This device uses high frequency (50–100 MHz) ultrasound transducers that provide a depth in the human eye

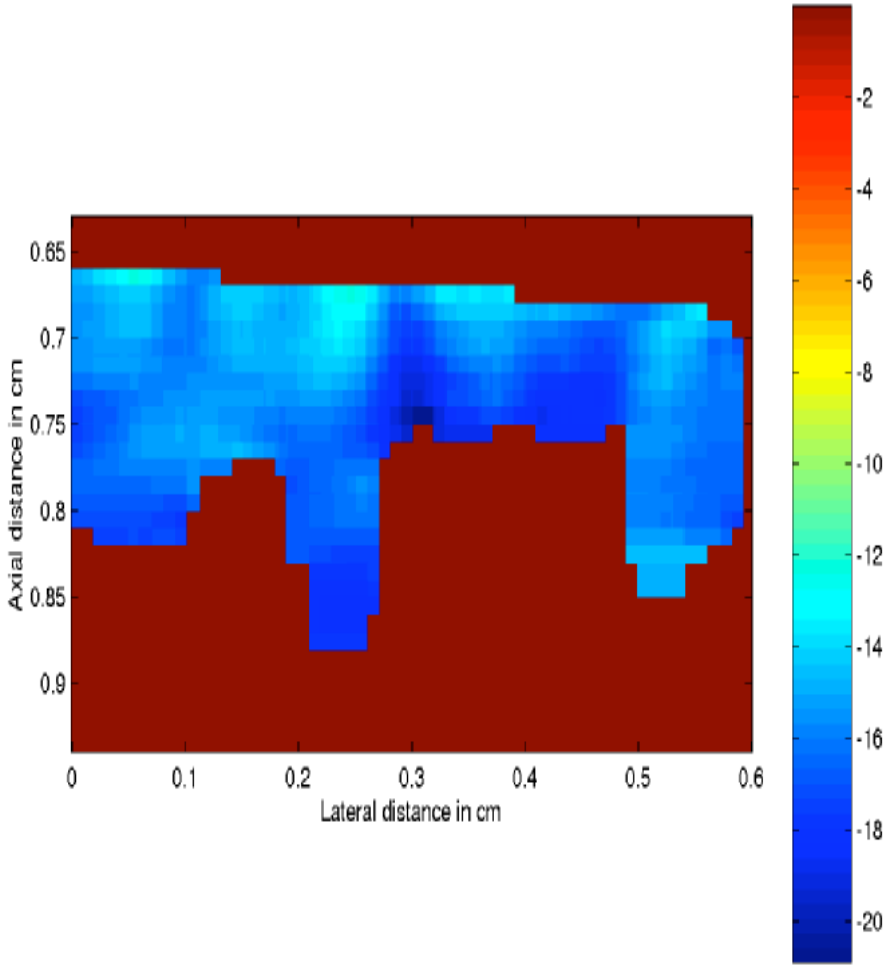

Figure 5. Quantitative ultrasound image of a day 17 pregnant cervix displaying scatterer mean regions of interest. The scale bar indicates that the regions of interest are in the range of 12–19 μm^3.

of 4 mm and an axial resolution of 20 microns (Pavlin, Harasiewicz, Sherar, & Foster, 1991). Typical clinical ultrasound imaging without backscatter technology has an axial resolution of 1 mm.

Ultrasound biomicroscopy has been used to evaluate progression of ocular melanomas (Maberly, Pavlin, McGowan, Foster, & Simpson, 1997) and for the diagnosis of retinopathy, which can lead to blindness in premature infants (Maberly et al., 1997). Ultrasound backscatter technology has also been used to evaluate myocardial function after a myocardial infarction (O'Brien, Sagar, Warltier, & Rhyne, 1995). The use of backscatter technology is attractive for *in vivo* use in parts of the body where biopsy would not be feasible. The cervix in pregnancy has the potential to be an ideal application for this technology. Currently, there is not a reliable method to detect cervical ripening during pregnancy until the process is so advanced that it is irreversible.

E. Research Questions

The purpose of this study was to test a new ultrasound methodology that is hypothesized to evaluate and quantify cervical ripening *ex vivo* in pregnant rats. The long-term goal will be to apply this technology in humans to predict preterm cervical ripening and birth. Presently there is no noninvasive instrument to diagnose cervical ripening until it is quite advanced. The specific aims of the proposed research were as follows:

1. To identify and quantify with enhanced B-mode ultrasound the microstructural changes in the cervix of the pregnant rat associated with cervical ripening over the course of gestation. The working hypothesis for this research questions states, Ultrasound-detected microstructural changes due to gestational

changes in cervical collagen and water content are detectable and quantifiable from the processed frequency-dependent ultrasound backscatterer signals (enhanced B-mode ultrasound).

2. To determine whether there is a relationship between gestational age, ultrasound scatterer diameter, scatterer acoustic concentration, scatterer strength factor and per cent hydroxyproline and percentage of water in the cervix. The working hypothesis for this research questions states, Quantitative ultrasound measurements of the cervix will be correlated with hydroxyproline and water content of the cervix reflecting cervical ripening.

3. To determine whether a statistical model will significantly predict group classification (gestational age) from the ultrasound variables of scatterer diameter, acoustic concentration, and scatterer strength factor.

F. Summary

Presently there is no technique available to accurately measure cervical ripening. After the first trimester of pregnancy, unless a woman is experiencing pelvic pressure or contractions, digital cervical examinations are not usually performed. Thus the process of premature cervical ripening may go undetected. Furthermore, transvaginal ultrasound cervical length examinations have determined that only the outer half of the cervix is appreciated during a digital cervical examination (McFarlin et al., 2000a, 2000b). The cervix ripens from the internal os to the external os, thus, digital examinations may miss the early ripening process.

Developing an *in vivo* and noninvasive ultrasound technique to quantify cervical ripening is attractive for several reasons: (a) it is noninvasive and has a record of safety

in pregnancy; (b) it is acceptable to women; and (d) it is available, as most obstetrics practices have ultrasound equipment in their offices. Having a noninvasive technique to quantify cervical ripening in humans would also be an important research instrument. The proposed backscatter ultrasound technology makes use of information that is essentially processed out of most commercial ultrasound equipment. It would be possible to add this feature to the computer processing of commercially available equipment in the future. Furthermore, this research seeks to validate ultrasound findings of cervical ripening with physiologic markers of collagen and water content of cervical biopsy specimens.

Experimental studies indicate that parturition has two major phases: the long conditioning phase which involves cervical ripening before the active phase and activation of the fetal membranes (progesterone dependent), and the active phase of extensive uterine activity and cervical dilation (Chwalisz & Garfield, 1998b). This last phase may be truly irreversible, as historical experience with tocolysis has found. Years of trying to arrest contractions in preterm labor have not resulted in fewer preterm births.

Efforts to arrest the acute phase event, the preterm contractions, have been unsuccessful, except to delay birth for the 48 hours necessary to promote fetal lung maturation. There has been little monitoring of the cervical ripening process that precedes the extensive phase of uterine activity by weeks. Clinicians and researchers have had little understanding of the cervical ripening process, no objective instruments to measure the process, and no pharmacological interventions that would delay cervical ripening.

Cervical softening occurs in every mammalian species studied to date, and the microstructural and biochemical studies in all species indicate the dissociation of the normally tightly woven collagen fibers and bundles, and the disorganization of the normally well-aligned fibrillar collagen network (Leppert et al., 2000). However, the precise molecular basis of cervical softening is incompletely understood. Although the etiologies of preterm labor are heterogeneous, it is clear that more information is needed about the role of cervical anatomy and physiology in preterm labor. Research and clinical efforts have been hampered by the inability to quantify changes in the cervix in pregnancy.

II. LITERATURE REVIEW

This section presents the theoretical concepts related to the anatomy of the human and rat cervix as well as physiological factors involved with cervical ripening. A review of the methods to detect cervical ripening is presented.

A. Challenges of Studying Cervical Ripening

Almost all of the scientific studies of cervical ripening have been conducted on animal models and the results extrapolated to humans (Leppert, 1995). The rat cervix has been found to be an appropriate animal model for conducting studies of cervical ripening physiology due to the similarity of the types of collagen in the human and rat cervices (Leppert et al., 2000). Genetically altered mice have also been used in several of the studies due to the availability of stem cells to genetically alter the mice to study a particular question. Presently stem cells are not readily available to alter larger animals to use as models. Several studies have been conducted with human tissue at the time of delivery; however, obtaining biopsy specimens of human cervices during pregnancy presents methodological and ethical problems (Leppert, 1995; Stjernholm et al., 1996; Stjernholm et al., 1997; Wang, Stjernholm, Ekman, Eriksson, & Sahlin, 2001). Small biopsies may not be representative of the whole cervix, thus complicating our understanding of cervical ripening (Leppert, 1995). Many studies have also been conducted in the laboratory either with human tissue or animal tissue *in vitro.* Some of the reasons for conducting *in vitro* studies, besides the ethical problems associated with conducting them in humans, are the amount of control and manipulation of possible influencing factors, which is not possible in clinical trials outside of a controlled laboratory environment.

B. The Anatomy of the Human Cervix

In a human adult multiparous woman, the corpus of the uterus occupies two thirds of the uterus and the cervix the lower one third. In very young nulliparous women this relationship may be half corpus and half cervix. The transition from the mostly smooth muscle corpus to the mostly collagenous and connective tissue cervix occurs over a 10 mm length in the lower segment of the uterus (Cunningham et al., 1993). Figure 6 displays a saggittal image of a pregnant uterus and cervix. The average nulliparous uterus is 6–8 cm long with an average weight of 50–70g (Leppert, 1995). The average size of the multiparous uterus is 9–10 cm with a weight of 80 g (Leppert, 1995). The part of the cervix that protrudes into the vagina is called the *portio vaginalis*

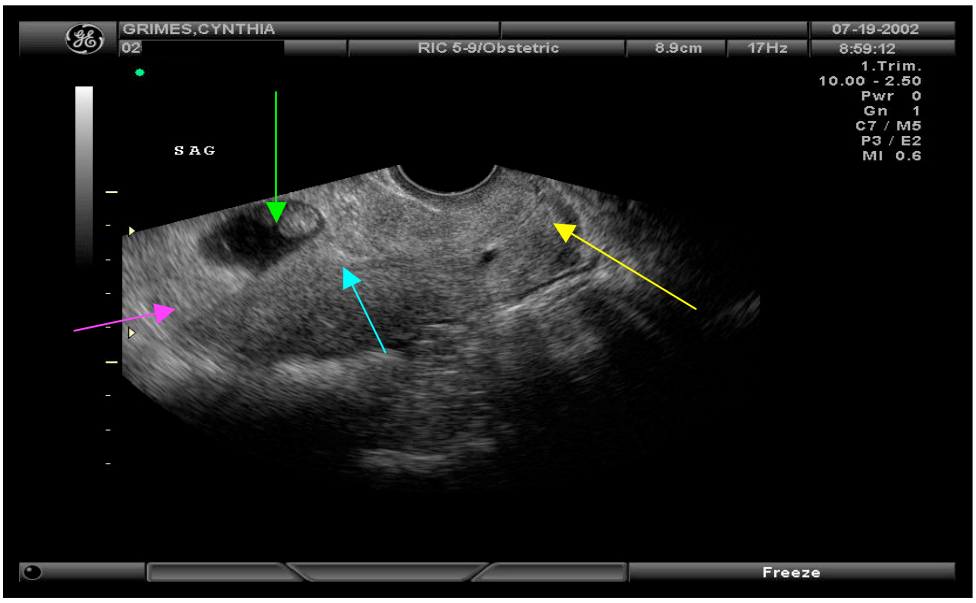

Figure 6. Saggittal transvaginal ultrasound image of the pregnant uterus and cervix. The green arrow indicates the gestational sac, the pink arrow the corpus, or body of the uterus, the turquoise arrow the internal os of the cervix and the yellow arrow the external os of the cervix.

(Cunningham et al., 1993). The *portio vaginalis* is approximately 3 cm long and 2.5 cm wide, although the size and shape varies with hormonal status, age, and parity. Cervical length studies with ultrasound during pregnancy have taught us that there is a wide variation of what is a “normal” cervical length. The cervix is also dynamic, meaning that contractions may change the cervix during the measurement period (McFarlin et al., 2000a, 2000c; Owen, Yost, Berghella, et al., 2001). The portion of the cervix that connects to the lower segment of the uterus is the internal os of the cervix. The internal os connects the external os by way of the endocervix, which is lined with endocervical mucosal columnar epithelial cells. The ectocervix is lined with smooth squamous epithelium.

The blood supply to the cervix is from the internal iliac arteries, which lead into the uterine arteries, and are divided at the supravaginal portion of the cervix. The lateral branches descend at the 3 o’clock and 9 o’clock lateral aspects of the cervix (Leppert, 1995).

There are sensory, parasympathetic, and sympathetic nerve fibers in the cervix. Instrumentation, such as dilation of the cervix is capable of causing a vasovagal response (Cunningham et al., 2005; Droegemuller, Herbst, Michell, & Stendever, 1987). Ferguson suggested that stretching of the cervix in labor is a stimulus for oxytocin release from the posterior pituitary gland where it is synthesized (Ferguson, 1941), thus suggesting that the stimulation of nerve fibers in the cervix stimulated the pituitary gland. Ferguson’s reflex was never proven; however, this effect has often been noted clinically. Women often have contractions after a cervical exam. Evidence of a possible neural signal from the cervix to the pituitary was found by investigators measuring

prolactin during instrumentation of the cervix during termination of pregnancy and hysteroscopy (Fernandes, Boroditsky, Roberts, Wodzicki, & McCoshen, 1999). A similar rise in prolactin was also found in the second stage of labor, but not in the first stage of labor.

C. The Rat Cervix

The Sprague-Dawley rat has been used as a model to study cervical ripening due to the similarity in the type and amount of each type of collagen in the cervix (Leppert et al., 2000). The rat, however, has two uterine horns and two canals within one cervix (Harkness & Harkness, 1959). The cephalad portion of the cervix consists of approximately 40–50% smooth muscle whereas the lower two thirds consists mainly of connective tissue and only 20–40% smooth muscle (Harkness & Harkness, 1959). From gestational day 11 to 12, the rat cervix progressively increases in wet weight and total collagen content but the concentration of collagen decreases (Harkness & Harkness, 1959). The decreased concentration of collagen in the rat cervix as pregnancy progressed was associated with increased water, decorin, and hyaluronan and a decrease in the percentage of smooth muscle (Golichkowski et al., 1980; Harkness & Harkness, 1959).

D. The Composition of the Cervix

The connective tissue stroma of the cervix has a cellular component and an extracellular matrix. The major cellular components of the connective tissue of the cervix are fibroblasts, and to a lesser extent, smooth muscle cells (5–10%). Fibroblasts are cells of mesodermal origin that have the ability to give rise to other cells of mesodermal origin (fat, bone, cartilage, smooth muscle), collagen, and elastin fibers,

various glycoproteins, and ground substance. Smooth muscle cells are closely aligned with collagen and play a role in collagen rearrangement (Thomson, Norman, et al., 1997). Apoptosis, or programmed cell death, in the rat cervix is associated with an increase in enzymes that break down collagen, and cytokines, which are associated with cervical ripening (Leppert, 1995). Almost 50% of the dry weight of the cervix and 80% of the total protein content consists of collagen. Approximately 70% of the collagen is type I, and 30% is type III in the cervix, and the small amount of type IV collagen comprises the basement membranes (Thomson, Norman, et al., 1997; Winkler & Rath, 1999).

In the nonpregnant state, water comprises 75–80% of the extracellular matrix of the cervix. The water content of the cervix increases to about 86% in pregnancy, and changes more with ripening or softening of the cervix (Thomson, Norman, et al., 1997; Winkler & Rath, 1999). The water content in the cervix is very important for the enzymes, proteins, and elastin to function later during ripening. Without hydration elastin would not achieve its suppleness and stretch. Table II summarizes the composition of the cervix.

1. Collagen

The collagen content in the human uterus increases by 8–10 fold during pregnancy (Jeffrey, 1991). Collagen is produced by the fibroblasts in the cervix. Collagen molecules are ordered fibrils embedded in the connective tissue matrix. The structural unit of collagen is tropocollagen, which is a helix of three individual collagen α-chains of approximately 100,000 MW each. The three α-chains are coiled around each other in a right handed fashion, which provides strength. Collagen is the

TABLE II

COMPOSITION OF THE HUMAN CERVIX

Cells	Extracellular Matrix
Surface epithelium: squamous	Collagen:
Endocervical cells: columnar	Type I: 70%
	Type III: 30%
	Type IV: small amount
Smooth muscle cells	Elastin
Fibroblasts:	Water
White cells:	Glycosaminoglycans:
Macrophage	Hyaluronan
Eosinophils	Decorin
	Enzymes:
	MMP-1,8,13
	Cytokines:
	IL-1, IL-6, IL-8, TNF-α

substance in the cervix that makes it tough and strong (Leppert, 1995). High amounts of proline and glycine are contained in the collagen α-chains (Huszar & Walsh, 1991). Hydroxyproline is an amino acid residue unique to collagen and can be used to measure collagen content of the tissues (Huszar & Walsh, 1991).

Peptidyl lysine oxidase is an enzyme responsible for cross-linking collagen. The cross-links give collagen its strength. Several factors can modify the activity of peptidyl lysine oxidase. Cigarette smoking inhibits peptidyl lysine oxidase (Schectman, 1993). Abnormalities of copper metabolism result in a decrease in peptidyl lysine activity (Kuivaniemi, Peltonen, & Kivirikko, 1985). Copper deficiency is associated with

connective tissue abnormalities such as Type IX Ehlers-Danlos syndrome (Kuivaniemi et al., 1985; Myllyharju & Kivirikko, 2001). Ehlers-Danos syndrome is an X-linked disorder that is characterized by abnormalities in copper metabolism. Women with Ehlers-Danos syndrome have a 21% incidence of preterm delivery (Lind & Wallenburg, 2002).

Table III summarizes cervical collagen content studies. Total collagen content in the rat cervix has been found to increase during pregnancy and peaks just before labor, while the concentration of collagen decreases (Alberts et al., 2002; Golichkowski et al., 1980). A feature of cervical ripening in rats, is that the collagen becomes disorganized and the fibers become rearranged without a loss of tensile strength (Kokenyesi & Woessner, 1991; Leppert et al., 2000). The collagen fibers reorient and reorganize by 48 hours after delivery (Kokenyesi & Woessner, 1991; Leppert et al., 2000). It is thought that the mechanism for the disorganization and rearrangement of the collagen fibers is due to the interaction of decorin with collagen (Leppert et al., 2000).

Women with a history of cervical incompetence were found to have lower amount of cervical collagen, as compared to women without a history of cervical incompetence (Petersen & Uldbjerg, 1996). The exact etiology of these differences is not clear (Petersen & Uldbjerg, 1996).

Ascorbic acid (vitamin C) is a water-soluble vitamin that humans do not synthesize, therefore a steady dietary intake of vitamin C is required to maintain necessary amounts in the tissues. Vitamin C has a necessary role in collagen metabolism as it strengthens and stabilizes collagen. When there is vitamin C

deficiency, the procollagen α chains fail to form the stable triple helix and are degraded within the cell, leading to collagen loss and fragile tissue, which can easily tear

TABLE III

SUMMARY OF THE STUDIES MEASURING HYDROXYPROLINE IN THE CERVIX

Investigators	Method	Animal Model	Collagen concentration nonpregnant (wet weight)	Water Content nonpregnant (%)	Collagen concentration @ term (wet weight)	Water Content of Cervix @ term (%)	Findings
(Harkness & Harkness, 1959)	(Neuman & Logan, 1950)	Albino rats	5.6 ± 0.59 g/100 g	85 ± 9 mg	4.52 ± 0.21 g/100g	158 ± 5 mg	Fall in the concentration of collagen in the cervix as it ripens. No ripening changes occur in the rat cervix until the 11-12th day of pregnancy.
(Danforth, Buckingham, & Roddick, 1960)	(C. J. Martin & Axelrod, 1953)	Humans	10.2 % ± 1.8 of dry weight		4.7 % ± 1.5 dry weight		Biopsies at cesarean hysterectomies. Changes in the cervix in labor may be due to the ground substance rather than the collagen.
(Danforth et al., 1974)	(Danforth et al., 1974)	Humans		74.4%		78.4%	Human biopsies after delivery. Water content of cervix cannot account for wide scattering of collagen fibrils. Striking tissue remodeling.
(Kleissl, van der Rest, Naftolin, Glorieux, & De Leon, 1978)	(van Der Rest, Cole, & Glorieux, 1977)	Humans	28.1 ± 6.8 residues/1000 amino acid residues	73%	22.5 ± 5.1 residues/ 1000 amino acid residues	80%	Biopsies after delivery. Mostly type I collagen and 20-38% type III.
(Golichkowski et al., 1980)	(Woessner, 1961)	Sprague-Dawley rats	Graph approximation 3 nmol/mg dry wt (0.39 mg/ mg cervix)		Graph approximation 2 nmol/mg dry wt (0.26mg/mg cervix)		Hyaluronate content in the cervix increased 17-fold and a ratio of hyaluronate to hydroxyproline increased 10-fold by term. There is an accumulation of hyaluronate in the interstices of the collagen fibers along with water resulting in softening.
(Williams, Hollingsworth , & Dixon, 1982)	(Woessner, 1976)	Sprague-Dawley rats	5.6 ± 0.5 mg/g	72.5% ± 0.2	9.5 ± 0.6 mg/g	80.4% ± 0.8	Cervical softening related to tissue controlled tissue hydration. Collagen content increased by term pregnancy.

