Tensile Mechanical Properties and Dynamic Collagen Fiber Re-Alignment of the Murine Cervix Are Dramatically Altered Throughout Pregnancy

The cervix is a unique organ able to dramatically change its shape and function by serving as a physical barrier for the growing fetus and then undergoing dramatic dilation allowing for delivery of a term infant. As a result, the cervix endures changing mechanical forces from the growing fetus. There is an emerging concept that the cervix may change or remodel “early” in many cases of spontaneous preterm birth (sPTB). However, the mechanical role of the cervix in both normal and preterm birth remains unclear.

Therefore, the primary objective of this study was to determine the mechanical and structural responses of murine cervical tissue throughout a normal gestational time course. In this study, both tissue structural and material properties were determined via a quasi-static tensile load-to-failure test, while simultaneously obtaining dynamic collagen fiber re-alignment via cross-polarization imaging. This study demonstrated that the majority of the mechanical properties evaluated decreased at midgestation and not just at term, while collagen fiber re-alignment occurred earlier in the loading curve for cervices at term. This suggests that although structural changes in the cervix occur throughout gestation, the differences in material properties function in combination with collagen fiber re-alignment as mechanical precursors to regulate term gestation. This work lays a foundation for investigating cervical biomechanics and the role of the cervix in preterm birth.

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Introduction

Preterm birth (PTB) is one of the primary causes of infant morbidity and mortality in the U.S. affecting 11.2% of live births [1] and costing $26 billion per year. Moreover, preterm children have an inherently higher risk for future medical complications [2].

Many factors have been used to identify women with a higher risk for PTB including: multiple pregnancies, race, a prepregnancy body mass index less than 18, prior preterm birth, and drug use. However, these risk factors fail to identify the majority of the women who present with a spontaneous PTB [3–5]. The inability to accurately predict which women will have a spontaneous preterm birth (sPTB) lies in our lack of understanding of the precise pathogenesis by which this adverse event occurs.

Recent studies suggest that the cervix plays a critical role in both normal parturition and spontaneous PTB [6–8]. During
pregnancy, the primary role of the cervix is to maintain the load of the growing fetus within the uterus. At parturition, the cervix radically switches its function, allowing delivery of the fetus via dilation [9,10]. Failure of the cervix to maintain its structure previously labeled as “cervical incompetence or insufficiency” is proposed to be the loss of mechanical integrity of the cervix and has been cited as one of the causes of PTB [11–13]. Few studies have examined the changes of the human cervix during pregnancy, partially due to limitations in obtaining adequate samples [14–17]. Previous research has examined gene expression pathways before and after spontaneous parturition and showed drastic changes in chemokines, cell adhesion, and extracellular matrix (ECM) proteins, as well as in other signaling pathways [15,16].

While another study demonstrated that a distinct micro ribonucleic acid (microRNA) profile in cervical cells was associated with eventual sPTB, suggesting a molecular change in the cervix during the second trimester of the pregnancy prior to any clinical signs or symptoms of PTB [8,17]. Despite these efforts, understanding of how the cervix drastically changes during normal gestation and how this is modified in sPTB during human pregnancy remains limited [18]. The current deficit of knowledge makes the use of animal models necessary [19].

Mouse studies have shown that multiple mechanisms contribute to the drastic physical changes the cervix undergoes during pregnancy [11,12,20]. The two most prominent theories postulate that cervical changes during gestation are triggered either by progesterone withdrawal or by an inflammatory response [21–24]. Despite this previous research, the mechanisms of cervical change or remodeling during normal gestation and PTB do not adequately explain the complex structural response of the cervix during pregnancy [22]. Additional understanding of these mechanical adaptations, as well as how the cervical ECM affects these mechanical properties during gestation is warranted [16,25,26].

As a load-bearing tissue [11], cervical strength and function are directly related to ECM, and one of its major components is collagen [9,20]. Fibrous collagen is the main structural protein known to affect tensile properties of the cervix [27]. Changes in collagen have been shown to affect the normal function of many different organ systems [28–31]. However, the role of collagen fibers within the cervix has only recently been investigated [32–35]. In order to further understand the role of the cervix, it is imperative to study not only the molecular changes occurring during gestation and PTB, but also the biomechanical changes, and how components such as collagen fibers may alter the cervical function.

Multiple research groups have investigated the mechanical response of the cervix [22,27,36,37]. A classic study evaluated the tensile mechanical response of rat cervical tissue via a ring test, an approach which resolves the typical difficulties associated with the gripping biological tissue in a mechanical testing frame [38]. This testing methodology has been recently employed to investigate the mechanical properties of murine cervixes [32,39]. However, a ring test makes calculating the true cross-sectional area (CSA) of the test sample difficult, and therefore, reporting material properties is not possible. Despite this, the previous work shows important correlations between collagen crosslinks and both stiffness and ultimate strength [32]. Moreover, although most previous experimental studies have only reported structural properties such as load and stiffness, a recent study estimated material properties such as modulus through a mathematical model [39].