TABLE III (continued)

SUMMARY OF THE STUDIES MEASURING HYDROXYPROLINE IN THE CERVIX

Investigators	Method	Animal Model	Collagen concentration nonpregnant (wet weight)	Water Content nonpregnant (%)	Collagen concentration @ term (wet weight)	Water Content of Cervix @ term (%)	Findings
(Uldbjerg, Ekman, Malmstrom, Olsson, & Ulmsten, 1983)	(Stegemann & Stalder, 1967)	Humans	19.5 ± 1.8 μg/mg	80.8%	5.0 ± 1.4 μg/mg	85.9%	Early pregnant biopsies taken from women seeking abortions, term pregnancies from cesarean sections.
(Uldbjerg, Malmstrom, Ekman, & Ulmsten, 1985)	(Stegemann & Stalder, 1967)	Humans	15.8 μg/mg		5.8 μg/mg term 4.8 μg/mg after delivery		Three women at cesarean section, three after term delivery, three at hysterectomy. No qualitative change in collagen in labor remains intact.
(Fosang & Handley, 1988)	(Stegemann & Stalder, 1967)	Ewes	12.9 ± 2.5 mg/g content	79.5%	7.2 ± 1.5 mg/g content	86%	Amount of hydroxyproline decreases per wet weight in pregnancy.
(Kokenyesi & Woessner, 1990)	(Woessner, 1961) Assay	Sprague-Dawley rats	53 ±4 μg/mg		24 ± 2 μg/mg		Correlation of collagen concentration and small dematan sulfate.
(M. Norman, Ekman, & Malmstrom, 1993)	(Stegemann & Stalder, 1967)	Humans	20.8 ± 2.9 μg/mg		9.0 ± 3.4 μg/mg		Tissue samples taken immediately after normal delivery and at hysterectomy. Cervical ripening occurs in two stages in humans, a slow connective tissue change in pregnancy and then an active process before or in labor.

TABLE III (CONTINUED)

SUMMARY OF THE STUDIES MEASURING HYDROXYPROLINE IN THE CERVIX

Investigators	Method	Animal Model	Collagen concentration nonpregnant (wet weight)	Water Content nonpregnant (%)	Collagen concentration @ term (wet weight)	Water Content of Cervix @ term (%)	Findings
(Regassa & Noakes, 2001)	Semi-automated process from chemistry lab, known hydroxyproline controls	Ewes	130.24 mg/g	76%	79 mg/g	80%	Cervical changes in ewes are similar to other species.
(Breeveld-Dwarkasing et al., 2003)	HPLC (Bank, Jansen, Beekman, & te Koppele, 1996)	Cow			300-380μg/mg dry weight	82-85%	Collagen content expressed as dry weight. Similar to human cervix.

(Alberts et al., 2002). Vitamin C is a requirement for the regulation of lysyl hydroxlase and prolyl hydroxylase, which are necessary enzymes for the synthesis of hydroxyproline and hydroxylysine (Kivirikko & Pihlajaniemi, 1998; Wu et al., 2000). Hydroxlase and prolyl hydroxylase which are necessary enzymes for the synthesis of hydroxyproline and hydroxylysine (Kivirikko & Pihlajaniemi, 1998; Wu et al., 2000). Hydroxyproline and hydroxylysine are requirements of collagen to form the cross bridges across the triple helix, providing stability to collagen (Nakata & Maeda, 2002; Woods, Plessinger, & Miller, 2001). Vitamin C also stimulates collagen production by increasing the steady-state in procollagen mRNA, (Chojkier, Houglum, Solis-Herruzo, & Brenner, 1989; Pinnel, Murad, & Darr, 1987) and expression of metalloproteinase-2 (Pfeffer et al., 1998). Metalloproteinase-2 expression has been shown to be an important factor in cervical collagen ripening by breaking down collagen (Ledingham, Denison, Riley, & Norman, 1999; Stygar et al., 2002).

Ascorbic acid has been shown to be important in stabilizing atherosclerothic plaque collagen in mice. When the mice were Vitamin C deficient, the plaque was more likely to rupture than it was in those with adequate vitamin C (Nakata & Maeda, 2002). Women who smoke (Schectman, 1993) and take oral contraceptives have lower serum ascorbic acid levels (Rivers & Devine, 1975). The National Health and Nutrition Examination Survey (NHANES) found significantly lower serum levels of ascorbic acid in African American participants as compared to Latino (second highest values) and Caucasions, who had the highest serum levels (Chen, He, Ogden, Batuman, & Whelton, 2002). As pregnancy advances, maternal serum levels of ascorbic acid decline in the third trimester and are higher in the fetus than in the mother (Woods et al.,

2001). There has been an association of premature rupture of the membranes and low maternal serum ascorbic acid levels (Plessinger, Woods, & Miller, 2000). Guinea pigs that were fed a vitamin C deficient diet during pregnancy had significantly reduced hydroxyproline content in the uterus and fetus compared to animals that were fed normal diets (Rivers & Devine, 1975). Whether this association of low ascorbic acid levels could affect cervical collagen integrity is unknown and has not been studied.

2. Elastin

Elastin is produced from fibroblast cells in the extracellular matrix of the cervix. Collagen is responsible for the tensile strength of the cervix, but elastin is responsible for making the cervix stretchy. Elastin fibers are interspersed and run parallel to collagen fibers. Elastin fibers surround the collagen in the extracellular matrix to provide both strength and elasticity. When elastin fibers are stretched they still have cross-linked bonds which allow the fibers to return to their unstretched state. Elastin fibers are thought to be very resilient in that they can rebound to their original extensibility, size, and shape (Sandberg, Soskel, & Leslie, 1981). There is a greater ratio of elastin to collagen at the level of the internal os of the cervix (Leppert, 1995). This would accommodate the need for the cervix to stretch and maintain its strength without tearing (tensile strength) during the process of labor. The elastin fibers are arranged in a band 20-30 μm thick that runs in a parallel plane from the internal to the external os (Leppert, Cerreta, & Mandl, 1986). Water molecules surrounding elastin are essential for the fibers to stretch and relax (Sandberg et al., 1981). Without water molecules, the elastin fibers are quite rigid (Sandberg et al., 1981). There has been an

association between a decreased amount of elastin in the cervix and cervical incompetence (Leppert, Yu, Keller, Cerreta, & Mandl, 1987).

3. Proteoglycans

Proteoglycans are core proteins with a polysaccharide chain attached (Rath, Osmers, Szeverenyi, Stuhlsatz, & Kuhn, 1991). These are called glycosaminoglycans (GAGs) due to the carbohydrate chain (Rath et al., 1991). Proteoglycans are defined by their protein core (decorin, biglycan, fibromodulin, lumican, chondroadherin) and classes of proteoglycans characterized by their glycosaminoslycans chains are heparin, heparan sulfate, chontroitin sulfate, dermatin sulfate, and keratan sulfate. Not all of these proteoglycans have been studied yet in relation to their significance to cervical ripening. Proteoglycans bind to other molecules on the surfaces of cells or in the extracellular matrix (Neame & Kay, 2000).

4. Hyaluronan

Hyaluronan (formerly called hyaluronic acid), is a GAG without a protein core and exists as a free glycosaminoglycan (Uldbjerg & Malmstrom, 1991). Hyaluronan has an important role in the way it affects cell behavior by creating a cell-free space so that substances and cells can move, divide, and migrate within the extracellular matrix of tissues (Toole, 2001). It does this through binding proteins (hyaladherins), hydrating the pericellular matrices, and interacting with cell surface receptors (Toole, 2001).

Hyaluronan is produced by the fibroblasts in the cervix. Its production is stimulated by IL-1, IL-8, and prostaglandins (Leppert, 1995; Ludmir & Sehdev, 2000; Winkler & Rath, 1999). During pregnancy the concentration of hyaluronan remains stable until the onset of labor, when there is a 12-fold (Rath et al., 1991) to a 17-fold

increase in the extracellular matrix of the cervix (Golichkowski et al., 1980). Hyaluronan has an important role in cervical ripening, as it occupies the extracellular matrix and increases the water content of the cervix, which leads to loosening of the collagen fibrils and also softening of elastin (Obara et al., 2001). Hyaluronan has a very high capacity to bind water molecules and contributes 12–33% of the total GAG content of the cervix (Leppert, 1995; Winkler & Rath, 1999). Hyaluronan has also been shown to be responsible for neovascularization, which is an important process in cervical ripening (Ludmir & Sehdev, 2000).

5. Decorin

Decorin is a small dermatan sulfate proteoglycan that binds to the surface of collagen fibrils in the cervix, preventing the fibrils from sliding away from each other (Winkler & Rath, 1999). Decorin is produced by the fibroblasts and the smooth muscle cells in the cervix (Leppert et al., 2000). The mechanical tensile strength of collagen has been related to decorin (Kokenyesi & Woessner, 1990; Pins, Christiansen, Patel, & Silver, 1997). Genetically altered decorin-null mice have very fragile skin that tears easily, which suggests decorin's role in providing strength to collagen (Danielson et al., 1997). Stretching of collagen has resulted in a loss of decorin in collagen (Pins et al., 1997). There is increased expression of decorin in resting smooth muscle and fibroblast cells (Leppert et al., 2000).

Decorin accounts for about 66% of the GAG content of the cervix (Winkler & Rath, 1999). The role of decorin in cervical ripening seems to be that increased expression results in collagen disorganization (Leppert et al., 2000). Decorin regulates the intrafibrillar distance of collagen (Leppert et al., 2000). Decorin degrades quite

rapidly postpartum, which allows reorganization of the collagen fibrils (Kokenyesi & Woessner, 1991). Factors that influence the expression of decorin are retinoic acid, which stabilizes the mRNA (Pearson & Sasse, 1992) and TNFα, which reduces decorin expression (Mauviel, Santra, Chen, Uitto, & Iozzo, 1995). The amount of decorin in rats was increased by prostaglandin $F_{2\alpha}$, suggesting a role for hormonal control of decorin expression (Kokenyesi & Woessner, 1991).

6. Enzymes

The enzymes involved in degrading collagen have been called collagenase. Their names have recently been changed to metalloproteinases (MMPs). Metalloproteinases are zinc-dependent enzymes, of which there are at least 26. Metalloproteinases are capable of degrading extracellular proteins such as the proteoglycans (Ludmir & Sehdev, 2000; Yoshida et al., 2002). Metalloproteinase-1, -8, and -13 are enzymes associated with collagen (Stygar et al., 2002). Metalloproteinase-2 and -9 have activity with type IV collagen, elastin, proteoglycans, and fibronectin (Salamonsen, 1996). These enzymes are key enzymes in the process of cervical ripening. The MMPs are expressed from fibroblasts and smooth muscle cells. MMP-1, -8, and -13 have been shown to be associated with collagen types I and III, which are predominant in the cervix (Yoshida et al., 2002). Metalloproteinase-1 has been found to be produced in the fibroblasts cells of the cervix, and its regulation is thought to be mediated by prostaglandin $F_{2\alpha}$, IL-1, and mechanical stretching (Yoshida et al., 2002).

There seems to be a fine balance between the MMPs and their inhibitors, which are called tissue inhibitor of matrix protease (TIMP). It is thought that TIMPs can either prevent or stop the collagenase activity of MMPs (Jeffrey, 1991). Progesterone has an

inhibitory effect on collagenase, and prevents activation of the enzyme (Jeffrey, 1991). This makes sense in that progesterone has been the hormone responsible for maintaining a quiescent uterus and the pregnancy (Chwalisz & Garfield, 1994; Chwalisz, Hegele-Hartung, & Schultz, 1991).

7. Summary

Collagen, elastin, smooth muscle cells, decorin, and hyaluronan are produced by the fibroblasts in the cervix. The primary component of the cervix is collagen, which unlike the smooth muscle of the corpus of the uterus, is firm connective tissue with very little contractile function. Thus, the cervix functions to provide a strong narrow opening to the corpus of the uterus until a complex series of event occur in pregnancy to ripen the cervix and initiate the process to open the cervix. The changes to start the process to soften the collagen occur with an increase in decorin and hyaluronan as pregnancy advances. The enzymes involved in cervical ripening are important in remodeling the collagen. Hyaluronan binds water molecules, which in turn softens elastin and collagen. Decorin organizes the collagen fibrils, regulates the distance between the collagen fibers, and provides strength to the collagen.

E. Hormones Involved in Cervical Ripening

Hormones have historically been used to induce cervical ripening and labor (Baulieu, Ulmann, & Philibert, 1987; Bishop, 1955, , 1964; Calder & Greer, 1992; Chwalisz & Garfield, 1994; Csapo, 1956; Cunningham et al., 2005). Only recently has the role of homones been considered in inhibiting cervical ripening in pregnancy (da Fonseca, Bittar, Carvalho, & Zugaib, 2003). This section reviews what is known about

the hormonal influence on cervical ripening. What is not known is what signals the onset of the ripening process.

1. Progesterone

Progesterone is produced in the corpus luteum of the ovary until about 10 weeks' gestation. Between the 7th and 10th weeks of gestation is a transition time between the corpus luteum and the placenta in the production of progesterone, resulting in a slight decrease in serum levels (Thomson, Lunan, et al., 1997). Maternal serum progesterone levels gradually increase to 100–200 ng/mL at term, with the placenta producing approximately 250 mg per day (Speroff & Fritz, 2005). Progesterone is also produced and metabolized in the human decidua and membranes (Speroff & Fritz, 2005). Progesterone receptors are found in the cervix and their concentrations decrease with cervical ripening (Stjernholm et al., 1997).

Progesterone is important for the maintenance of human pregnancy. Although maternal and fetal blood levels do not decline before the onset of labor, the number of receptors probably changes (Challis & Lye, 1994; Chwalisz & Garfield, 1994; Olson, Mijovic, & Sadowsky, 1995). Progesterone suppositories (100 mg vaginally every day) from 24–34 weeks of pregnancy significantly reduced the incidence of preterm birth in a clinical trial, suggesting a role once again for progesterone withdrawal as a mechanism for the initiation of parturition (da Fonseca et al., 2003).

Removal of the corpus luteum early in pregnancy results in abortion. Antiprogestins, such as RU-486, are capable of enhancing myometrial responsiveness to prostaglandins at any age of pregnancy and induce cervical ripening (Chwalisz & Garfield, 1994). Studies of RU-486 in animal models and humans, pregnant and

nonpregnant, have confirmed a cervical ripening effect of the antiprogesterone (Durlot, Dubois, Brunerie, & Frydman, 1988; Gupta & Johnson, 1990; Radestad, Christensen, & Stromberg, 1988; Urquhart & Templeton, 1990; "The use of mifepristone (RU 486)", 1990). Cervices treated with antiprogesterones exhibit increased amounts of hyaluronan and decorin (Ludmir & Sehdev, 2000).

Progesterone catabolism late in pregnancy in mice has been shown to be influenced by an enzyme, steroid 5α-reductase type-1. In mice lacking this enzyme, the level of progesterone in the cervix remains high at term, resulting in a lack of cervical ripening (Mahendroo et al., 1999).

2. Estrogen

The effect of estrogen in target tissues is mediated by binding to estrogen receptors (Gronemeyer, 1992). In humans, estrogen receptors in the cervix are lower in term pregnant and postpartum women than in nonpregnant women (Stjernholm et al., 1997). Estrogen has not been found to be responsible for remodeling of collagen fibers in the cervix of the rat at term (Vogel, Glavind-Kristensen, Thorsen, Armbruster, & Uldbjerg, 2002). However, estrogen was found to modulate the infiltration eosinophils into the cervix and cervical collagen remodeling in the rat cervix (Ramos et al., 2000). This effect was tested and confirmed with rats being treated with estrogen versus tamoxifen (an estrogen antagonist), RU-486 (progestin antagonist), and actinomycin-D (inhibits RNA synthesis) (Ramos et al., 2000). In this study, progesterone inhibited the infiltration of eosinophils into the cervix. These findings suggest an indirect role for estrogen in the process of cervical ripening. Although estrogen may not have a direct effect on the collagen, leukocyte invasion of the cervix is an important feature of cervical

ripening. It has been suggested that leukocyte infiltration of the cervix along with the increased permeability of the tissue may be a mechanism to allow rapid passage of hormones and enzymes that are responsible for collagen remodeling and cervical softening (Luque, Munoz de Toro, Ramos, Rodriguez, & Sherwood, 1998).

Eosinophils have traditionally had roles in allergy and parasitic infections. However, there is evidence from other parts of the body that activated eosinophils can cause intense acute inflammation with vascular permeability and tissue edema (Sell, 1996). Eosinophils are seen in the cervix during cervical ripening and in cases of cervical carcinoma, which suggests a similar role in producing intense inflammation (Luque, Ramos, Rodriguez, & Munoz de Toro, 1996).

3. Prostaglandins

Prostaglandins have been used extensively to artificially induce cervical ripening in the first trimester of pregnancy and at term. However, the role of prostaglandins in ripening the cervix has increasingly come into question. Chwalisz (1997) has shown that cervical ripening in normal pregnancy in guinea pigs and rats starts long before prostaglandins become dominant at the onset of labor. Further evidence against prostaglandins as being the final mediator of cervical ripening is evident when indomethecin or the enzyme cyclooxygenase (COX-1) inhibitors were used. These inhibitors were able to suppress the formation of prostaglandins but not the cervical ripening effects of anti-progestins on the cervix (Radestad & Bygdeman, 1992; Shi, Diel, & Fitzemeier, 1966), suggesting a role for progesterone in preventing cervical ripening. The cyclooxygenase enzyme is responsible for converting arachidonic acid to prostaglandins, prostacyclin, and thromboxane A_2 (Ledingham, Denison, Kelly, Young,

& Norman, 1999). There was hope that the use of prostaglandins to induce cervical ripening before labor would reduce the cesarean section rate due to cervical factors. However this has not occurred. There is still a higher rate of cesarean section with induction of labor, even when the cervix is considered “ripe” by the Bishop score (Boulvain, Marcoux, Bureau, Fortier, & Fraser, 2001; Horrigan, 2001; Keirse, 1992). Also, the effect of prostaglandin often promotes tetanic uterine contractions, which can cause adverse effects on the fetus and potentiate the risk of uterine rupture in women with a previous cesarean section (Romero, 1998).

4. Relaxin

Relaxin is a peptide hormone produced in the corpus luteum during pregnancy. Relaxin has also been identified in the placenta, decidua, and chorion in the human and the rabbit (P. A. Fields & Larkin, 1981; P. A. Fields, Larkin, & Pardo, 1982). Serum relaxin concentrations increase until 10–12 weeks of gestation, then decrease between 12–24 weeks, and then remain constant throughout the rest of pregnancy (Vogel et al., 2002). It is thought that relaxin is responsible for softening the connective tissues and ligaments of the reproductive tract during pregnancy in many species (Speroff & Fritz, 2005).

Relaxin has been found to have a direct effect on remodeling collagen in the rat cervix (Luque et al., 1998) and a role in controlling the central release of oxytocin (Summerlee, O'Byrne, Paisley, Breeze, & Porter, 1984). In a study of cervical ripening in rats, porcine relaxin combined with the administration of RU-486 (mifepristone) resulted in faster labors and more live pups than the placebo control group (Zhao & Sherwood,

2004). Mifepristone is an antiprogesterone agent that binds progesterone receptors with greater affinity than progesterone (Zhao & Sherwood, 2004).

In the pregnant pig, relaxin increases the expression of matrix metalloproteinase-2 and -9 , which play an important role in collagen breakdown and reorganization in the cervix (Lenhart, Ryan, Ohleth, Palmer, & Bagnell, 2001). Fibroblasts have relaxin receptors, which may play a role in control of the metalloproteinase activity in the cervix (McMurtry, Floersheim, & Bryant-Greenwood, 1980).

Porcine relaxin has been successfully used for cervical ripening in women (Evans et al., 1983; MacLennan, Green, Bryant-Greenwood, Greenwood, & Seamark, 1980; MacLennan, Green, Grant, & Nicolson, 1986). However, a recombinant human relaxin had no effect on cervical ripening, even at varying doses (Bell et al., 1993; Brennand et al., 1997). Exactly why there was a discrepancy between the two preparations is not clear. The amino acid sequencing structure (protein expression) of human relaxin differs from porcine relaxin (Gunnersen, Fu, Roche, & Tregear, 1996). Whether this difference accounted for the lack of effect of human relaxin on cervical ripening or if it was due to another factor in the preparation is unknown.

Elevated serum relaxin levels have been used as a marker for preterm labor (Vogel, Salvig, Secher, & Uldbjerg, 2001). In women with preterm labor symptoms, the odds ratio of preterm delivery and an elevated serum relaxin was 4.8 (Vogel et al., 2002). It was hypothesized that an elevated serum relaxin may signal or cause preterm delivery in symptomatic women (Vogel et al., 2002). Women with the highest serum relaxin levels had fewer tears and less bleeding during delivery than women with lower

levels of serum relaxin, suggesting a role for preparing the genital tract for birth (Vogel et al., 2002).

5. Oxytocin

Oxytocin most likely does not have a great role in cervical ripening, as oxytocin stimulates smooth muscle cells to contract. The corpus of the uterus is composed of smooth muscle, which has a function to contract in labor. Approximately 90% of the human cervix is composed of connective tissue, which has very little contractile function in labor (Danforth, 1947).

Maternal serum levels of oxytocin are very low during pregnancy (Keelan, Myatt, & Mitchell, 1997). Around the time of labor, there are more frequent pulses of oxytocin at night, which coincide with uterine contractions (Keelan, Myatt, et al., 1997). Oxytocin mRNA has been found in the human amnion, chorion, and deciduas, and along with maternal pituitary and fetal contribution (Gimpl & Fahrenholz, 2001; Keelan, Myatt, et al., 1997). Oxytocin may be important for stimulating prostaglandin synthesis, which is important in cervical ripening (Keelan, Myatt, et al., 1997).

There are oxytocin receptors in myometrial smooth muscle cells and in the cervix (Gimpl & Fahrenholz, 2001). Myometrial cells exposed to oxytocin for 20 hours resulted in an almost 10-fold reduction in oxytocin binding capacity, suggesting down regulation of the oxytocin receptors (Phaneuf et al., 1997). This is an effect that is noted clinically in cases of long labor that are induced with synthetic oxytocin, Pitocin®. Frequently after Pitocin® is infused for many hours to induce labor, a dose-response effect with higher doses of Pitocin® is no longer achieved. This may be why studies have not necessarily

reported improved results with high dose Pitocin® protocols for induction of labor, probably due to the receptor sites being occupied.

Maternal serum oxytocin levels rise with stimulation of the cervix during an examination, instrumentation of the cervix, or stretching of the cervix, an effect called Ferguson's reflex (Gimpl & Fahrenholz, 2001; Leppert, 1995). Analgesic effects of oxytocin have been observed in labor and after intrathecal injection of oxytocin for back pain (Gimpl & Fahrenholz, 2001).

6. Prolactin

Prolactin is a hormone mainly secreted by the anterior pituitary gland. There have been reports of a possible role for prolactin in labor (Fernandes et al., 1999; Fernandes, Koodoo, Wodzicki, Allardice, & McCoshen, 1997). Cervical dilation, cervical examination, or instrumentation resulted in increased maternal serum prolactin levels (Fernandes et al., 1999; Fernandes et al., 1997). Maternal prolactin levels were static or decreased during the first stage of labor and increased markedly during the second stage of labor, suggesting a neural signal from the cervix. Exactly why prolactin increases is not clear. The role for prolactin in cervical ripening is not clear, but it is interesting that there are neural signals in the cervix for prolactin secretion.

7. Summary

Hormones have historically been used to induce labor and only recently has their role been considered in inhibiting labor and possibly cervical ripening. Cervices treated with antiprogesterones exhibited increased amounts of hyaluronan and decorin (Ludmir & Sehdev, 2000), thus suggesting a role for progesterone in stimulating the process of cervical ripening. Estrogen was found to be associated with leukocyte

infiltration of the cervix, which is important for increased tissue permeability to allow rapid passage of cytokines and hormones to act on the collagen (Ramos et al., 2000). Prostaglandins are the most common hormones, which are used clinically for cervical ripening. Evidence against prostaglandins as being the final mediator of cervical ripening is evident when studies of prostaglandin inhibitors were able to suppress the formation of prostaglandins but not the cervical ripening effects. Oxytocin is the most commonly used hormone to induce labor; however, it most effective when the cervix is ripe, as it stimulates the smooth muscles cells to contract. Oxytocin receptors increase as pregnancy advance.

F. Inflammatory Factors

Leukocyte invasion of the cervix at term has been thought to have an important function in cervical ripening (Vogel et al., 2002). When polymorphonuclear leukocytes invade the extracellular matrix of the cervix, the tissue exhibits an increased permeability to other substances and hormones that are necessary for cervical ripening (Vogel et al., 2002). Pro-inflammatory cytokines, interleukins (IL-1β, IL-6, IL-8), and tumor necrosis factor α (TNFα) have been identified in reproductive tissues in labor (Young et al., 2002). Cytokine is a term for a large group of proteins that are produced by many cells in the body to either stimulate or inhibit the functions of other cells. They do this by altering the behavior and the metabolism of cells by phosphorylation of proteins by cellular kinases, which are their signaling mechanisms for cell function. This usually happens on a local level and the cytokines are transient as the half life of cytokines is very short. So in the case of cervical ripening, there may be intense cytokine activity in the cervix, without systemic effects of inflammation.

1. Interleukins

Interleukins are cytokines that have active roles in cervical ripening. Interleukin-1β (IL-1) is produced in the decidual membranes during pregnancy (Romero et al., 1989). Interleukin-1 is produced by macrophages and fibroblast cells. Macrophages are mature monocytes. Together with neutrophils, macrophages are the phagocytes in the body responsible for consuming dead and damaged cells and microorganisms (Alberts et al., 2002). Interleukin-1 induces inflammatory responses as a pro-inflammatory cytokine, making the cervix more edematous. Interleukin-1 also promotes the production of prostaglandins, IL-2, and the infiltration of leukocytes (Young et al., 2002).

Interleukin 6 (IL-6), a proinflammatory cytokine, has also been found in the cervix and implicated with cervical ripening (Young et al., 2002). The major source of IL-6 in the cervix is from infiltrating leukocytes and fibroblasts (Young et al., 2002). Interleukin-6 has synergistic effects with IL-1 and TNFα (Alberts et al., 2002). The presence of IL-6 in the cervical and vaginal secretions has been found to be predictive of preterm delivery (Coleman, Keelan, McCowan, Townend, & Mitchell, 2001).

Interleukin-8 (IL-8) is produced by macrophages and has functions of attracting neutrophils. Interleukin-8 is thought to have a synergistic interaction with prostaglandin E_2 (el Maradny et al., 1995). Interleukin-8 has been found in leukocytes in cervical cells and has been implicated as a mediator of cervical ripening in humans (Sennstrom et al., 1997). All of these factors have been implicated in promoting cervical ripening.

2. Tumor necrosis factor α

Tumor necrosis factor α (TNFα) was discovered due to its ability to necrose tumors in experimental animals. Tumor necrosis factor α is produced primarily by monocytes and macrophages. Tumor necrosis factor α has been localized in the cervix in infiltrating leukocytes and cervical epithelial cells and has a role in tissue remodeling, angiogenesis and leukocyte invasion of tissue. TNFα stimulates prostaglandin E_2 synthesis (Winkler & Rath, 1999), IL-1 and MMP- 9 production (Young et al., 2002), all important factors in the process of cervical ripening. The exact role of pro-inflammatory cytokines in labor is not entirely clear. As their concentrations increase markedly in labor, it is thought that they stimulate uterine contractions, increase prostaglandin production, attract additional leukocytes, and have a role in tissue remodeling (Young et al., 2002).

3. Nitric oxide

Nitric oxide (NO) is a free radical and a potent inflammatory mediator (Calder, 1998). Free radicals are molecules that have an unpaired electron. Nitric oxide is one of the most widespread free radicals in the body. Nitric oxide, when produced by the endothelial cells, is essential for vascular smooth muscle relaxation, platelet aggregation, leukocyte adhesion, angiogenesis, vascular tone, and thrombosis (Fang, Yang, & Wu, 2002). Nitric oxide is also responsible for killing pathogens activated by macrophages (Fang et al., 2002). Vitamins C and E have antioxidant effects in that they inhibit the inflammatory effects of NO production (Fang et al., 2002). Nitric oxide may be an important mediator during the transition period between the quiescent phase before labor begins until active labor. Nitric oxide, along with progesterone is thought to

regulate uterine quiescence and the rigidity of the cervix (Chwalisz & Garfield, 1998a). Nitric oxide is thought to stimulate cervical ripening by its pro-inflammatory stimulation of cytokines and enzymes.

The actions of NO donors on the uterus and cervix depend on the route of administration. A NO donor, sodium nitroprusside, has been used locally on the cervix to promote cervical ripening with the added benefit of not inducing contractions (Chwalisz et al., 1997). Transdermal nitroglycerine is being studied for its use as a tocolytic due to its effect of relaxing smooth muscles in the uterus (Chwalisz & Garfield, 1998a; Rowlands, Trudinger, & Visva-Lingam, 1996). Tocolytics are medications used to stop uterine contractions. Intravenous nitroglycerine has been used for manual removal of the placenta as it causes the relaxation of the myometrium and is very effective for the procedure (DeSimone, Norris, & Leighton, 1990). When NO donors were compared to prostaglandins to induce cervical ripening in the first trimester of pregnancy, NO donors were associated with less abdominal pain and pre-operative bleeding than prostaglandins. The postpartum side effects of NO donors used for cervical ripening, were increased blood loss and more headache compared to prostaglandins (J. E. Norman et al., 1998).

4. Summary

Cervical ripening is an inflammatory process. There is intense cytokine activity in the process of cervical ripening and in labor. Cytokines, which have a synergistic effect, are important to stimulate the invasion of white cells into the cervix as well as prostaglandins and stimulating the enzymes which remodel collagen.

G. Techniques to Measure Cervical Ripening

Presently there is no known non-invasive clinical method to objectively quantify cervical ripening. Cervical ripening is a gradual process that occurs in the last half of pregnancy before cervical effacement and dilatation. In conditions where cervical ripening is accelerated, such as in preterm birth or incompetent cervix, it would be useful to have a diagnostic instrument to inform the clinician.

1. The Bishop score

Indirect measures of cervical ripening have been studied. One of the oldest and least technological methods is the use of the Bishop score which is displayed in Table IV (Bishop, 1964). The Bishop score was developed to evaluate cervical ripening for induction of labor at term in multiparas. Although the Bishop score is used for both multiparas and nulliparas, 835 of the women in Bishop's original study at term, were multiparas and only 165 were nulliparas. The mean duration of labor was

TABLE IV

COMPONENTS OF THE BISHOP SCORE TO ASSESS READINESS OF THE CERVIX FOR LABOR[a]

	Bishop Score			
Component	0	1	2	3
Dilation (cm)	0	1–2	3–4	5–6
Effacement (%)	0–30	40–50	60–70%	80
Station	-3	-2	-1 or 0	+1 or+2
Consistency	Firm	Medium	Soft	
Position	Posterior	Mid	Anterior	

[a] (Bishop, 1964).

significantly different between the nulliparas and the multiparas. Bishop cautioned about using his cervical ripening assessment instrument for nulliparas.

Interobserver agreement of the Bishop score was studied in 156 pregnant women at term (Faltin-Traub, Boulvain, Faltin, & Extermann, 2004). Perfect agreement between examiners was found in 28% of the cases (Faltin-Traub et al., 2004). The Kappa coefficient found a fair to substantial agreement between examiners (.35 to .69) (Faltin-Traub et al., 2004). In addition, the Bishop score has not been validated for predicting preterm cervical ripening. In a study of 2915 low risk pregnant women, a Bishop Score ≥ 6 had a positive predictive value of 38.5% and a negative predictive value of 96% to predict preterm birth before 35 weeks of gestation (Iams et al., 1996). Clinically, this instrument is freely used by clinicians regardless of parity and gestational age to assess cervical ripening (Pitkin, 2003).

The problems with digital examinations for assessing the cervix are many. The examinations are subjective and women find them uncomfortable and invasive. After the first trimester, unless the pregnant woman is experiencing pressure or contractions, digital examinations are usually not performed until the end of pregnancy, thus the process of premature cervical ripening may go undetected. Transvaginal ultrasound examinations of the cervix have indicated that the cervix opens from the internal os to the external os (see Figure 7). While performing a digital examination of the cervix, clinicians only appreciate the outer half length of the cervix (Figure 7), which may limit the ability to detect cervical shortening very early in the process (McFarlin et al., 2000a, 2000c).

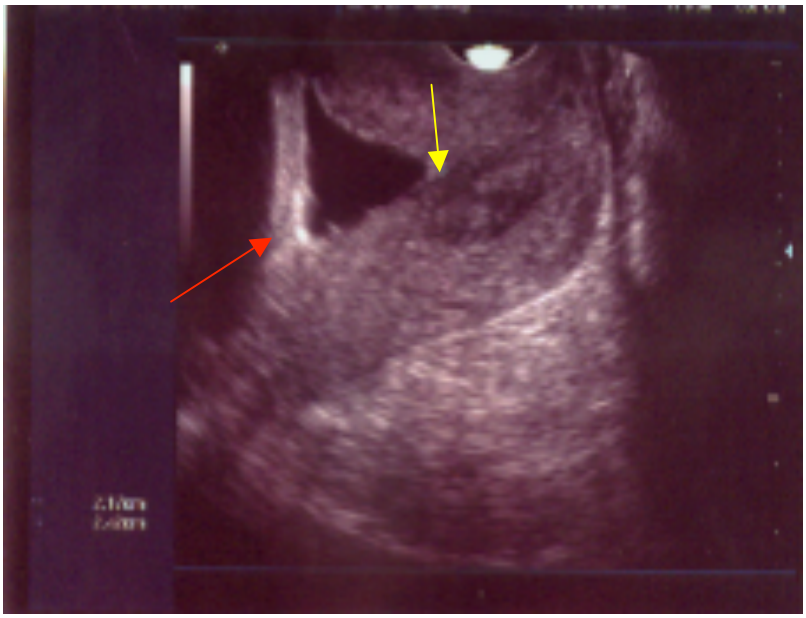

Figure 7. Transvaginal ultrasound of a shortened cervix with a funnel. The red arrow (left) is indicating the fetal head at the open internal os of the cervix. The yellow arrow (pointing down) is indicating the lower portion of the funnel and the cervical canal which is closed.

2. Ultrasound measurement of cervical length

Transvaginal ultrasound measurements of the length of the cervix have been used as a measure of cervical ripening and predicting preterm birth (Iams et al., 1996; Owen et al., 2004; Owen, Yost, Berghella, et al., 2001). As the cervix prepares for labor, or ripens, it not only softens in consistency but shortens in length, which is the process of effacement. As the cervix ripens, the internal os opens before the external os opens. Digital examination of the cervix compared to transvaginal ultrasound assessment of cervical length was studied in 59 women admitted to the hospital in preterm labor (Gomez et al., 1994). Digital examination of the cervix was not significantly associated with preterm birth (Gomez et al., 1994). Ultrasound assessment

of cervical length and funnel was associated with a 6.4 relative risk of preterm birth (95% confidence interval: 2.8 to 14.7) (Gomez et al., 1994). These findings suggest that ultrasound assessment provided valuable information regarding the cervix leading to preterm birth, which was not detectable with digital examinations (Gomez et al., 1994).

Amniotic *sludge* or debris has been observed at the internal os of the cervix during ultrasound assessment of cervical length in 84 women in preterm labor (Espinoza et al., 2005). Women who had the sludge at the internal os were significantly more likely to have shorter cervical lengths, positive amniotic cultures, histologic amnionitis, and impending preterm birth compared to women who presented in preterm labor without the sludge, suggesting a role of infection with preterm labor (Espinoza et al., 2005). An intact closed cervix and cervical mucus plug is an important factor to prevent ascending bacterial invasion of the uterus by providing a mechanical and direct antibacterial barrier between the vagina and the uterus (Romero, Gomez, Araneda, Ramirez, & Cotton, 1993).

A multicenter trial studied 2,915 women at 24 weeks of pregnancy and 2,531 women at 28 weeks of pregnancy to assess the risk of preterm birth and a short ultrasound cervical length (Iams et al., 1996). Although a shortened cervix was significantly associated with preterm birth, it was difficult to predict which women would actually deliver early (Iams et al., 1996). Another multicenter trial studied 183 women at high risk for preterm birth with ultrasound cervical length ultrasound scans every two weeks from 16 weeks of pregnancy to 24 weeks (Owen et al., 2004). Of the women with a cervical length of < 25 mm cervical length in the midtrimester, 37% delivered at < 26 weeks, and 50% delivered at ≥ 35 weeks of pregnancy.

Studies of ultrasound assessment of cervical length have increased understanding of factors involved with cervical ripening and preterm labor. However, the precision with which cervical sonography can predict cervical ripening leading to preterm delivery remains poor (Iams, 2003). Currently the use of ultrasound assessment of the cervix is only recommended for its negative predictive value in women at high risk for preterm birth (Iams, 2003). It is not recommended as a screening method for women at low risk for preterm birth (Iams, 2003).

3. Investigational devices

Three investigational devices have been used to detect cervical ripening; none is commercially available for use with humans. Garfield developed a device called the collascope, which uses light-induced fluorescence to quantify the amount of collagen in the cervix (Garfield et al., 2001). Another device used a piezoelectric transducer and a vibration pick-up element to measure the stiffness in the cervix (Kaga et al., 1996). The amount of stiffness was correlated to histologic specimens of cervical collagen in mice (Kaga et al., 1996). A method to quantify the gray scale level in a region of interest of the human cervix on a transvaginal ultrasound was developed to predict women at risk for preterm birth (Takesin, Meyer-Wittkopf, Sierra, & Schmidt, 2002). A mean gray value of < 6.54 had a positive predictive value of 67.6% and a negative predictive value of 85.3% (Takesin et al., 2002).

H. Summary of Cervical Ripening

Experimental studies indicate that parturition has two major phases: the long conditioning phase, which involves cervical ripening before the active phase and activation of the fetal membranes (progesterone dependent), and the active phase of

extensive uterine activity and cervical dilation (Chwalisz & Garfield, 1998b). This last phase may be irreversible, as experience with tocolysis has found. Efforts to modify the acute phase event, the preterm contractions, have been largely unsuccessful except for delaying birth for 48 hours. Cervical ripening is a complex process that involves inflammatory mechanisms that make the cervix more edematous, which in turn softens the collagen and elastin. Little clinical effort has taken place to monitor pregnant women during the ripening process that precedes the acute event by weeks. Changing the theoretical framework of understanding parturition into a phasic approach may help change the rationale for research and clinical programs. Currently there are no pharmacologic agents to prevent premature cervical ripening. Before interventions to modify the course of cervical ripening leading to preterm labor can be explored, instruments to aid in the early diagnosis and investigation of the process of cervical ripening must developed. It is through understanding these complex series of events that it may possible to develop effective interventions that are based upon the physiological events which precede labor.

III. METHODOLOGY

The design, sample, research site, instruments, procedures for data collection, and data analysis are presented in this chapter. This study described ultrasound characteristics of the microstructure of the rat cervix (scatterer size, acoustic concentration, and ultrasound scatterer strength factor) with percentage of water and hydroxyproline content of the cervix as a measure of cervical ripening, for six groups of rats (one nonpregnant and five pregnant groups).

A. Sample

This was a prospective study of five timed–pregnant groups and one nonpregnant group of Sprague-Dawley rats (Harlan, Indianapolis, IN) to determine whether the ultrasound variables of scatterer diameter, acoustic concentration, and ultrasound scatterer strength factor could predict group assignment. The cervices of Sprague-Dawley rats were scanned in the nonpregnant rat and at day 15, 17, 19, 20, and 21 of pregnancy. The length of pregnancy for the rat is 21–22 days long. There were 13 rats in each group. The order in which they were entered into the study was by randomization of the six groups.

1. Sample size

This was a pilot study. Previous studies measuring scatterer diameter, acoustic concentration, and scatterer strength factor have not been conducted on pregnant or nonpregnant rat cervices. Thirteen rats per group were chosen for the sample size for this study based on past experience with backscatter research at the Bioacoustics Research Laboratory (BRL). Additionally, the ability to detect the backscatter differences at different gestational ages was unknown. Previous laboratory

and histopathology reports have documented marked changes in collagen content, water content, and collagen dissociation during the cervical ripening process over the course of pregnancy in rodents.(Kaga et al., 1996; Leppert et al., 2000). Based on calculations with power analysis software (nQuery Advisor, Statistical Solutions, Saugus, MA), the following power and effect sizes were expected for this study assuming six groups, 13 rats in each group, using a one-way ANOVA (2-tailed test), and α = .05. Table V displays the power analysis.

Effect size is the "degree to which the phenomenon is present in the population" (Cohen, 1988, p. 9). Effect size reflects the mean variability between groups compared to the variability within groups. The chart can be interpreted as follows: With a sample size of 13 rats in each group, the effect size would have to be at least .23 to have a power of .90 (usually a power of .80 is the minimum goal). Previous work by Goilichowski (1980) found marked increases in the collagen and water content

TABLE V

POWER ANALYSIS

Effect Size	Group Size	Power
.36	7	.80
.27	9	.80
.21	11	.80
.18	13	.80
.46	7	.90
.34	9	.90
.27	11	.90
.23	13	.90

of the pre-labor cervix in pregnant Sprague-Dawley rats; therefore, a sample size of 13 rats in each group was selected for this study.

2. Randomization procedures

Rats were randomly selected based upon the gestational age of the rats (nonpregnant, days 15, 17, 19, 20, and 21) for order of testing. The randomization was blocked to assure 13 rats in each gestational age group at the end of the study. The randomization was determined by a computer software program obtained on http://www.randomization.com, Tufts University (McLeod, 1985; Wichman & Hill, 1982). Fluctuations in the availability of a specific gestational age led to having to change the randomization sequence periodically. The actual sequence that the rats were entered into the study is listed in Table VI. A superscript notes where the sequence of randomization was changed.

3. Type of rats

Timed pregnant, first time pregnant, Sprague-Dawley rats were selected for this research. Virgin Sprague-Dawley rats were selected for the nonpregnant group. Timed pregnant rats minimized housing during the gestational period and guaranteed exact gestational age of pregnancy to conduct the experiments. First time pregnant, timed-mated Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Sprague-Dawley rats were chosen based on their use in previous studies by other investigators on cervical collagen and ripening during pregnancy (Golichkowski et al., 1980; Leppert et al., 2000). Additionally, the collagen content (type I and III) of the Sprague-Dawley rat cervix is very similar to the human cervix and has a long history of being used as a model to conduct cervical ripening research.

TABLE VI

SEQUENCE THAT RATS WERE ACTUALLY ENTERED INTO STUDY

Rat number/Gestational age at scan	Rat number/Gestational age at scan	Rat number/Gestational age at scan	Rat number/Gestational age at scan
1. day 20	21. nonpregnant	41. day 19	61. nonpregnant
2. day15	22. day 20	42. day 15	62. day 19[a]
3. day 21	23. day 17	43. day 19	63. nonpregnant
4. nonpregnant	24. day20	44. day 20	64. day 19
5. day 19	25. day 21	45. day 15	65. day 21
6. day 17	26. day15	46. day 15	66. day 20
7. day 19	27. day 21	47. day 17	67. day 15
8. day 20	28. day 21	48. nonpregnant	68. day 15[a]
9. day 15	29. day 19	49. day 17[a]	69. day 15
10. day 21	30. day 20	50. day 17	70. day 17
11. day 19	31. day 17	51. day 17	71. day 17
12. day 19	32. day 17	52. day 19	72. nonpregnant
13. day 21[a]	33. day 19	53. day 19[a]	73. day 17
14. nonpregnant	34. day 20	54. nonpregnant	74. day 20
15. day 15	35. day 15	55. day 20	75. nonpregnant
16. day15	36. day 21	56. day 21	76. day 19
17. nonpregnant	37. day 21	57. day 20	77. day 20
18. day17	38. day 17	58. day 15	78. day 21
19. day17	39. nonpregnant	59. day 21	79. day 21
20. nonpregnant	40. day 20	60. nonpregnant	80. day 21

[a] Order varied from original randomized order due to fluctuations in availability of rats.

4. Animal protocol

The animal protocol was approved by the Laboratory Animal Care Advisory Committee at the University of Illinois at Urbana-Champaign (UIUC Animal Care Protocol #03170) and the University of Illinois at Chicago (UIC Animal Care Protocol #03-200, Appendix A) for 3 years. The University of Illinois at Chicago has Animal Welfare Assurance Number A3460.01 with the Office of Laboratory Animal Welfare at the National Institutes of Health. All of the investigators and laboratory personnel completed the required animal care and ethical training required at the Chicago (UIC) and Urbana-Champaign (UIUC) campuses.

B. Research Site

The research was conducted at the Bioacoustics Research Laboratory (BRL) at the Beckman Institute for Advanced Science and Technology at UIUC at Urbana, Illinois. The histology samples were analyzed at Veterinary Pathology, Veterinary Medicine at UIUC at Urbana, Illinois. The compositional analysis was conducted at the Department of Animal Sciences at UIUC at Urbana, Illinois.

C. Instruments

1. Selection of ultrasound transducers

An ultrasound transducer is a device that converts electrical energy to mechanical energy and visa versa. The ultrasound transducer both transmits and receives acoustic energy. Three ultrasound transducers were used in the development of the methodology for this study: two miniature unfocused pin vaginal probe transducers that could be used *in vivo* and one focused immersible single element transducer that could be used in a water tank *ex vivo.* The original goal of this research

was to scan the cervix of the anesthetized live animal with a vaginal probe transducer, which would be similar to the procedure used in human women. By scanning the animal alive, the tissues would continue to be perfused by the live animal, which it was hypothesized would minimize error in measuring the water content of the cervix. However, due to methodological problems with the miniature pin transducers, the cervix tissue had to be scanned *ex vivo*.

When combined with the processing strategy, very high frequency transducers possess characteristics to determine very small details, or structures in the tissue microstructure that are less than the wavelength. Collagen fibers are approximately 0.5–3 μm in diameter and are tightly packed in bundles (Alberts et al., 2002). Collagen fibers contain multiple collagen fibrils, which measure 10–300 nm in diameter (Alberts et al., 2002). Although it is not possible to resolve the individual collagen fibers with the frequency of ultrasound used in this study, it was hypothesized that it might be possible to resolve in a statistical sense the bundles of collagen by modeling the ultrasound scatterers in the microstructure of the tissue. To determine the maximal information from the ultrasound in the tissues of the cervix, it was important that the ultrasound transducer have the following characteristics: (a) broad bandwidth, (b) short pulse duration, (c) no ringdown, (d) high frequency, (e) be immersible in water, and (f) miniature size that will fit into the rat vagina next to the cervix.

The range of frequencies in an individual pulse of ultrasound is called the bandwidth (Shung & Thieme, 1993). A schematic example of bandwidth is displayed in Figure 8. A broad bandwidth transducer has the advantage of resolving information in the tissues from a wide range of frequencies. Structures in tissues have different sizes,

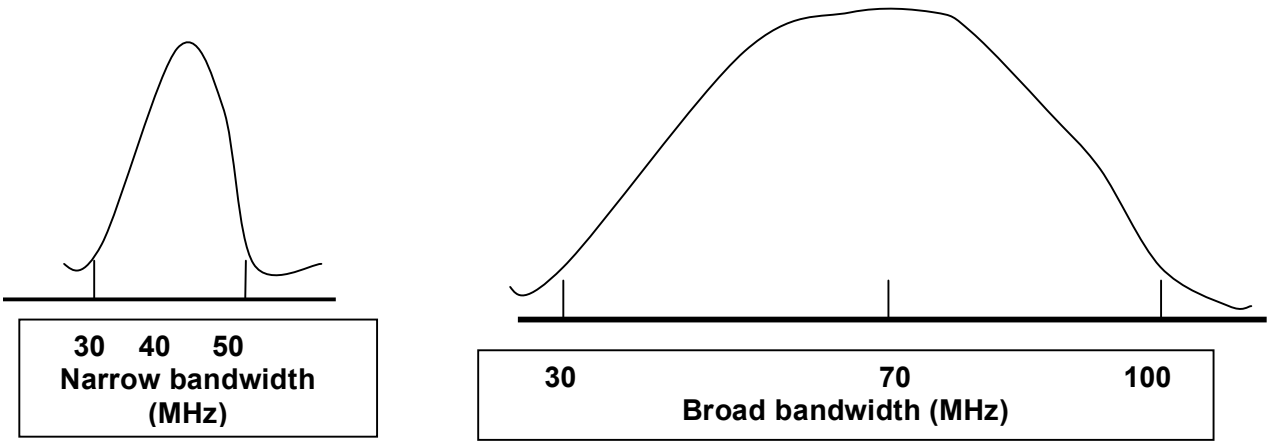

Figure 8. Examples of the effect of narrow and broad bandwidth on range of frequencies in and ultrasound pulse.

shapes, densities, and distances from the transducer; thus, a wide range of frequencies has the potential to resolve a wide variety of these microstructures in the tissues. If a narrow bandwidth of frequencies were used for a transducer, only structures within this narrow range of frequency could be resolved in the tissues, thus possibly losing microstructure information.

A short ultrasound pulse is an important characteristic of the transducer, which improves the bandwidth and the axial resolution of a transducer. Long ultrasound pulses can be a result of a ringing or ringdown effect, which in turn reduces axial resolution and bandwidth. The ringing effect of the first ceramic pin transducer made it difficult to obtain a signal from the tissue, as the signal from the tissue was within the ringing from the transducer. The pulse duration is controlled by the design of the transducer. By applying backing material behind the transducer element, vibration is dampened or controlled by absorbing energy from the back of the transducer element. A variety of damping

materials can be used, which include metal powder, plastic, epoxy, or materials with microbubbles to dampen the pulsed ultrasound signals (Cannata, Ritter, Chen, Silverman, & Shung, 2003).

Detection of small structures in the tissues depends upon the frequency of the ultrasound transducer. Short acoustic wavelengths associated with very high frequency transducers have the potential to improve ultrasound resolution in tissues as compared to lower frequency transducers. Although very high frequency transducers improve the detection of small structures in the tissues, they display weaker signals from the tissues and suffer from less depth penetration into the tissues than lower frequency transducers. A high frequency transducer was well suited for this research project as the structures in the cervix (collagen bundles separated by water, cells) are very small and the rat cervix itself measures only 5–6 mm in length and 4 mm transverse diameter. The depth of penetration of the high frequency ultrasound signal was not a limiting factor for this research project, as the depth of the rat cervix ranges from 2.5–3.5 mm thick. Amplification of the ultrasound signal can be a concern if the amplification is associated with excessive background noise, making it difficult to extract the signal from the cervical tissue.

Figure 9 displays the basic design of a single element transducer. The piezoelectric element can be made of a variety of materials. The piezoelectric effect is the property of some materials (ceramics, quartz, sapphires) to change their physical dimensions when an electrical field or pressure is applied (Shung, 1992). This phenomenon can be explained by the effect of pressure or electricity being applied to

many electric dipoles in the material, which causes the material to change shape (Shung, 1992). Lower frequency transducer elements are commonly made of

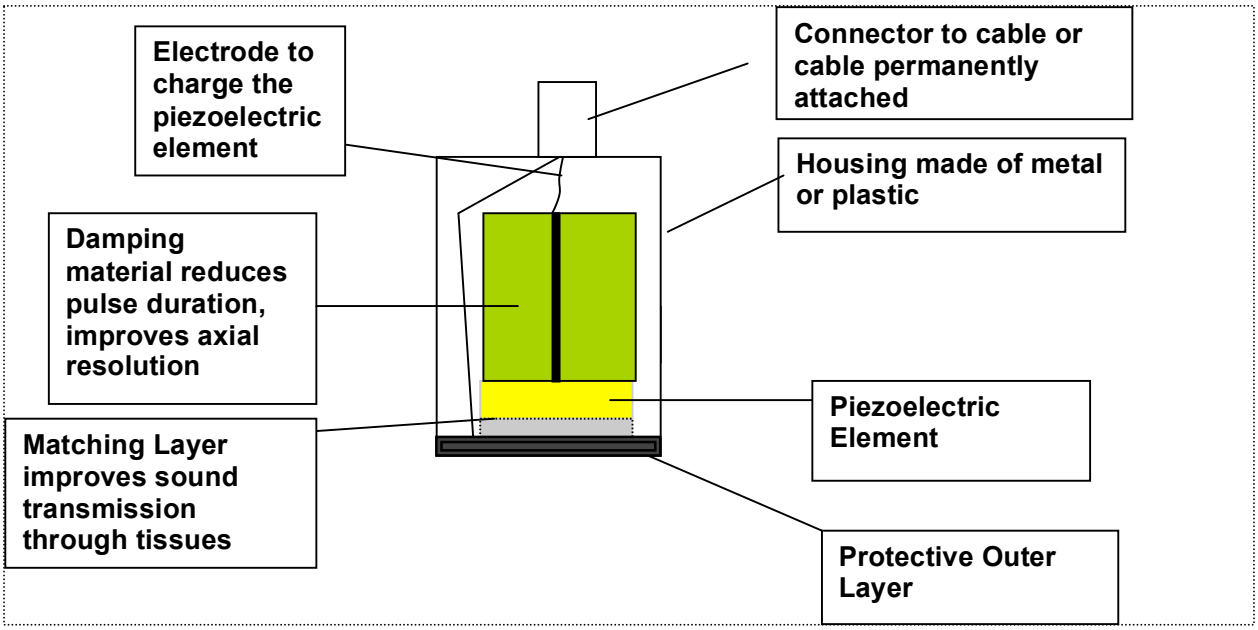

Figure 9. Single element transducer design.

ceramics, which are very sensitive (requiring little power or amplification to obtain a signal from the tissues) but suffer from a narrow bandwidth and longer pulse durations. Therefore, other materials have been developed to improve the ultrasound characteristics, especially for high frequency medical imaging (Cannata et al., 2003).

2. <u>Miniature pin transducers</u>

The first miniature pin vaginal probe transducer tested for this project was a custom-made, unfocused ceramic piezoelectric element transducer with a 46 MHz center frequency (Valpey-Fisher, Hopkinton, MA). This transducer suffered from two design flaws: a long pulse duration and ringdown effect. The returned ultrasound signal from the tissues was within this ringdown (see the vertical red line near the center of the

image in Figure 10), making it difficult to detect unique backscattered ultrasound signals from the tissues. The ringdown effect could be minimized with features of better

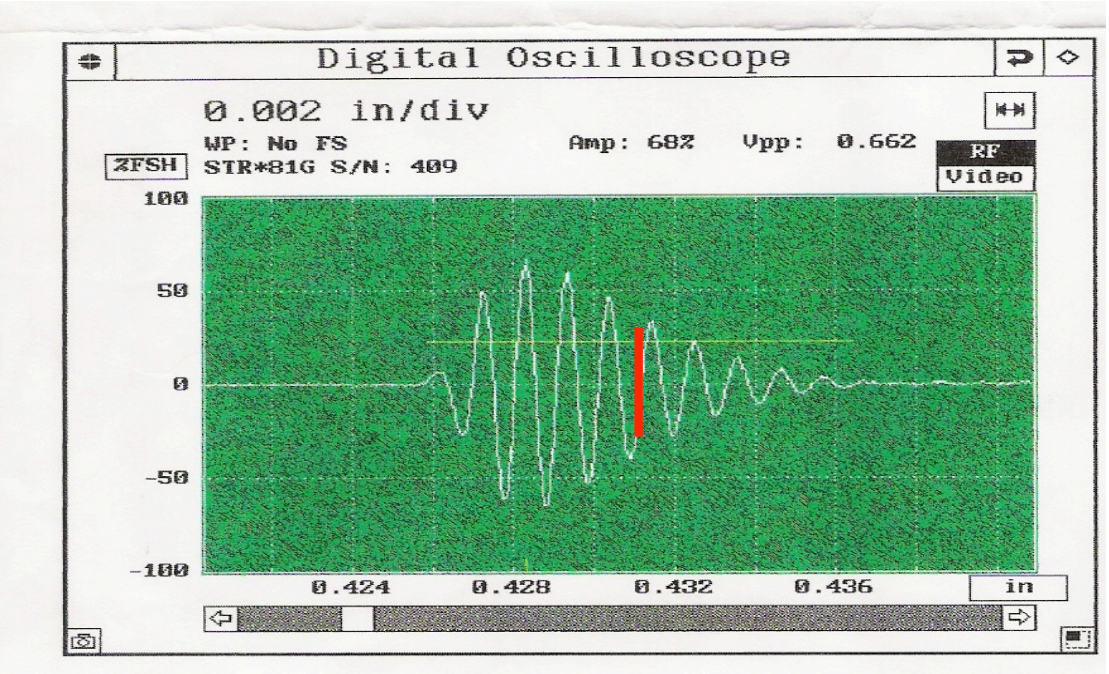

Figure 10. Oscilloscope signal from first Valpey Fisher miniature vaginal probe transducer. Red vertical (solid) line represents signal from cervix tissue.

transducer design, which includes using backing material behind the transducer element and using a different material to manufacture the transducer element.

The second miniature pin transducer was a custom-made transducer with a center frequency of 50 MHz, specifically designed and manufactured for this research project by the National Institutes of Health Transducer Resource Center (University of Southern California, Los Angeles). The aperture diameter measured 1.3 mm, the outer diameter of the housing of the transducer was 3.14 mm, and the length of the transducer was 4.03 cm. The 9 micron polyvinylidine fluoride-trifluoroethylene (PVDF-

TrFE) piezoelectric element for use in the transducer was custom made (Ktech Corporation, Albuquerque, NM). A 45° angle mirror was designed and manufactured in the machine shop at the Department of Electrical and Computer Engineering at UIUC.

Polyvinylidine fluoride-trifluoroethylene is a plastic material used to fabricate ultrasound transducer elements. Polyvinylidine fluoride-trifluoroethylene was chosen for the transducer element for the following reasons: (a) it had a low acoustic impedance that was ideal for use in human tissues (Cannata et al., 2003), (b) it produced a broad bandwidth that was ideal for identifying the microstructure of tissues, and (c) it could be produced very thin, less than 25 μm, to produce very high frequency transducers. Grinding piezoelectric materials thin enough to produce quality high frequency transducers has been a limiting factor in medical imaging (Lopath et al., 1999). A negative feature of PVDF-TrFE is that it has a poor coupling factor, thus requiring a large amount of electrical energy to create an ultrasound signal from tissues (Cannata et al., 2003). The 45° angle mirror that was attached to the transducer was not useful to scan the rat cervix as there was inadequate space adjacent to the rat cervix to maneuver the transducer to aim the ultrasound beam at the cervix (Figure 11). The PVDF-TrFE transducer failed to produce a signal from the rat cervix tissue.

The third and final transducer tested for this research project was a transducer that performed well for rat cervix tissue. However, it could be used only *ex vivo* due to its size and design. The transducer element was made of lithium niobate, with a center frequency of 70MHz, and possessed the characteristics of a short pulse duration, low ringdown effect, and broad bandwidth. An additional feature of the transducer was that it had good electromagnetic coupling, which means it was efficient at converting electrical

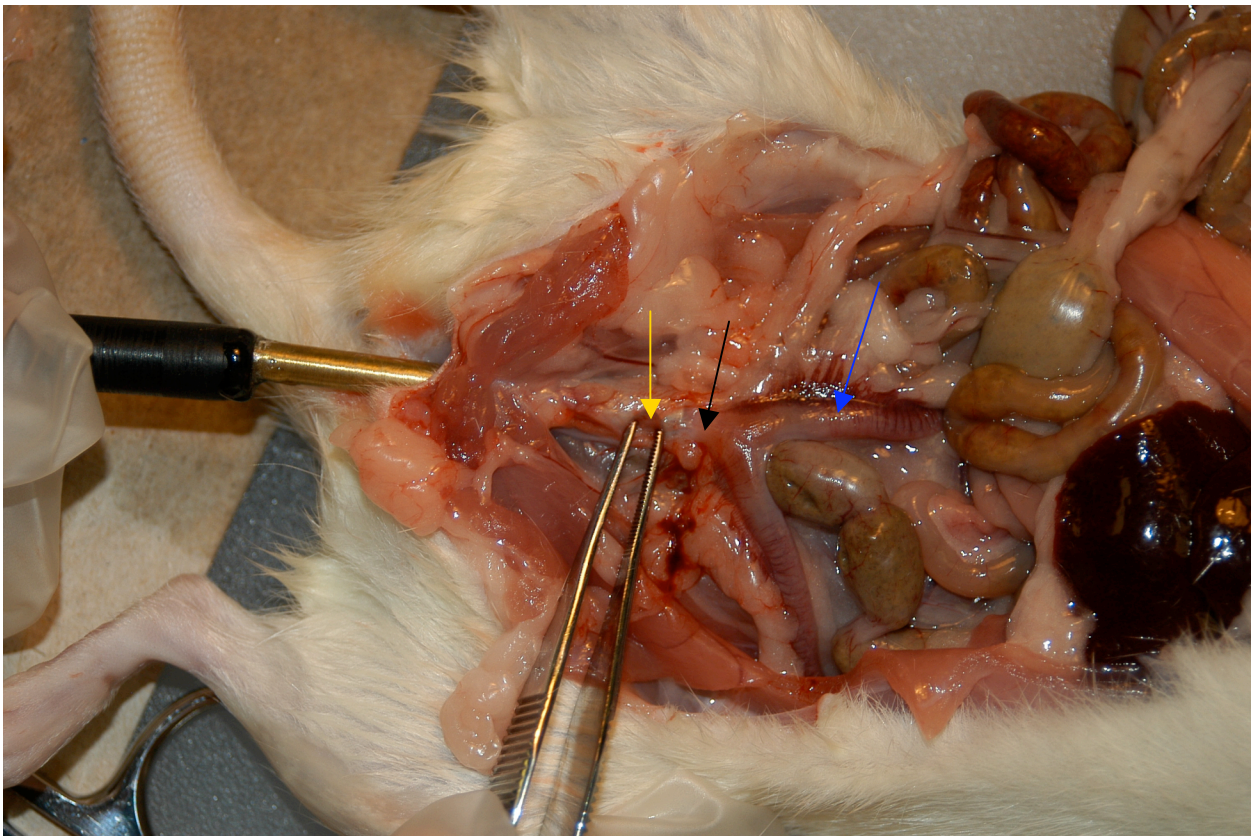

Figure 11. The PVDF-TrFE transducer is placed in the nonpregnant rat vagina. The tip of the forceps shows that with the 45° mirror attached to the transducer, one would be scanning the vagina (left, or yellow, arrow) and not the cervix (middle, or black, arrow). The right, or blue, arrow points to the right horn of the uterus.

energy into mechanical energy, thus making it sensitive. This lithium niobate transducer was designed and manufactured by the National Institutes of Health Transducer Resource Center (J. Cannetta, Penn State University, 1999).

3. Characterization of transducers

The characteristics of the ultrasound transducer were measured in the BRL to determine the spatial and temporal field distribution of the acoustic energy. Table VII lists the beam characteristics. The characteristics of the ultrasound transducer can change depending upon the integrity of the transducer as well as the manner and environment in which it is used. For example, the manufacturer of the transducer listed the frequency at 65MHz; however, the frequency measured in this laboratory was 70.5 MHz. The technique to measure the spatial and temporal pulse-echo acoustic field distribution was to scan a target, a tungsten wire that had the diameter less than the wavelength, across the acoustic beam in a tank of degassed water.

TABLE VII

CHARACTERISTICS OF LITHIUM NIOBATE TRANSDUCER AT THE BIOACOUSTICS RESEARCH LABORATORY

Lithium Niobate Transducer Parameters	Bioacoustics Research Laboratory
Center frequency (MHz)	70.50
~3 dB bandwidth (MHz)	11.00
Fractional bandwidth (%)	15.60
Wavelength in water (μm)	21.07
~20dB pulse duration (μs)	57.60
Focal length (mm)	6.75
f1 (MHz)	65.00
f2 (MHz)	76.00

The bandwidth is the range of frequencies contained within a pulse of ultrasound. The fractional bandwidth is the bandwidth divided by the operating frequency (Kremkau, 1998). Figure 12 displays an image of the beam profile of the transducer. The beam is the region through which the sound pulse propagates (Kremkau, 1998).

D. Backscatter Theory and Measurement

Standard B-mode (brightness-mode) ultrasound images of living tissues created with a clinical ultrasound system are processed from radio frequency (RF) echo signals. The RF echoes are created by reflections from interfaces between acoustically different regions and by incoherent scattering from tissue microstructures. The RF echoes contain frequency-dependent information about the smaller-scale structures (less than the wavelength of sound) in the tissues. Conventional B-mode ultrasound processing removes the frequency-dependent information. Clinical B-mode ultrasound images are good at displaying information about larger scale structures (those that are larger than

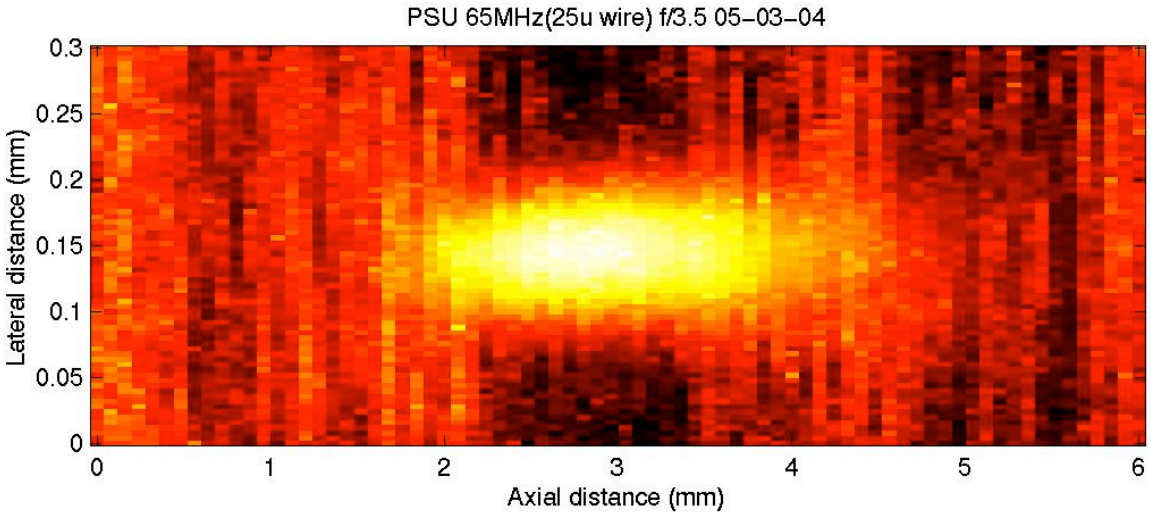

Figure 12. Characterization of ultrasound beam with lithium niobate transducer.

the wavelength). In order to resolve and quantify smaller scale structures in tissues, the frequency dependent information must be utilized.

Scattering occurs when a sound wave (acoustic wave) moves across tissues or fluids of different impedances (Oelze, Zachary, & O'Brien, 2002b). Backscatter theory assumes that tissues can be modeled as complex fluids (Insana, 1995). The magnitude of the frequencies reflected back to the source depends on the size and shape of the underlying scatterers (Oelze et al., 2002b). Scatterer property estimates can be made by comparing the sample backscattering spectrum from each region of interest (ROI) in the tissues being evaluated, to a theoretical backscatter power spectrum. The formula for the theoretical backscatter spectrum is as follows (Insana, Wood, & Hall, 1991):

$$W_{theor}(f) = C(a_{eff},n_z)f^4F(f,a_{eff})$$

where

f is the frequency in megahertz;

C is a constant that depends on the average effective radius, $a_{eff,}$ of the scatterers in millimeters;

n_z is the average acoustic concentration of scatterers per mm^3 times the square of the fractional impedance difference between the scatterer and background; and

F is the form factor.

The Gaussian form factor was used for this study (Oelze et al., 2002b). The Gaussian form factor assumes that the scatterers are spherical and the impedence varies continuously rather than abruptly with the surrounding tissues (Oelze et al., 2002b). The average effective scatterer radius, $a_{eff,}$ denotes the distance the impedance decreases to one half its maximum impedance value.

Typically, three measurements are measured from the backscatter tissue volumes: scatterer diameter, acoustic concentration, and scatterer strength factor. Scatterer diameter is the correlational length of the scatterer measured in μm. Acoustic concentration is the number of scatterers/mm^3 × (average impedence of scatterers)^2. Impedance is the density of a media × the sound propagation speed (Kremkau, 1998). If impedances are the same in a media, there will be no reflection of sound. Scatterer strength factor is acoustic concentration × (average radius of scatterers)^6.

Adjustments to the backscatter formula are made for tissue-specific insertion loss of acoustic energy. Insertion loss is the loss of energy that results when inserting the sample into the sound beam. Linear regression was used to fit a slope and intercept value to the measured insertion loss for each group. These data were used in the calculation of the backscatter coefficients for each group. This method assumes that insertion loss increases linearly with increased frequency (Garra, 1993). The insertion loss of the cervical tissue from three rats in each of the six groups was measured. To accomplish this, a plexiglass plate was placed at the focal zone and the tissue sample was placed between the transducer and the plexiglass plate. The plexiglass acts to reflect the ultrasound back to the transducer. The experiment set up is displayed in Figure 13. The acoustic amplitude is measured with and without the specimen between the plexiglass.

Backscatter measurements were taken from a specific tissue volume ROI that consisted of many parallel ultrasound A-lines of the same length. A backscatter power

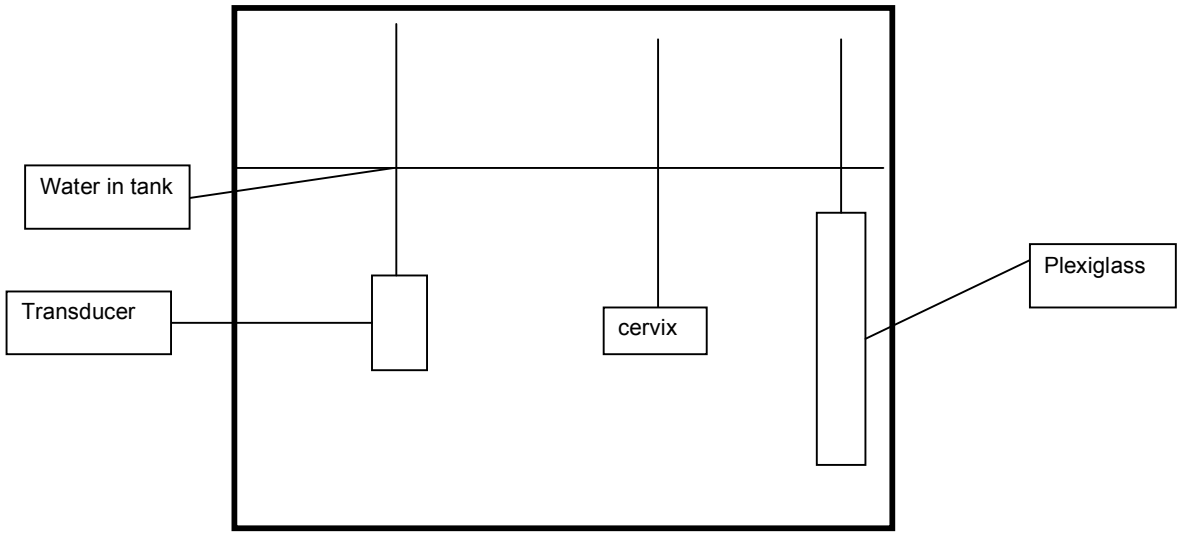

Figure 13. Diagram of the insertion loss experiment setup.

spectrum is then calculated for each scan line by gating the time signal corresponding to the chosen ROI (Oelze et al., 2002b). The total power spectrum for the ROI was measured by averaging the power spectra from each individual scan line corresponding to the ROI. The optimal size of each ROI was calculated from 10 times the wavelength and 5 times the beam widths of the 70 MHz transducer (Oelze & O'Brien 2004). Estimates of the average scatterer diameter, acoustic concentration, and scatterer strength factor were obtained for each ROI by comparing the measured power spectrum with the theoretical power spectrum. The scatterer strength factor is defined as the acoustic concentration times the average effective radius to the 6^{th} power and is proportional to the scattered power. Each ROI was converted into a color pixel that represented the average scatterer diameter and scatterer strength factor.

E. Data Acquisition

The ultrasound transducer was connected to a micro-precision motion control system and placed in a water tank of degassed water. The Deadal® (Deadal®, Irwin, PA) micro-precision control system allows movement along the x, y, and z axes and around two angular axes with an accuracy of 2 micrometers and 0.02 degrees, respectively. The Deadal® is controlled by one of several computer programs for positioning and for scanning. The transducer was connected via a custom-made coaxial cable that has the appropriate impedance (50 Ω) and 1 meter length for the transducer, and to an ultrasound pulser/receiver in the pulse-echo mode (Panametrics 5900, Waltham, MA). The pulser/receiver transmits a single monopole pulse to excite the transducer. The Panametrics 5900 also receives and amplifies the signal back from the tissue and then the signals are displayed on a digital oscilloscope. The echoes of each A-line of ultrasound of the cervix were recorded and digitized on an oscilloscope (9354 TM; LeCroy, Chestnut Ridge, NY) at 500 MHz and processed on a Linux 2 GHz workstation using Matlab (Mathworks, Inc.,Natick, MA). Each A-line represents one pulse of ultrasound. The ultrasound image is formed from multiple parallel A-lines every 20μm across the length of the tissue. The Matlab codes to measure the backscatter (scatterer diameter, acoustic concentration, and scatterer strength factor) and attenuation were written by M. L. Oelze, PhD. Figure 14 is a block diagram displaying the data acquisition system.

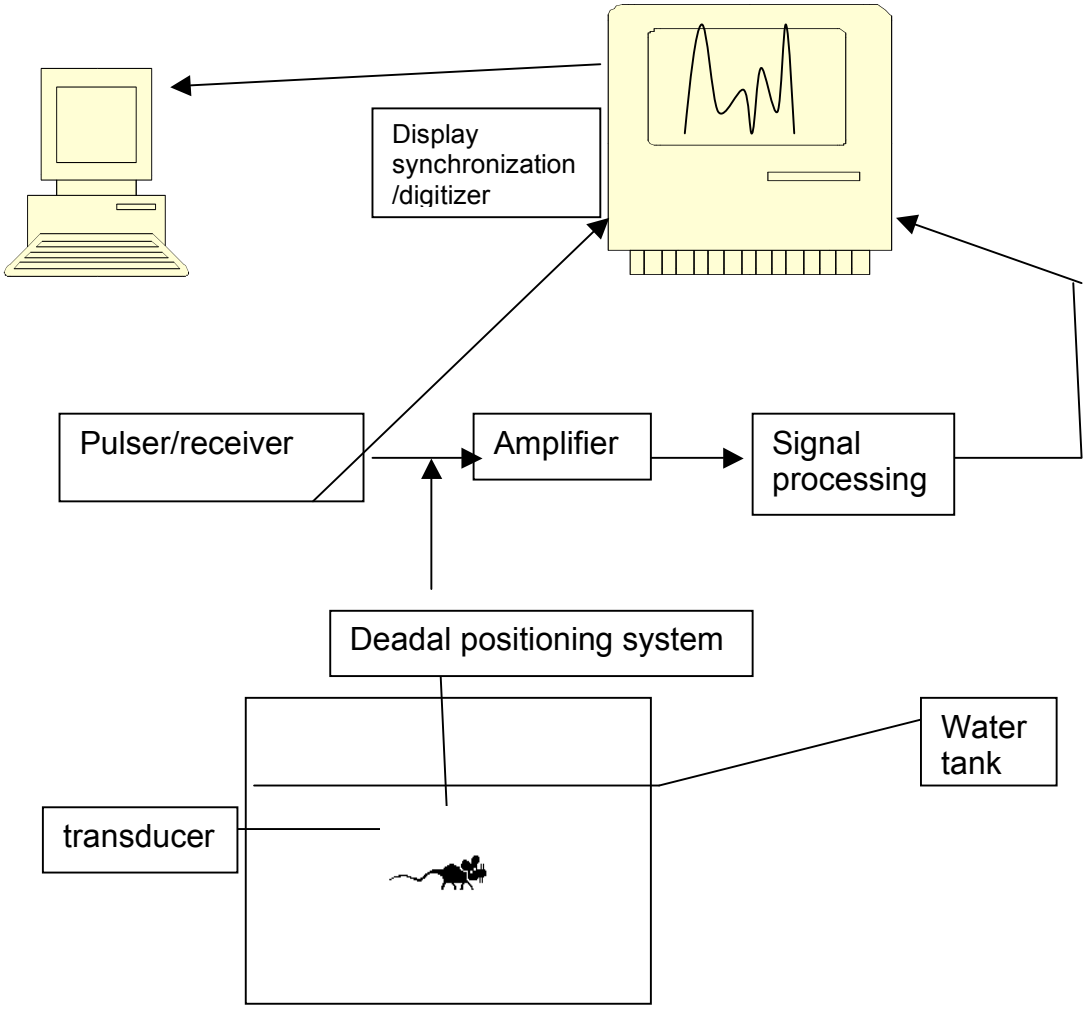

Figure 14. Diagram of backscatter experiment set up and data acquisition system.

F. Weights and Measures

1. Rats

The rats were ordered from Harlan Inc. (Indianapolis, IN) once a week and delivered on Wednesdays. Timed pregnant rats were used for this study in order to be sure of the gestational age at the time of scanning. The rats were housed at the Beckman animal care facilities, fully accredited by AAALAC. The animals were fed a standard diet of rodent chow (Teklad 8604, Madison, WI) and given tap water *ad lib*. The rats were housed in solid bottom polycarbonate containers in a room where the lights are on from 7 AM to 7 PM Central Standard Time (CST) and off from 7 PM to 7 AM CST each day. The rats were housed in the polycarbonate containers by gestational age. Up to 3 rats were housed in a container at a time. The temperature in the room was maintained between 18 to 24 °C and there were 15 to 20 air exchanges per hour in the room.

The rats were individually euthanized humanely in the animal care facility immediately before the cervix was to be dissected. The rats were euthanized in their polycarbonate cage, which was placed in a larger plastic container attached to a tank of carbon dioxide. The carbon dioxide was turned on for 4 minutes and then the cage was left in the carbon dioxide container for another 5 minutes. Necropsy was conducted in the BRL immediately after euthanasia.

The rats were weighed in the BRL before the scanning procedure. An Acculab Model GSI 2001 scale (Acculab, Newton, PA) was used to weigh the rats before the scanning procedure (purchased 3-05-03). This scale had a weight capacity to 2000 g, with accuracy to ± 3 g. It was manually calibrated with known weights and zeroed prior

to each experiment. The reliability of this device is reported to be 0.1 g by the manufacturer. The rat was weighed on this scale in a 10-cup Rubbermaid® bowl. The cervices of the euthanized rats were dissected, trimmed, and weighed. Each cervix was weighed *ex vivo* with a Denver Instrument, XE-50 Precision Balance Scale (Denver Instruments, Ananda, CO), with a capacity to 50 g, readability to 0.1 g, and reliability of 0.1 g. The scale was leveled and calibrated with known calibration weights.

Before each cervix was weighed, the scale was calibrated to zero with a piece of weighing paper placed on the scale. The length, width, and depth of each cervix was measured with a digital caliper (Model CD-6" CS, Mitutoyo Corporation, Japan) that had an accuracy of ±10 μm and a measuring range of 0–150 mm. The length, transverse, and anterior-posterior diameters of the cervix were measured by a single examiner, the author of this study. A section to the left side of the cervix at the level of the internal os to the external os was cut free-hand on an oblique angle with a razor blade, placed in formalin, and sent for histology and stained with Masson's Trichrome stain for collagen. The rest of the cervix was immediately flash frozen in liquid nitrogen and stored in a scientific freezer at -70 °C until analyzed for collagen and water content in the animal sciences laboratory at UIUC at the end of the study. The analysis for water and collagen content was not conducted until all of the specimens were collected at the end of the study.

2. <u>Wet and dry weight determination for water content of cervix</u>

Water content of the cervix was calculated by Standard Method 934.01 of the *Official Methods of Analysis of the Association of Official Analytical Chemists* (Association of Official Analytical Chemsts [AOAC], 2002). After the rat was euthanized

the cervix was dissected and trimmed of any excess tissue. The cervix was weighed and measured in the necropsy room at the BRL. Immediately after measuring and weighing the cervix, the portion that was evaluated for water content, hydroxyproline, and total nitrogen was flash frozen in liquid nitrogen until it formed a solid pellet. The cervix was wrapped in aluminum foil with the edges folded over twice and placed in a plastic zip-lock bag, labeled, and placed in a −70 °C Forma Bio Freezer (Forma Scientific, Marietta, Ohio) in the BRL. All of the samples remained in this freezer until the time of analysis at the end of the study. The number and weights of the pups from each pregnant rat were recorded.

The procedure for obtaining the water content of tissues has been validated for tissue samples as small as 5 mg (AOAC, 2002). The samples were placed in porcelain crucibles in a 105 °C oven over night. The crucible was removed from the oven with tongs and the specimen desiccated. The crucible was not touched with the hands as moisture on the hands would add to the weight. Possible sources of error for measuring the water content in tissues could be due to exposure of the tissue to ambient air and the loss of perfusion of the tissues due to the experiment's being carried out *ex vivo*. The time from euthanization until flash freezing the tissue was recorded for each animal. The cervix was weighed immediately after dissection and then again at the end of the experiment to determine any loss or gain of weight. The cervix specimen was protected from water exposure in the scanning tank by wrapping it in plastic wrap, folding the edges twice, and taping the specimen to a holder in the water tank for scanning. Figure 15 displays the specimen in the holder.

Figure 15. Cervix specimen wrapped in plastic wrap and taped to holder to be placed in water tank.

3. Hydroxyproline and total nitrogen analysis

After the specimen was analyzed for wet and dry weight, in duplicate, 0.01 g of the desiccated sample was weighed, placed in 13 × 100 mm screw cap test tubes, 0.1 mL 6N HCL added to each tube, vortexed, and allowed to hydrolyze in a 105 °C oven for 16 hours. Hydroxyproline method 990.26 and total nitrogen content method 992.15 of the Association of Official Analytic Chemists were used for this study (AOAC, 2002). The protocols for these methods are listed in Appendix B and Appendix C, respectively. The method of the Association of Official Analytic Chemists was used for this study (AOAC, 2002). The method was tested and validated before the study began on 4 nonpregnant postpartum Sprague-Dawley rats. Table VIII lists the demographics, characteristics and hydroxyproline results for these rats.

TABLE VIII

VALIDATION SAMPLE OF HYDROXYPROLINE AND WATER CONTENT FROM PILOT NONPREGNANT RATS

Sprague Dawley Rat Number	Rat Weight (g)	Cervix Weight (mg)	Cervix length (mm)	Cervix transverse (mm)	Cervix depth, AP (mm)	Hydroxy-poline (mg/g cervix dry weight)	Cervix Water Content %
#21 (2352)	303	63.5	6.05	3.50	3.62	45.6	62.30%
#22 (2353)	312	62.5	4.86	3.47	3.34	54.4	70.59%
#31 (2354)	272	57.8	5.90	3.04	2.48	30.2	66.67%
#32 (2355)	260	54.8	6.20	3.86	2.04	47.4	64.62%

4. Histology analysis

The portion of the cervix to be evaluated by histology was fixed by immersion in 10% neutral-buffered formalin. Following fixation, cervices were trimmed and processed for histopathology, embedded in paraffin, and sectioned at 5 μm sections. The specimen on the slide was then stained with Masson's Trichrome (MT) for quantitative identification and evaluation of muscle and connective tissues layers. Masson's Trichrome stain utilizes three dyes (Biebrich Scarlet, Weigert's Hematoxylin, and Aniline Blue) to selectively stain muscle, collagen fibers, fibrin, and erythrocytes. Masson's Trichrome dye stains the collagen fibers blue.

Quantitative measurements of the layers of the cervices were made using a Nikon microscopic imaging workstation consisting of a Nikon E600 research microscope. A transverse and a longitudinal section of each cervix were photographed at 20X magnification and saved as digital images. The images were then colorized with the Adobe® Photoshop software program. A blue stained area of collagen was selected with the magic brush feature in the Adobe® Photoshop program. With the feature "select

similar," all blue areas on the histology slide were selected with a tolerance of 24 shades lighter or darker than the selected blue and were colorized to black. Then the parts of the slide that were not colorized black were changed to white. The number of black and white pixels on each slide was counted with a Matlab program written by Jonathan Mamou, Ph.D. The total number of black and white pixels was recorded as well as the black to white ratio of pixels in an attempt to develop a method to quantify the amount of collagen in the cervix samples from histology slides. Figure 16 displays an example of the process of colorizing a histology slide to count the black and white digital pixels.

G. Procedure for Data Collection

Methodology development, testing, and characterization of the transducers were conducted between January and August 2004. During this time 48 experiments were conducted and 14 nonpregnant rats were scanned. The nonpregnant rats used during

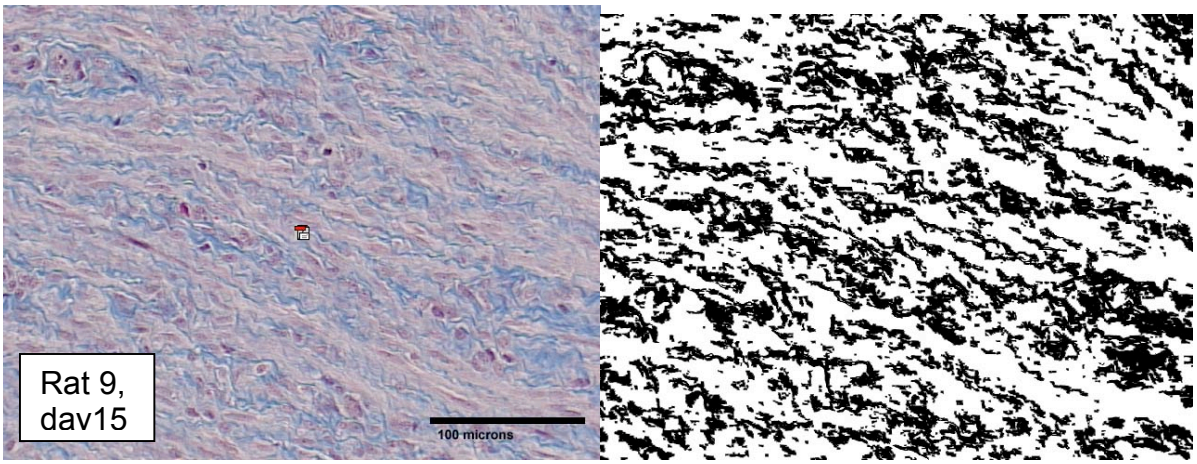

Figure 16. Histology slide of a day 15 pregnant Masson's stained cervix slide which was colorized in an attempt to objectively quantify the amount of collagen on each slide.

this period of methodology development were either going to be euthanized or used in another investigator's study. Data for the study were collected between August and December 2004. Analysis of the histology slides occurred between February and March 2005. The following sequence was followed for data gathering:

1. The rats were ordered every week from Harlan (Indianapolis, IN) and delivered to the animal care facility every Wednesday morning. Depending on gestational age availability, one to three rats were scanned per day.

2. Before humanely euthanizing the animals, the experiment was set up by placing degassed water in the tank and setting up the transducer and the pulser/receiver. The same settings on the pulser/receiver were used to collect all of the ultrasound data. The settings on the pulser/receiver were set as follows: 1 μJ of energy, 0 dB of attenuation, 40 dB of gain, 50 ohms damping, low pass filter 100 MHz, and high pass filter 3 MHz. To control for noise, averaging of the signal was set at 40. The temperature of the water in the tank was room temperature and recorded for each scan.

3. Each rat was humanely euthanized with carbon dioxide in the animal care facility at the Beckman Institute immediately before necropsy, which was performed in the BRL.

4. Each rat was weighed before necropsy and then the cervix was dissected, trimmed, and weighed. The cervix was immediately placed and sealed in plastic wrap, with the edges folded over twice and taped to the holder that would be placed in the tank of degassed water. The pups were dissected from the uterus, counted, and weighed.

5. The focal length of the transducer was 6.75 mm. The cervix specimen in the holder was placed at the approximate focal zone and adjustments in distance from the transducer were made with the Deadal® (Deadal®, Irwin, PA) micro-precision control system until a maximal signal from the tissue on the oscilloscope was obtained. Care was taken to ensure that the specimen and holder were exactly parallel to the transducer. Each cervix specimen was scanned longitudinally for 7 mm with a step size for each A-line of 20 μm. A B-mode image of each cervix specimen was evaluated for adequacy after each scan. The water temperature was recorded for each scan.

6. The data from each scan were recorded and stored in a Linux computer and were not analyzed until all of the animals in the study had been scanned.

7. After the cervix specimen had been scanned, it was again weighed and approximately one fifth of the cervix was cut free-hand with a razor blade longitudinally from the internal os to the external os on the left side of the cervix for histology. This section was immediately placed in a labeled glass container with formalin and the rest of the cervix specimen was flash frozen with liquid nitrogen. After flash freezing, the specimen was wrapped in aluminum foil with its edges folded over twice, placed in a zip-lock small plastic bag and placed in the -70 °C freezer in the BRL.

8. The following times were recorded: time of euthanasia, time the specimen was placed in the plastic wrap, and the time the specimen was frozen.

H. Ethical Considerations

Ethical considerations in conducting animal research include appropriate training of personnel handling laboratory animals, using appropriate numbers of animals, using the appropriate animal model to answer the research questions, and adhering to

accepted research protocols in approved facilities. Personnel conducting research with live animals were required to complete a 1-hour theory course at UIC entitled "Essentials for Animal Research" (GC470). This course provided classroom training in the ethical conduct in the use of live animals for research, animal model selection, laboratory animal anesthesia theory, euthanasia, protocol development, and federal and institutional rules and regulations. At UIUC an online course in the ethical treatment of animals was completed. An additional course was completed (GC471) at UIC, which provided hands-on supervised training with experimental techniques with laboratory animals. This training included theory and clinical sessions on animal model selection, care and practice safe handling of laboratory animals, administering anesthesia and sedation, surgery, and necropsy.

The animal facilities at UIUC and the Beckman Institute are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care-International (AAALAC) and comply with guidelines with the Office for Laboratory Animal Welfare (OLAW), which is required for any research involving National Institutes of Health funds. Both UIC and UIUC have animal welfare assurances with the Office of Laboratory Animal Welfare at the National Institutes of Health. Approved research protocols were obtained at UIC and UIUC by the Institutional Animal Care Committees. The BRL employs a full-time veterinarian to supervise and assist investigators conducting animal research.

Timed-pregnant Sprague Dawley rats were chosen as the animal model to study cervical ripening in pregnancy due to the similarity with the collagen type and content of the human cervix.(Harkness & Harkness, 1959; Kokenyesi & Woessner, 1990, 1991;

Leppert et al., 2000) A power analysis estimated the number of animals needed to answer the research questions.

I. Statistical Analysis

The data were analyzed using the computer program SPSS/PC, version 13.0. The dependent variable, gestational age, was used as a surrogate for cervical ripening. The independent variables are divided into the following categories: animal demographics, procedure, ultrasound measures, and laboratory measures. The variables associated with animal characteristics were animal age, animal weight, pre- and postscan cervix weight, number pf pups, and weight of pups. The variables associated with the procedure were time of euthanasia and duration of the scan. The variables associated with the ultrasound measures were attenuation slope and intercept coefficients for each gestational age group, scatterer diameter, scatterer acoustic concentration, and scatterer strength factor. The laboratory variables were percent of hydroxyproline, μg hydroxyproline/mg wet weight of cervix, percent of water in the cervix, and quantification (number and ratio of black and white pixels from colorized slides) of collagen from the Masson's Trichrome stained histology slides.

Descriptive statistics were calculated for all of the dependent variables and analyzed by group. The level for statistical significance was set at alpha = .05. The statistical tests for each of the research questions were as follows:

1. To identify and quantify with enhanced B-mode ultrasound the microstructural changes in the cervix of the pregnant rat associated with cervical ripening over the course of gestation. Working hypothesis: Ultrasound-detected microstructural changes due to gestational changes in cervical collagen and water

content are detectable and quantifiable from the processed frequency-dependent ultrasound backscatterer signals (enhanced B-mode ultrasound).

Ultrasound attenuation was analyzed over the power spectrum, with linear regression and coefficients for the slope and intercept for each gestational age group calculated. One way ANOVA (2-tailed tests) were conducted on ultrasound scatterer diameter, scatterer acoustic concentration, and scatterer strength factor to determine whether there were significant differences in the means of the independent variables with gestational age. Tukey post hoc tests were conducted on all ANOVA tests to determine which groups were significantly different from each other.

2. To determine whether there is a relationship between gestational age, ultrasound scatterer diameter, scatterer acoustic concentration, scatterer strength factor and per cent hydroxyproline and percentage of water in the cervix. Working hypothesis: Quantitative ultrasound measurements of the cervix will be correlated with hydroxyproline and water content of the cervix, reflecting cervical ripening. One way ANOVA (2 tailed tests) were conducted on ultrasound scatterer diameter, scatterer acoustic concentration, and scatterer strength factor to determine whether there was significant relationship with gestational age. Tukey post hoc tests were conducted on all ANOVA tests to determine which groups were significantly different from each other. Pearson correlations were calculated to assess the correlations among ultrasound scatterer diameter, scatterer acoustic concentration, scatterer strength factor, and gestational age group, water content, and percentage of hydroxyproline.

3. To determine whether a statistical model will significantly predict group classification (gestational age) from the ultrasound variables of scatterer diameter,

acoustic concentration, and scatterer strength factor. The statistically significant variables from the ANOVA (ultrasound scatterer diameter, scatterer acoustic concentration, scatterer strength factor, and per cent hydroxyproline) were entered into a discriminant function analysis equation. The data entered into the function were evaluated for normality, equality of group means, equality of covariance, and contribution of each variable to the discriminant function. Percent correct classification of gestational age groups was calculated from the variables entered into the discriminant function. Discriminant function coefficients of the independent variables were used to create a model to predict gestational age.

IV. RESULTS

A. Description of the Sample

Eighty Sprague-Dawley rats were entered into the study, including 67 virgin timed-pregnant rats and 13 virgin nonpregnant Sprague-Dawley rats. The 13 nonpregnant rats were a group of rats that were scheduled to be euthanized from another investigator's study, thus were significantly older in age than the timed-pregnant rats purchased specifically for this study, $F(5, 72) = 78.26$, $p < .0001$. Two rats were excluded from the analysis. One rat, number 38, was 17 days pregnant and had 11 early aborted fetuses in the uterus and 7 live fetuses. None of the other rats in the study had any aborted rat fetuses at necropsy. The other excluded rat was a day 21 rat. During the necropsy, a pup was delivering through the cervix, thus the rat was not used in the analysis.

Complete data on 78 rats were collected for this study, 13 rats in each of six gestational age groups. The ages of the groups were as follows: nonpregnant, day 15, day 17, day 19, day 20, and day 21 of pregnancy. Two additional day 21 rats, one day 20, and two day 19 rats delivered before they could be euthanized, scanned, or entered into the study. Backscatter data was collected on all 13 rats in each group. Insertion loss data, in addition to backscatter data, was collected on 3 rats in each group for the last 18 rats in the study.

Table IX displays the characteristics of the sample by groups. Rats gained a significant amount of weight from nonpregnant to day 21 of pregnancy, $F(5, 72) = 14.86$, $p < .0001$. There were no statistically significant differences in the number of pups per

TABLE IX

CHARACTERISTICS OF THE SAMPLE BY GROUPS

Gestational Age of Rat	*N*	Rat Age, Days		Rat Weight, g		Number of Pups		Pup Weight, g		Duration of Experiment, Minutes		Water Temperature °C	
		Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Nonpregnant	13	215	(25)	301	(17)	—	—	—	—	99	(51)	21.5	(.4)
Day 15	13	108	(14)	286	(15)	13	(1)	0.4	(.1)	90	(27)	21.6	(.5)
Day 17	13	108	(14)	298	(16)	12	(2)	1.1	(.2)	95	(33)	21.6	(.5)
Day 19	13	108	(12)	323	(39)	11	(3)	2.7	(.4)	108	(53)	21.6	(.8)
Day 20	13	110	(13)	349	(24)	13	(3)	4.2	(.3)	88	(32)	21.5	(.5)
Day 21	13	111	(11)	353	(36)	13	(2)	5.4	(.6)	105	(37)	21.4	(.5)

gestational age groups, $F(4, 60) = .84$, $p = .51$. As expected, pups were significantly heavier as pregnancy progressed, $F(4, 60) = 432.82$, $p < .0001$.

The time from euthanasia of the animal until the time the tissue was either frozen or placed in formalin is displayed in Table IX as duration of experiment. There were no significant differences in the duration of experiment between groups, $F(5, 72) = .51$, $p = .77$. The water temperature in the scanning tank was measured and recorded during each experiment. There were no significant differences in water temperature in the scanning tank between groups, $F(5, 72) = .29$, $p = .92$.

Table X displays the cervix weights and measures by groups. The mean and standard deviations of pre- and postscan cervix weights in Table X do not appear be very different; however, the related samples *t* test indicated that there may have been a trend toward significant differences, $t(77) = 1.78$, $p = .08$ (two-tailed). The differences between the pre- and postscan cervix weight were evaluated with a one-way ANOVA and not found to be significant, $F(5, 72) = 1.36$, $p = .25$. The nonpregnant rats had the lightest cervices and the day 21 rats had the heaviest cervices. There were significant differences in the length of the cervix between the groups, $F(5, 72) = 5.26$, $p < .0001$. The length of the cervix was significantly different between day 21 compared to groups 17 and 19. There were significant differences in the width of the cervix between the groups, $F(5, 72) = 9.82$, $p < .0001$. The width of the cervix was significantly different between day 21 and all of the other groups. Except for day 19, the cervix width was larger as pregnancy progressed. There were significant differences in the depth of the cervix between the groups, $F(5, 72) = 3.28$, $p = .01$. The depth of the cervix was significantly different between day 21 and the day 15 and nonpregnant groups.

TABLE X

CERVIX WEIGHTS AND MEASURES BY GROUPS

Gestational Age of Rat	*N*	Prescan Cervix Weight[a] Mean	(SD)	Postscan Cervix Weight[a] Mean	(SD)	Difference Pre- and Postscan Cervix Weight[a] Mean	(SD)	Cervix Length[b] Mean	(SD)	Cervix Width[b] Mean	(SD)	Cervix Depth[b] Mean	(SD)
Nonpregnant	13	66	(13)	65	(12)	-0.4	(1.8)	5.9	(.6)	4.4	(.4)	2.9	(.4)
Day 15	13	74	(10)	74	(10)	0.5	(2.6)	6.4	(.5)	4.7	(.5)	3.1	(.5)
Day 17	13	88	(11)	87	(11)	-0.7	(2.1)	6.7	(.6)	4.7	(.7)	3.4	(.4)
Day 19	13	97	(11)	97	(11)	-0.03	(1.8)	6.8	(.8)	4.7	(.7)	3.2	(.6)
Day 20	13	99	(18)	99	(20)	-0.5	(2.6)	5.8	(.8)	5.1	(.4)	3.3	(.5)
Day 21	13	114	(23)	112	(23)	-1.7	(2.4)	5.8	(.7)	6.1	(1.1)	3.7	(.6)

[a] Weight in mg.

[b] Measured in mm.

B. Ultrasound Measures of the Cervix

Three ultrasound measures obtained for each cervix sample were acoustic scatterer diameter, acoustic concentration, and scatterer strength factor. Table XI displays the results of the ultrasound measures by groups. There were significant differences in scatterer diameter between the groups, $F(5, 72) = 10.72$, $p < .0001$. Except for the day 17 group, there was a trend toward an increase in scatterer diameter from day 15 to day 21. The day 17 group had a significantly larger scatterer diameter than both the day 21 and nonpregnant groups. Scatterer acoustic concentration represents the concentration of scatterers in the tissues. There were significant differences in acoustic concentration between the groups, $F(5, 72) = 19.47$, $p < .0001$. Acoustic concentration was significantly different from day 21 compared to day19, 15, and the nonpregnant group. Acoustic concentration was the highest in the nonpregnant group and the lowest in the day 21 group. The day 17 group had very similar acoustic concentration to day 21. Scatterer strength factor was significantly different between groups, $F(5, 72) = 26.47$, $p < .0001$. The nonpregnant group had the highest value for scatterer strength factor and the day 21 group had the lowest value. Scatterer strength factor was significantly different from day 21 compared to the nonpregnant group.

C. Compositional Measures of the Cervix

Compositional measures of cervical ripening by group are displayed in Table XII. There was a significant decrease in the percent of hydroxyproline per cervix from the nonpregnant group to the day 21 of pregnancy, $F(5,72) = 107.45$, $p<.0001$. These findings suggest that the concentration of hydroxyproline was the greatest in the nonpregnant cervix and the least at the end of pregnancy. The percent of

TABLE XI

ULTRASOUND MEASURES OF THE CERVIX

		Scatterer diameter[a] μm		Acoustic concentration[b] dB/mm^3		Scatterer Strength Factor[c] dB/mm^3	
Gestational Age of Rat	*N*	Mean	(SD)	Mean	(SD)	Mean	(SD)
Nonpregnant	13	10.4	(1.6)	-118.4	(5.9)	2.8	(1.2)
Day 15	13	8.2	(1.3)	-130.4	(7.0)	-1.5	(1.2)
Day 17	13	11.2	(0.8)	-143.7	(6.9)	-2.3	(1.7)
Day 19	13	8.6	(1.2)	-130.4	(5.1)	-1.2	(0.8)
Day 20	13	8.8	(1.0)	-136.0	(8.1)	-2.2	(1.6)
Day 21	13	10.0	(1.7)	-141.3	(10.6)	-2.7	(1.9)

[a] Scatterer diameter = Correlational length of the scatterer.

[b] Acoustic concentration = Number of scatterers/mm^3 × (average impedence of scatterers)^2.

[c] Scatterer Strength Factor = Acoustic concentration × (average radius of scatterers)^6.

TABLE XII

COMPOSITIONAL MEASURES OF THE CERVIX

Gestational Age of Rat	*N*	% Hydroxyproline/Cervix		µg Hydroxyproline/ mg Cervix		% Water/Cervix		Histology Collagen Stain, Pixels[a]		Histology Collagen/Cell Ratio[b]	
		Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Nonpregnant	13	.88	(.1)	567.7	(75)	68	(5)	270500	(53200)	.83	(.3)
Davy 15	13	.72	(.1)	532.3	(83)	69	(8)	242400	(55750)	.69	(.3)
Day 17	13	.68	(.1)	603.1	(103)	68	(5)	226700	(54600)	.61	(.2)
Day 19	13	.62	(.1)	605.4	(75)	69	(6)	249000	(37700)	.69	(.2)
Day 20	13	.64	(.04)	640.0	(131)	69	(4)	218270	(50090)	.58	(.2)
Day 21	13	.62	(.1)	706.9	(153)	68	(6)	244130	(63600)	.70	(.3)

[a] Represents the number of collagen stained pixels per digital histology slide at 20X magnification.

[b] Represents the ratio of collagen stained pixels compared to stained cells (smooth muscle, white cells) per digital histology slide at 20X magnification.

hydroxyproline per cervix was significantly different in the day 21 group compared to the day 15 and nonpregnant groups. There was a significant difference in the μg hydroxyproline/mg cervix between the groups, $F(5, 72) = 4.09$, $p < .003$. The μg hydroxyproline/mg cervix was significantly different in the day 21 group as compared to the day 15 and nonpregnant groups. The percentage of water was not significantly different between any of the groups, $F(5, 72) = .24$, $p = .94$. The histology collagen stain pixels was not significantly different between the groups, $F(5, 72) = 1.54$, $p = .19$. The histology collagen ratio was not significantly different between any of the groups, $F(5, 72) = 1.61$, $p = .17$.

D. Insertion Loss of the Cervix

Figure 17 displays the loss of energy that results when inserting the sample into the sound beam. One would expect a loss of energy to increase, even for water, as frequency increases. The insertion loss acoustic energy of the 21 day group was closest to the water path for all frequencies displayed. Table XIII displays the correlations between the insertion loss for frequencies between 30–90MHz and the water path. All of the results suggest a significant correlation between gestational age and the water path, although the correlation was stronger in the pregnant groups rather than the nonpregnant group. The nonpregnant cervix group had the greatest amount of insertion loss of energy of all of the groups. This suggests that the nonpregnant group cervix tissue, which is denser, has more loss of acoustic energy across the power spectrum of frequencies as compared to the day 21 pregnant group of cervices. Table XIV displays the attenuation coefficients obtained from linear regression analysis of the power spectrum of the attenuation experiments. The day 21 cervix slopes are similar for all of

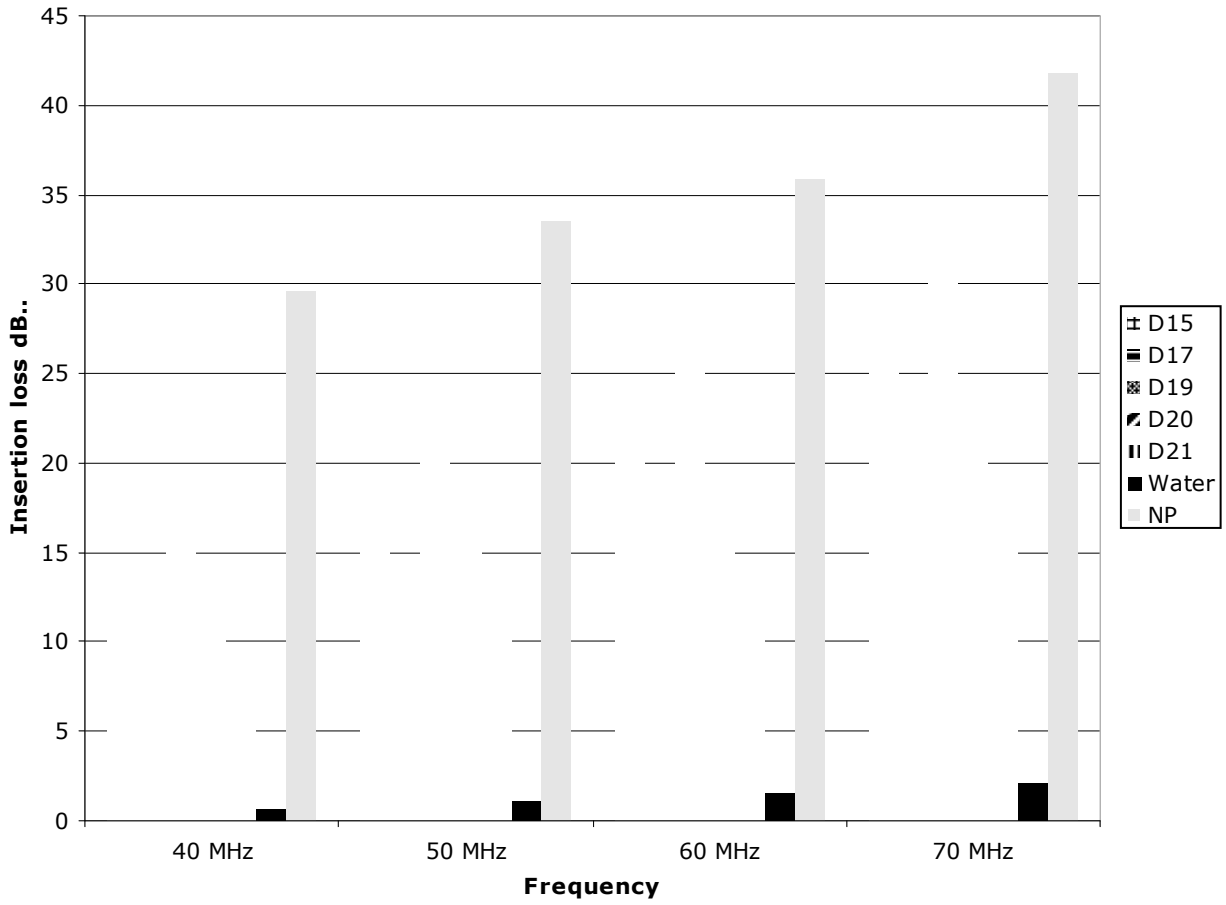

Figure 17. Insertion loss for cervix samples by groups (Temperature 21.8 °C) as compared to insertion loss of water (Temperature 22 °C) for four different frequency ranges.

TABLE XIII

TWO-TAILED PEARSON CORRELATIONS BETWEEN MEAN INSERTION LOSS OF CERVIX SAMPLES AND WATER PATH, 30–90 MHZ (*n* = 493)

	Nonpregnant	Day 15	Day 17	Day 19	Day 20	Day 21
Pearson Correlation	.82*	.95*	.97*	.97*	.97*	.96*

* p < .0001.

TABLE XIV

MEAN ATTENUATION COEFFICIENTS OF THE CERVIX BY GROUPS

	n	Nonpregnant	Day 15	Day 17	Day 19	Day20	Day 21
Slope (dB/MHz/cm)	3	2.46	2.37	1.50	2.45	2.03	1.73
Intercept (dB/cm)	3	131.73	8.32	0.29	10.21	-10.99	-12.60
r^2	3	.98	.97	.98	.98	.99	.97

the groups except for the day 17 and day 21 groups, while the intercepts are significantly different. The day 21 cervix was the closest to the power spectrum of the water path. These results suggest that there are marked insertion loss changes associated with gestation age.

E. Histology Slides

Transverse and longitudinal black pixels on the histology slides which represented collagen staining were not significantly different by group, $F(5, 72) = 1.54$, $p = .19$. The ratio of collagen staining to the staining of the smooth muscle cells on the histology slide was not significantly different by group, $F(5, 72) = 1.61$, $p = .17$.

F. Correlations

Table XV displays the Pearson correlations between ultrasound and compositional variables in the study. The percentage of hydroxyproline per cervix was significantly correlated with gestational age group, acoustic concentration, acoustic scatterer diameter, and scatterer strength factor. The ultrasound measures were significantly correlated with each other as expected. Acoustic concentration was significantly correlated with scatterer diameter and scatterer strength factor. Histology collagen stained pixels ratio were significantly correlated to acoustic concentration and scatterer strength factor but not percentage of hydroxyproline.

G. Discriminant Analysis

Discriminant analysis was employed to develop a model to predict gestational age in future studies and to determine classification of group assignment from the present data. For the purposes of this study gestational age was used as a surrogate for cervical ripening. The significant variables determined on univariate analysis were entered into the model, which were: hydroxyproline percent, acoustic concentration, scatterer strength factor, and scatterer diameter. The data were examined for linearity, normality, and outliers. Discriminant analysis first evaluated the entire model, which was found to be significant, Wilks' Lambda = .089, $\chi^2(20)$ = 173.97, $p < .0001$. The first discriminant function had an eigenvalue of 4.37 and 82.1% of the variance was explained by the model. The eigenvalue reflects the ratio of the importance of the variables in the model which classified the dependent variable. The unique contribution of each variable to the discriminant function was displayed by the pooled within-groups correlations. The percentage of hydroxyproline per cervix had the highest correlation in

TABLE XV

PEARSON CORRELATIONS OF ULTRASOUND AND COMPOSITIONAL VARIABLES

	Scatterer Diameter	Acoustic concentration	Scatterer strength factor	Hydroxy-proline %	Water %	Histology collagen ratio
Scatterer diameter						
Acoustic concentration	-.38**					
Scatterer strength factor	.09	.89**				
Hydroxyproline%	.23*	.39**	.53**			
Water %	.02	-.05	-.04	-.12		
Histology collagen ratio	.03	.39**	.35**	.14	-.17	
Gestational age group	-.08	-.52**	-.60**	-.70**	-.007	-.17

*$p \leq .01$.

**$p \leq .001$.

the model (.64), followed by scatterer strength factor (.63), then acoustic concentration (.47), and lastly scatterer diameter (.12). Entering all of these significant variables into the model, the following discriminant function equation can be used to calculate gestational age (GA).

GA = (scatterer diameter * -.63) + (acoustic concentration * -1.24) +

(scatterer strength factor * 1.82) + (%hydroxyproline * .789)

A second discriminant function analysis was conducted to evaluate only the ultrasound variables entering the significant ultrasound parameters of scatterer diameter, scatterer strength factor and acoustic concentration. The model was found to be significant, Wilks' Lambda = .187, $\chi^2(15)$ = 121.617, $p < .0001$. The first discriminant function had an eigenvalue of 1.889 and 68.6% of the variance was explained by the model. The unique contribution of each variable to the discriminant function was displayed by the pooled within-groups correlations. The scatterer strength factor (.98) had the highest correlation in the discriminant function, followed by acoustic concentration (.72), and lastly scatterer diameter (.19).

Entering all of these significant ultrasound variables into the model, the following discriminant function equation can be used to calculate gestational age:

GA = (Acoustic concentration * -.82) + (Scatterer strength factor * 1.671) +

(Scatterer Diameter *-233)

H. Group Classification and Cross-valididation

Table XVI summarizes the results of group classification and misclassification by discriminant analysis and the four variables of scatterer diameter, scatterer strength factor, acoustic concentration and percent hydroxyproline. Overall, 65.4% of the cases

TABLE XVI

GROUP CLASSIFICATION FOR ORIGINAL DATA AND CROSS-VALIDATED DATA BASED UPON SCATTER DIAMETER, ACOUSTIC CONCENTRATION, SCATTERER STRENGTH FACTOR, AND % HYDROXYPROLINE

	Nonpregnant		Day 15		Day 17		Day 19		Day 20		Day 21	
Group	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Original Sample												
Nonpregnant	13	**(100.0)**	0		0		0		0		0	
Day 15	0		9	**(69.2)**	1	(7.7)	1	(7.7)	2	(15.4)	0	
Day 17	0		0		11	**(84.6)**	0		0		2	(15.4)
Day 19	0		2	(15.4)	1	(7.7)	7	**(53.8)**	1	(7.7)	2	(15.4)
Day 20	0		1	(7.7)	1	(7.7)	3	(23.1)	5	**(38.5)**	3	(23.1)
Day 21	0		0		3	(23.1)	2	(15.4)	2	(15.4)	6	**(46.2)**
Cross-validated Sample												
Nonpregnant	13	**(100.0)**	0		0		0		0		0	
Day 15	0		9	**(69.2)**	1	(7.7)	1	(7.7)	2	(15.4)	0	
Day 17	0		1	(7.7)	9	**(69.2)**	0		0		3	(23.1)
Day 19	0		3	(23.1)	1	(7.7)	6	**(46.2)**	1	(7.7)	2	(15.4)
Day 20	0		1	(7.7)	1	(7.7)	3	(23.1)	5	**(38.5)**	3	(23.1)
Day 21	0		0		3	(23.1)	2	(15.4)	3	(23.1)	5	**(38.5)**

were correctly classified. However, 100% of the nonpregnant cases were correctly classified and 84.6% of day 17 but only 46.2% of day 21 cases were correctly classified. Although there were significant errors in the misclassification of gestational age, none of the pregnant groups were classified nonpregnant and it appeared that the errors were close to the gestational age group that was intended. For example, if the intended group were day 21, 23.1% were classified day 20, 15.4% day 19, and none day 15. Conversely, for the day 15 group, 69.2% were classified correctly, 15.4 % were incorrectly classified as day 19 and none as day 21. The model did a better job of correctly classifying gestational age in the nonpregnant groups and day 15 and 17 groups than the rest of pregnancy.

The results of the cross-validation of the discriminant analysis are listed in Table XVI. The cross-validated sample is a calculation that is run by the SPSS statistical program on a portion of the data. There was no change in correct classification of group assignment for the nonpregnant group and the day 15, day 17, and day 20 groups. The day 19 and day 21 groups decreased slightly in correct assignment.

Figure 18 displays the discriminant function plots of functions 1 and 2, displaying the distances between group classification and group centroids. Discriminant functions 1 and 2 explained 96.9% of the variance of the model. Note that there is a definite distance between the nonpregnant group and all of the pregnant groups. The day 21 group also has quite some distance from the day 15 group.

Table XVII summarizes the results of group classification and misclassification by discriminant analysis and the ultrasound variables of scatterer diameter, scatterer strength factor, and acoustic concentration. This analysis evaluated only the ultrasound variables that could be measured during an ultrasound examination during pregnancy. Overall 53.8% of the cases were correctly classified. However, 100% of the nonpregnant cases were correctly classified and 61.5% of day 17 but only 15.4% of the day 21 cases were correctly classified. This time two cases of day 17 pregnant rats were classified nonpregnant and it appeared that the errors were close to the gestational age group that was intended. For example, if the intended group was day 21, 23.1% were classified day 20, 7.7% day 19, and 38.5% day 17. Conversely, for the day 15 group, 61.5% were classified correctly, 7.7 % were incorrectly classified as day 19 and none as day 21. The model did a better job of correctly classifying gestational age in the nonpregnant groups and day 15 and 17 groups than the rest of pregnancy.

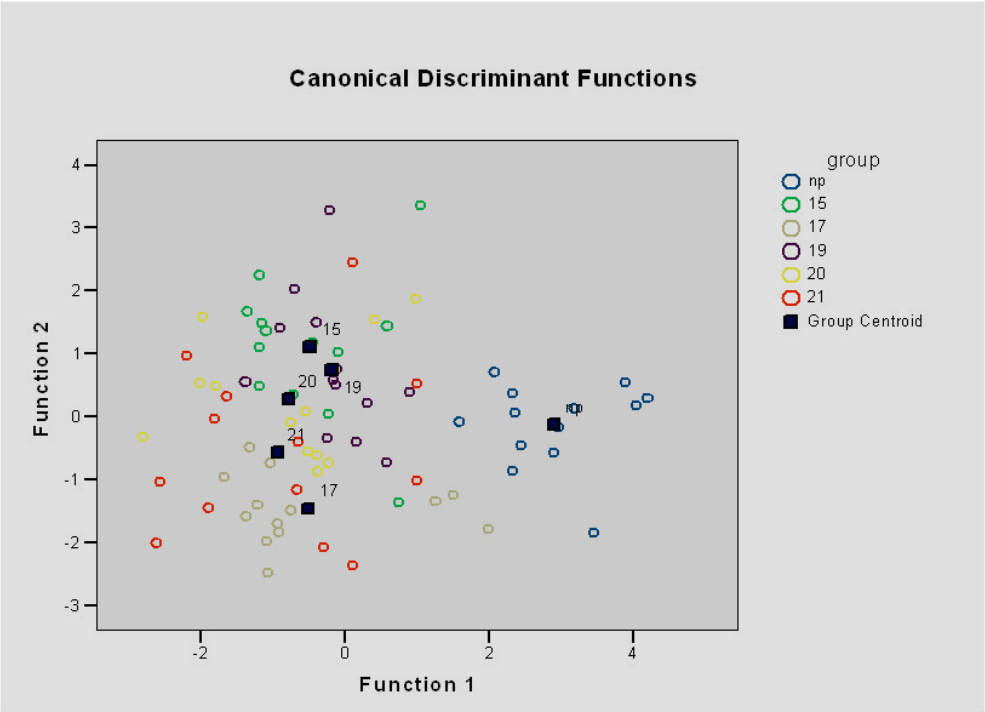

Figure 18. Discriminant function plot of scatterer diameter, scatterer strength factor, acoustic concentration and hydroxyproline percent. Plot of functions 1 and 2 with group centroids. The plot illustrates the distances between group assignments.

TABLE XVII

GROUP CLASSIFICATION FOR ORIGINAL DATA AND CROSS-VALIDATED DATA BASED UPON ULTRASOUND MEASURES OF SCATTER DIAMETER, ACOUSTIC CONCENTRATION, AND SCATTERER STRENGTH FACTOR

	Nonpregnant		Day 15		Day 17		Day 19		Day 20		Day 21	
Group	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Original Sample												
Nonpregnant	13	**(100.0)**	0		0		0		0		0	
Day 15	0		8	**(61.5)**	1	(7.7)	1	(7.7)	3	(23.1)	0	
Day 17	2	(15.4)	0		8	**(61.5)**	0		0		3	(23.1)
Day 19	0		4	(30.8)	1	(7.7)	5	**(38.5)**	3	(23.1)	0	
Day 20	0		2	(15.4)	0		2	(15.4)	6	**(46.2)**	3	(23.1)
Day 21	0		2	(15.4)	5	(38.5)	1	(7.7)	3	(23.1)	2	**(15.4)**
Cross-validated Sample												
Nonpregnant	12	**(92.3)**	0		1	(7.7)	0		0		0	
Day 15	0		6	**(46.2)**	1	(7.7)	3	(23.1)	3	(23.1)	0	
Day 17	2	(15.4)	0		7	**(53.8)**	0		0		4	(30.8)
Day 19	0		4	(30.8)	1	(7.7)	5	**(38.5)**	3	(23.1)	0	
Day 20	0		2	(15.4)	0		2	(15.4)	5	**(38.5)**	4	(30.8)
Day 21	0		2	(15.4)	6	(46.2)	1	(7.7)	3	(23.1)	1	**(7.7)**

Clearly removing the hydroxyproline variable from the model decreased the correct classification percentage of the model.

The results of the cross-validation of the discriminant analysis are listed in Table XVII. The cross-validation for the second model performed less accurately, with 46.2% of the cases correctly classified. Figure 19 displays the discriminant function plots of functions 1 and 2, displaying the distances between group classification and group centroids. Discriminant functions 1 and 2 explained 98.6% of the variance of the model. Note that there is a definite distance between the nonpregnant group and all of the pregnant groups. The day 21 group and the day 17 groups had a larger variation of values than those in Figure 19.

I. Summary

Gestational age differences in ultrasound and compositional measures of cervical ripening were measured and detected in this study. Significant relationships between gestational age and scatterer diameter, scatterer strength factor, acoustic concentration, and attenuation were found. Hydroxyproline was significantly correlated with gestational age group, scatterer diameter, scatterer strength factor, acoustic concentration, but not with histology slide collagen ratio. Significant gestational age differences in the insertion loss of energy over the power spectrum of frequencies were found. Discriminant analysis was correctly classified 100% of the non-pregnant group, 69.2% of day 15, 84.6% of day17, 53.8% of day 19, 38.5% of day 20 and 46.2% of day 21 group. Overall the model predicted 65.4% of group membership.

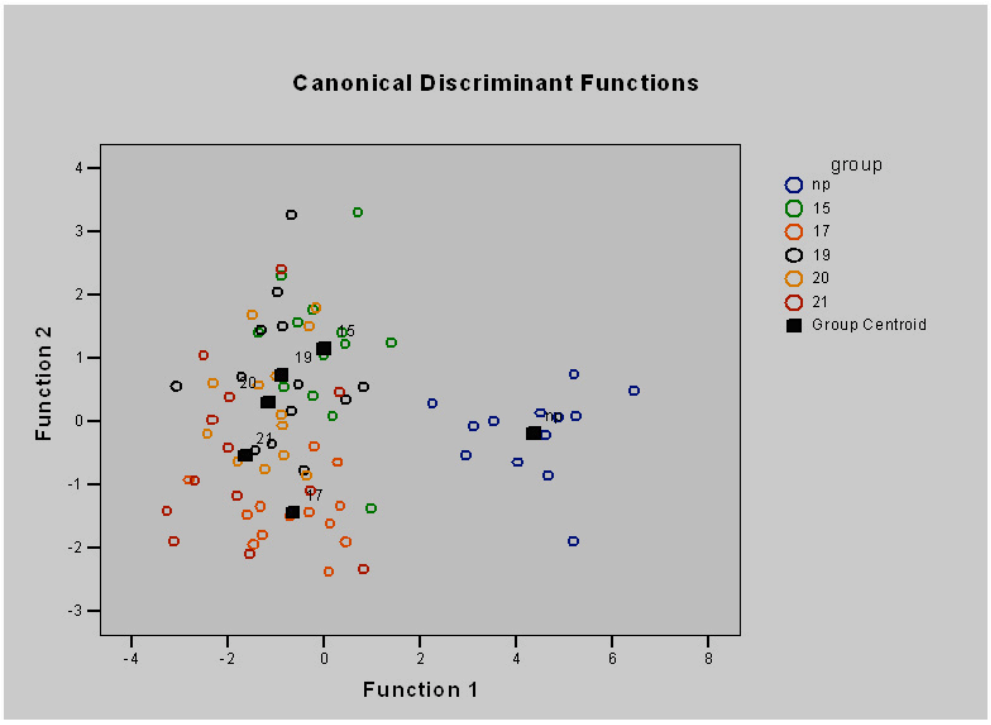

Figure 19. Discriminant function plot of scatterer diameter, scatterer strength factor, acoustic concentration. Plot of functions 1 and 2 with group centroids. The plot illustrates the distances between group assignments.

V. DISCUSSION

A. Concept Testing

The results of this study confirmed the following hypotheses of the conceptual framework of this study: (a) the hydroxyproline content (μg hydroxyproline/mg cervix) of the cervix increased as the pregnancy advanced in the rat; (b) the hydroxyproline concentration (% hydroxyproline) decreased as pregnancy advanced; (c) ultrasound acoustic concentration of the scatterers decreased as the pregnancy advanced, and (d) hydroxyproline had significant correlations with rat groups and ultrasound variables of scatterer diameter, acoustic concentration, and scatterer strength factor. Ultrasound insertion loss detected significant differences between the rat groups and the water path. Compositional analysis of the cervix tissues for water content did not detect a difference between the rat groups.

1. Ultrasound measurements of cervical ripening

a. Scatterer diameter and acoustic concentration

Scatterer diameter significantly differed between the nonpregnant and pregnant rat groups. Except for day 17, there was a trend for the scatterer diameter to increase as gestation advanced. It is not clear why the day 17 group scatterer diameter was larger than either the nonpregnant or the day 21. Also the day 21 scatterer diameter was similar to the nonpregnant group. Thus, there was not a linear change in the diameter of the scatterers as there was in the acoustic concentration, suggesting that the number of scatterers per unit volume changed more than the size of the scatterers as gestation advanced.

Acoustic concentration decreased from the nonpregnant group to day 21, thus suggesting there was more space between the scatterers as pregnancy advanced. It was hypothesized in the conceptual model that there would be a decrease in acoustic concentration of the scatterers due to more space between the collagen fibrils due to cervical ripening (Golichkowski et al., 1980; Leppert et al., 2000; Winkler & Rath, 1999). Substances which were not measured as part of this study, such as decorin and hyaluronan, are abundant in the extracellular matrix of the cervix during the ripening process (Golichkowski et al., 1980; Leppert, 1995; Leppert et al., 2000). The function of decorin in the cervical ripening process has been reported to orient and organize the collagen fibrils by regulating the fibrillar distance (Leppert et al., 2000). Other inflammatory factors such as white cells and cytokines could be responsible for the decrease in acoustic concentration.

In commercial ultrasound systems, resolution of structures in the tissues depends upon the frequency and the depth of resolving the intended structures. Only structures greater than the acoustic wavelengths for the bandwidth of frequencies (for example, for 70 MHz the wave length in water is 21.1 μm) can be resolved. Therefore, using broad bandwidth transducers has the potential to resolve a variety of structures of different sizes, shapes, and impedances. Resolution in backscatter ultrasound can be evaluated by the *ka* value (*k* = the number of waves, *a* = the average radius of the scatterers). The *ka* value = (2 * π)/wavelength * average radius of the scatterer. The *ka* values between 0.8 and 1.2 are optimal for the most sensitive resolution. The calculated ka for this study was 1.2 (*a* = 4.4, frequency = 70MHz). The calculation of *ka* takes into account the transducer properties (wavelength) as well as the tissue properties

(scatterer radius) to provide an indication of whether the appropriate instruments are being used and if the best resolution has been achieved. Very high frequency transducers were chosen for this study due to knowledge that the collagen fibers in bundles were very small, measuring in the range of 0.5–3 μm.

Third, it is possible that the form factor that was used to calculate the backscatter data was not a perfect fit for this type of tissue. For this study, the Gaussian form factor was used, which captures the geometry of the scattering. The Gaussian form factor assumes that the scatterers are spherical and randomly distributed (Oelze et al., 2002a). Once experience has been obtained with the acoustic and histologic properties of a specific tissue type, assumptions in the mathematical model can be modified for future studies.

Although differences in scatterer diameter were detected only between the day 21 group and the day 15 group, and the day 20 group from the day 17 and nonpregnant groups, the scatterer diameter (r = +.23) and acoustic concentration (r = −.39) were significantly correlated with percent of hydroxyproline in the cervix. These correlations did not account for all of the variance in the composition of the cervix. However, they make sense, as there are other structures (smooth muscle cells, white cells) and substances (enzymes, GAGs, water) occupying space in the cervix.

Other investigators have found correlations with hydroxyproline in other tissues in the body with ultrasound scattering, suggesting a strong role of collagen as a source of the scattering (Handley et al., 2003; Hoffmeister, Whitten, Kaste, & Rho, 2002; O'Donnell, Mimbs, & Miller, 1981; Pohlhammer & O'Brien, 1981). Ultrasound scatterer coefficients were significantly related to the percent wet weight of collagen in the brain,

liver, kidney, and tendon (Pohlhammer & O'Brien, 1981). The integrity of myocardial collagen has been quantitatively assessed with backscatter ultrasound, suggesting that the amount of scattering was related to collagen (Lucarini et al., 1994).

b. Scatterer strength factor

Scatterer strength factor is acoustic concentration × (average radius of scatterers)^6, which reflects the strength of the signal returning from the scatterers. Scatterer strength factor was significantly correlated to percent hydroxyproline (+.53). The nonpregnant group had the most densely packed tissue, having the least acoustic concentration. Thus, it stands to reason that the nonpregnant group had the highest value (2.8) for scatterer strength factor and the day 21 group had the lowest value (-2.7).

2. Compositional analysis

a. Hydroxyproline content of the cervix

The amount of hydroxyproline in the cervix was analyzed as the percentage of hydroxyproline per cervix specimen, representing the concentration of hydroxyproline in the cervix. The μg hydroxyproline/mg cervix was also calculated, representing the content of hydroxyproline in the cervix. Confirming other studies, the concentration of hydroxyproline per cervix decreased as pregnancy advanced and the amount of hydroxyproline per mg of cervix increased (Danforth & Buckingham, 1973; Golichkowski et al., 1980; Harkness & Harkness, 1959; Kokenyesi & Woessner, 1989). The correlation of percentage of hydroxyproline and μg hydroxyproline/mg cervix to group, scatterer diameter, acoustic concentration, and scatterer strength factor affirms the theoretical concepts of the model. Thus these findings confirm that ultrasonic

scattering detected the gestational age dependent changes in the cervix associated with cervical ripening.

b. Water content of the cervix

The water content of the cervix did not significantly change with gestational age group and was not correlated with any of the ultrasound measures of scatterer diameter, acoustic concentration, and scatterer strength factor. Other investigators have found a 5–8% increase in water content of the cervix from nonpregnant to day 21 (Golichkowski et al., 1980; Harkness & Harkness, 1959; Kokenyesi & Woessner, 1991; Leppert et al., 2000; J. E. Norman et al., 1998; Winkler & Rath, 1999; Woessner, 1976). This study detected 68–69% water content in the cervices in all of the groups. This was an unexpected finding. All of the animals and tissue samples were processed with similar methods. Except for the nonpregnant rats, there were no significant differences in rat weights, ages, or number of pups. Among all of the animals there were no significant differences in the duration of the experiment or water tank temperature.

There are several possible explanations why differences in water content were not detected between the groups. First, it is possible that there were no differences. Second, other substances may have been responsible for occupying the space in the extracellular matrix of the cervix that would account for the decrease in acoustic concentration and hydroxyproline concentration, such as hyaluronan, decorin, cytokines, and enzymes (Golichkowski et al., 1980; Jeffrey, 1991; Kobayashi, Sun, Tanaka, Kondo, & Terao, 1999; Kokenyesi & Woessner, 1991; Ledingham, Denison, Riley et al., 1999; Leppert et al., 2000; Luque et al., 1998). Glycosaminoglycans, such

as hyaluronan, increased 17-fold by the end of pregnancy in the rat cervix in one study (Golichkowski et al., 1980). The animal science laboratory that performed the compositional analysis has a long history of conducting such analyses, therefore significant errors were not expected. An interesting finding was that there was a significant correlation between the insertion loss for the cervix tissue samples and the insertion loss for water. Not only was the correlation high for all of the groups, but the correlation was higher for the pregnancy groups than the nonpregnant group. There was a trend that the insertion loss for the cervix samples was closer to that of water as pregnancy progressed. The relationship between water and insertion loss is intriguing and deserves more investigation.

B. Limitations of the Study

A limitation of the study was that the rats were not scanned alive. It was the initial goal of this research to scan the animals alive, similar to the method that would be used with human women. The animals were not scanned alive due to methodological problems with transducer design. During this process a tremendous amount of experience and information was gained. There is a lack of information in the engineering literature concerning the features of high frequency transducers that would be best suited for the properties of the cervix tissue. Through experience with this research, the ceramic transducer element and PVDF-TrFE transducer materials did not perform well for the cervix tissue. The lithium niobate transducer element was sensitive, had an adequate bandwidth, and had a low signal to noise ratio. For future studies, a miniature vaginal probe transducer for a rat, manufactured with a lithium niobate element, would be optimal for scanning the animal alive under anesthesia.

The other limitation was the number of animals used in the insertion loss experiments. The insertion loss experiment was a separate measurement and experiment in addition to collecting the backscatter data. The attenuation coefficient is a calculation used in the backscatter calculation. Through an error it was not collected in all of the cases.

The nonpregnant group was significantly older than the rest of the rats in the study. All of the pregnant rats were of similar age. The original design did not budget for a nonpregnant group. It was decided to collect data on this group as they were a group that was scheduled to be euthanized. Valuable information was learned by adding this group, regarding correct group classification, insertion loss, acoustic concentration, and scatterer strength factor. In future studies it will be important to budget for enough rats to ensure that all groups are similar in characteristics in order to make meaningful comparisons.

C. Recommendations for Research

1. Scan the animals *in vivo.* Scanning the animals alive will eliminate methodological concerns regarding the integrity the cervix tissue samples before processing.

2. Scan the animals over a wider range of gestational ages. The differences between the nonpregnant group and pregnancy were large. The differences between day 20 and 21 of pregnancy were small. Clinically one would like to detect differences in the cervix during pregnancy. In future studies it would be helpful to study a group in early pregnancy.

3. Scan the animals with a range of frequencies (lower frequencies). Presently human vaginal probe ultrasound transducers are commercially available only in the range of 5–12MHz. The human cervix is also 2–3 cm thick and 4 cm long. A very high frequency transducer would have limited penetration. Therefore, it would be appropriate to test the concept with a range of frequencies.

4. Measure hyaluronan, decorin, and possibly cytokines in the cervix samples as a measure of cervical ripening. By scanning the animal alive, the measurement of substances that may explain the decrease in acoustic concentration would be helpful to explain the model.

5. Develop several lithium niobate vaginal probe transducers in a range of frequencies for the rat and human. Methodology development in this study found the lithium niobate material for the transducer element performed well with cervix tissue. The transducer needs to be designed to be small enough to fit in the rat vagina. To scan humans, single element lithium niobate transducers of varying frequencies would be attached to a standard existing vaginal probe transducer.

6. Develop the methodology to measure insertion loss *in vivo.* The insertion loss of the cervix samples was obtained from an experiment that required the specimen to be excised from the rat and measured with the specimen in a water tank. Current research suggests than an *in vivo* measurement scheme may be feasible for estimating insertion loss when scanning the live animal and hence in humans (Bigelow & O'Brien, in press; Bigelow, Oelze, & O'Brien, 2005).

7. Improve the form factor model. Work with engineers to determine whether the form factor model can be improved upon. The nonpregnant group was more

accurately predicted than the day 21 group. Are there mathematical considerations to better predict the day 21 group?

8. Test the discriminant function equation. With subsequent data, test the present discriminant function equation.

9. Test the concept in humans and compare with cervical length measurements.

D. Implications for Practice

The results of this study indicate that it is possible to detect cervical ripening with backscatter ultrasound technology. The discriminant classification results are encouraging for a first attempt to test this research concept. Of interest is that the discriminant function analysis predicted group membership for 100% of the nonpregnant group. Exactly why the nonpregnant group was classified with higher accuracy than the other groups is unclear. Although the model has a high correlation with group and hydroxyproline, the correct classification of group was the lowest for the day 21 group. In Table XVI 9 of 13 group 21 cases were classified as either day 20 or 21. Correct classification of group membership when using the ultrasound variables, not just a high correlation, would be critical to make this technology useful for humans in clinical practice (Bland & Altman, 2003). Exactly how many incorrect group assignments would be tolerated depends upon the severity of the outcomes to patients and the consequences of treatments. Presently, no treatments are available to modify the course of premature cervical ripening. Obstetrics has had a history of accepting poorly tested interventions (bloodletting, episiotomy, thalidomide, diethylstilbestrol) and

technology (electronic fetal monitoring) into mainstream practice, resulting in harm or little benefit (Grimes, 1993).

The results of this study have the potential to impact the detection of premature cervical ripening humans, which is a significant public health problem. Solving this public health problem has the potential to save lives and to improve the quality of life by preventing the disabilities associated with the survival of preterm infants. It is only after cervical ripening can be detected that interventions to modulate the process can be developed. Unlike in the rat, in humans it is essential that the instrument used to detect cervical ripening in pregnancy be noninvasive.

The future holds the potential for targeted therapeutic advances based upon the understanding of structural, physiological, biochemical, and molecular mechanisms of health and disease. Imaging technologies have advanced from detecting structure to function to therapy. In this process, clinicians and researchers have gained insights to functions in parts of the body that were previously not possible. The cervix during pregnancy is an example of a part of the body that has been off-limits to therapeutic intervention. Intervention has been limited due to concern of the effects of intervention on the developing fetus. Presently, the pharmaceutical interventions to halt preterm labor are not FDA-approved. All of the medications to halt preterm labor are used “off-label.” The lack of FDA approval should not limit ingenuity to try to solve a major public health problem.

The findings of this study have the potential, with additional research, to impact the understanding and treatment of cervical ripening. Cervical ripening precedes preterm labor by weeks and sometimes months. Clinicians and researchers might learn

from other disciplines, borrowing modalities that could translate into obstetric practice. For example, targeted drug therapy that targets the specific mechanisms of cervical ripening could be investigated. In cardiology, investigators are evaluating ways of controlling the fibroblasts that play an important role in maintaining healthy normal heart collagen (R. D. Brown, Ambler, Mitchell, & Long, 2005). Fibroblasts have multiple functions in the process of cervical ripening, producing smooth muscle cells, hyaluronan, elastin, and other substances. Such a treatment approach is already being used in preventing transplant rejection, where multiple pathways of cytokine and immunological suppression are modulated rather than using nonselective agents (Hong & Kahan, 2000). Medications that neutralize TNF-α (Remicade®, Embrel®) are being used systemically for arthritis and other applications (de Boer, 2003). Tacrolimus ointment is currently available for topical use and has very little systemic absorption (Simpson & Noble, 2005). Tacrolimus is an immunomodulator which inhibits cytokines and has few adverse reactions except for local burning and tingling (Simpson & Noble, 2005). Other cytokine neutralizers, inhibitors, and antagonists are being developed (de Boer, 2003). Agents developed that may have the potential modulate cervical ripening by preventing remodeling of the extracellular matrix are matrix metalloproteinase inhibitors (S. Brown, Meroueh, Fridman, & Mobashery, 2004; Levin, 2004). Matrix metalloproteinase inhibitors have been tested for patients with arthritis and cancer and have been approved for periodontal disease (P. D. Brown, 2000; Levin, 2004). Ultrasound has the possibility in the future of targeting drug delivery via sonoporation, thus delivering medication only to the intended tissue. This is a process where

medication is carried to tissues in micro-bubbles that are released only when an ultrasound pulse is applied to the intended tissue.

In the future, maternal health care providers may not be using one medication to either stimulate labor or stop labor, but rather may be using a “cocktail” to make use of the multiple pathways that control the mechanisms involved with both cervical ripening and uterine contractility. By understanding the physiology one may develop new technologies to diagnose and treat cervical ripening in the human.

E. Conclusions

In 1947 Danforth was the first to describe the uterus as two separate structures, functionally and histologically (Danforth, 1947). Danforth described the structure of the corpus of the uterus, which has the active contractile role, as being composed of smooth muscle (Danforth, 1947). Whereas the passive cervix was composed of connective tissue (Danforth, 1947). Unfortunately, these important findings did not change practice from viewing the uterus as one organ. Dr. Danforth spent his career here in Chicago. Dr. Scott Fields and Professor Floyd Dunn, from the University of Illinois Bioacoustics Research Laboratory, were also the first to determine that the collagen content in tissues is responsible for ultrasound scattering (S. Fields & Dunn, 1973). Innovation takes time and collaboration between multiple disciplines to solve complex biological and engineering problems. Using backscatter technology to diagnose cervical ripening will bring these two important ideas together.

The findings of this study supported the conceptual model related to (a) the hydroxyproline content (μg hydroxyproline/mg cervix) of the cervix increased as the pregnancy advanced in the rat, (b) the hydroxyproline concentration (% hydroxyproline)

decreased as pregnancy advanced, (c) ultrasound acoustic concentration of the scatterers decreased as the pregnancy advanced, and (d) hydroxyproline had significant correlations with rat groups and ultrasound variables of scatterer diameter, acoustic concentration and scatterer strength factor. The findings of the study did not support the conceptual model in regards to the detection of water content in the cervix. Compositional analysis of the cervix tissues for water content did not detect a difference between the rat groups.

The following research questions were answered as follows:

1. Is there a significant gestational age difference in the rat cervix which can be detected with ultrasound scatterer diameter, scatterer acoustic concentration, and scatterer strength factor? There were significant differences in the nonpregnant and pregnant groups in ultrasound scatterer diameter, scatterer acoustic concentration, and scatterer strength factor. The gestational age of the rat was used as a surrogate for cervical ripening. Thus, ultrasound scatterer diameter, scatterer acoustic concentration, and scatterer strength factor were significantly associated with cervical ripening.

2. Is there a significant gestational age relationship between ultrasound scatterer diameter, scatterer acoustic concentration, scatterer strength factor, and per cent hydroxyproline and water content in the cervix? Significant correlations were found between ultrasound scatterer diameter, scatterer acoustic concentration, and scatterer strength factor and percent hydroxyproline. Water content in the cervix was not correlated with ultrasound scatterer diameter, scatterer acoustic concentration, scatterer strength factor, or percent hydroxyproline.

3. Can a statistical model predict gestational age? Two discriminant function equations were significantly associated with gestational age. The first model included ultrasound scatterer diameter, scatterer acoustic concentration, scatterer strength factor and percent hydroxyproline, with r^2 = .82. Overall 65.4% of the cases were correctly classified in this model. The second model included only the ultrasound variables of scatterer diameter, scatterer acoustic concentration, and scatterer strength factor, with r^2 = .67. Overall 46.2% of the cases were correctly classified in this model.

APPENDICES

APPENDIX A

ANIMAL CARE COMMITTEE APPROVAL IN 2003

UNIVERSITY OF ILLINOIS
AT CHICAGO

Animal Care (ACC) and Institutional Biosafety Committees (IBC) (MC 672)
Office for the Protection of Research Subjects
Office of the Vice Chancellor for Research
201 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

December 18, 2003

Rosemary White-Traut
Maternal Child Nursing
M/C 802

Dear Dr. White-Traut:

The protocol indicated below has been reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago and approved on **12/16/2003** for 3 years. *The protocol was not initiated until final clarifications were reviewed and approved on 12/18/03.*

Title of Application: B Mode Ultrasound Microstructure of the Pregnant Cervix

ACC Number: 03-200

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. Please transmit this letter of acceptable verification of your research protocol to your sponsor.

For your information, a copy of the UIC Animal Care Policy on Occupational Health Program for Personnel Caring For or Using Laboratory Animals is enclosed. Please refer to it for your responsibilities and procedures to be followed. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

William R. Law, PhD
Chair, Animal Care Committee
/Enclosure
WRL/mbb
cc: BRL, ACC File, Barbara L. McFarlin, William D. O'Brien.,James F. Zachary.

UIC

Phone (312) 996-7427 • Fax (312) 996-9088

APPENDIX A (continued)

ANIMAL CARE COMMITTEE AUTHORIZATION IN 2004

UNIVERSITY OF ILLINOIS
AT CHICAGO

Animal Care (ACC) and Institutional Biosafety Committees (IBC) (MC 672)
Office for the Protection of Research Subjects
Office of the Vice Chancellor for Research
201 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

September 23, 2004

Rosemary White-Traut
Maternal Child Nursing
M/C 802

Dear Dr. White-Traut:

The modifications requested in modification ***03-200-01*** pertaining to your approved protocol indicated below have been reviewed in accordance with the Animal Care Policies of the University of Illinois at Chicago and approved on ***September 21, 2004***.

Title of Application: **B Mode Ultrasound Microstructure of the Pregnant Cervix**

ACC Number: **03-200**

Modification Number: ***03-200-01***

Nature of Modification: ***Addition of 18 rats to obtain attenuation data on cervices of pregnant rats***

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. Please transmit this letter of acceptable verification of modification of your research protocol to your sponsor.

The records of the Animal Care Committee will be revised to reflect these changes. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely,

William R. Law, PhD
Chair, Animal Care Committee

WRL/ *mbb*
cc: BRL, ACC File, William D. O'Brien, James F. Zachary, Barbara L. McFarlin

UIC

APPENDIX B

COLLAGEN/HYDROXYPROLINE DETERMINATION

Reference:

Association of Official Analytical Chemists. (2002). Method #990.26. In W. Horwitz (Ed.), *Official methods of analysis of the Association of Official Analytical Chemists (*17th edition). Gaithersburg, MD: Author.

Reagents:

1. Hydrochloric Acid, 6N: Add 500 ml HCL to 500 ml distilled/deionized (dd) water.
2. Buffered Solution: Place 15.0 g citric acid monohydrate, 7.5 g NaOH and 27.1 g sodium acetate anhydrous (or 45.0 g sodium acetate trihydrate) in a 1 liter volumetric flask. Dissolve with 500 ml dd water. Add 145 ml 1-propanolol. Adjust pH to 6.0 with either NaOH or acetic acid. Bring up to volume with dd water. Store in a brown brottle in the refrigerator. Stable for 60 days.
3. Oxidant Solution: Place 0,705 g chloramines –T reagent in a 100 ml volumetric flask. Bring up to volume with buffer solution. Store in a brown bottle in the refrigerator. Stable for a week.
4. Color Reagent: Dissolve 10 g 4-dimethylaminobenzaldehyde in 35 ml perchloric acid (60% w/w). Slowly add, with constant agitation, 65 ml isopropanol. Not stable. Prepare immediately before use.
5. Hydroxyproline Stock Standard: Place 30 mg hydroxyproline in a 50 ml volumetric flask. Bring up to volume with dd water. 600 μg/ml. Store in refrigerator. Stable for 60 days.
6. Hydroxyproline Working Standard: Pipet 1 ml hydroxyprole stock standard into a 100 ml volumetric flask. Bring up to volume with dd water. 6 μg/ml. Not stable. Prepare on the day of use.

Procedure:

1. In duplicate, weight 10 mg samples to 13 x 100 mm screw cap test tubes.
2. Add 0.1 ml 6N HCL to each. Seal tightly, Vortex gently.
3. Hydrolyze for 16 hours in a 105 °C oven.
4. Add 0.05 ml dd water to each tube. Vortex gently.
5. Transfer the contents of each tube to a 10 ml volumetric flask using dd water.
6. Bring up to volume with dd water. Mix.
7. Filter an aliquot through Whatman 541 filter paper with activated charcoal.
   Note: Save all of your filtrate. If you need to rerun the colormetric portion of the assay you may start at step 8.
8. Dilute the aliquot of the filtrate 10fold.
9. Prepare the hydroxyproline standards.
   - 1.2 μg/ml = 1ml working standard + 4 ml dd water
   - 2.4 μg/ml = 2ml working standard + 3 ml dd water
   - 3.6 μg/ml = 3ml working standard + 2 ml dd water
   - 4.8 μg/ml = 4ml working standard + 1 ml dd water

APPENDIX B (continued)

COLLAGEN/HYDROXYPROLINE DETERMINATION (continued)

10. In duplicate, pipet 1 ml of water, standards or samples into 13x 100 mm disposable tests tubes.
11. Add 2 ml oxidant solution to each tube. Vortex.
12. Let stand 20 minutes. Do not allow to stand for more than 22 minutes.
13. Add 1 ml color reagent to each tube. Vortex. Parafilm the tubes.
14. Incubate the tubes for exactly 15 minutes in 60 °C water bath.
15. Cool tubes under running tap water for at least 3 minutes.
16. Read on spectrophotometer at 558nm.

APPENDIX C

DRY MATTER DETERMINATION

Reference:

Association of Official Analytical Chemists. (2002). Method #934.01. In W. Horwitz (Ed.), *Official methods of analysis of the Association of Official Analytical Chemists (*17th edition). Gaithersburg, MD: Author.

Procedure:

1. Place porcelain crucibles in a 105 °C oven overnight.
2. Remove the crucibles from the oven with tongs and place in the desiccator. Cool to room temperature.

Note: During this procedure, you must not touch the oven dried crucible with your hands prior to weighing it. Your skin is wet and will throw off your calculations. This is applicable to any assay requiring drying and weighing samples, beakers, crucibles, filter papers, etc.

3. Remove the crucible from the desiccator with tongs. Weigh the empty crucible. Record the weight.
4. Weigh 0.5-1.0 g ground sample into the crucible. Record the sample weight.
5. Dry overnight in a 105 °C oven.
6. Transfer samples from the oven to the desiccator with tongs. Cool to room temperature.
7. Remove the crucible + sample from the desiccator with tongs. Weigh the cooled crucible + sample. Record the dry crucible + sample weight.

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