Investigating the intrinsic material properties of the cervix directly provides valuable information regarding cervical tissue adaptations during pregnancy. Given the significant role of collagen in the cervix to mechanical properties, characterization of the dynamic fiber re-alignment during cervical loading could provide crucial insights. The purpose of this study was to measure the biomechanical and dynamic collagen fiber re-alignment of the cervix throughout normal pregnancy as well as postpartum (PP) using a mouse model. Since a growing fetus causes increased loading and deformation of the cervix, we hypothesized that a pregnant cervix will have decreased stiffness, modulus, maximum load, and maximum stress as well as less collagen re-alignment.

Materials and Methods

Fine Dissection. Nonpregnant (NP) and pregnant CD-1 mice at embryonic day (E) E10.5, E12.5, E14.5, E16.5, E18.5, and postpartum PP (PP, where samples were collected 24h after delivery) (n=10–16/group) were sacrificed and frozen until they were prepared for mechanical testing. The previous research has shown no effects of freezing on mechanical properties of the cervix [32,37,39]. At the time of testing, the reproductive tissue was carefully harvested (Fig. 1(a)), removing all musculature and surrounding soft tissue, and hydrated in phosphate-buffered saline (PBS) (Figs. 1(b) and 1(c)). During dissection, the vagina and cervix were cut open on the side opposite the bladder, and the bladder was removed. The cervix was dissected free of any extra soft tissue, and the uterus and vagina were carefully removed (Fig. 1(d) and 1(e)).

Mechanical Testing. The cervix was laid flat to expose the lumen. The ends were affixed between two pieces of sandpaper for gripping, such that a uniaxial tensile load on the grips would simulate dilation of the cervical canal (loading occurred perpendicular to the proximal–distal direction). The prepared sample was continually immersed in PBS till the start of mechanical testing. A custom laser device was used to measure the cross-sectional area at a minimum of two locations, which took less than 60 s [40]. The approximate variability of tissue cross section was within 20% of the average area. The cervix was then placed in custom fixtures to grip it at both ends. The cervix was then tested in tension using an Instron 5848 testing system (Instron Corp., Norwood, MA) using a standard protocol consisting of a preload of 0.005 N followed by a hold of 5 min and then a ramp to failure at 1 mm/min. Representative force–elongation plots from mechanical testing for each gestational time point are shown in Fig. 2(d). The entire test was performed in a saline bath at room temperature. The location of failure was recorded for each sample. Samples were excluded from further analysis if failure did not occur within the midsubstance of the tissue.

Collagen Re-Alignment. Collagen fiber alignment maps of the cervix were collected throughout the mechanical testing protocol using our established integrated cross-polarizer system, as described in Refs. [41–43]. This custom system consists of a linear backlight (Dolan-Jenner, Boxborough, MA), rotating polarized sheets offset by 90 deg (Edmund Optics, Barrington, NJ), and a camera (Fig. 2(a)). Custom software (National Instruments LabVIEW, Austin, TX) synchronized with analog output signals from the Instron triggered alignment maps to image capture at 5 s.

Fig. 1 Cervical mechanical testing dissection. (a) Schematic of cervix anatomy. (b) NP and (c) E18.5 cervixes before and b) and c) after dissection. During dissection the cervix was cut open on the side opposite to the bladder and then the bladder was removed. The cervix was then laid flat to expose the lumen, and then rotated. The ends were affixed between two pieces of sandpaper for gripping so that when the cervix was pulled in tension, this represented circumferential loading.
Corrected post hoc tests when appropriate. Mann–Whitney comparisons, while p time point. The significance was set at were used to evaluate differences in fiber re-alignment at each between the last three gestational ages (E14.5, E16.5, and E18.5). However, PP samples were significantly larger than all of the gestational time points tested as well as NP samples (Fig. 3(a)).

Tissue stiffness was decreased in all gestational samples (E12.5, E14.5, E16.5, and E18.5) compared to NP samples, with the exception of E10.5 samples. The E10.5 samples were also significantly stiffer compared to all other gestational ages (E12.5, E14.5, E16.5, and E18.5) and were no different when compared to NP samples (Fig. 4(a)). Interestingly, PP samples were also significantly stiffer than all other gestational ages except for E10.5.

Maximum load was significantly reduced in all gestational groups compared to NP samples (E10.5, E12.5, E14.5, E16.5, and E18.5). This was also observed in PP samples, which showed a significantly higher maximum load when compared to all the gestational samples, but was not significantly different when compared to NP samples (Fig. 4(b)). There were no differences found in maximum load between any of the gestational samples tested.

NP and E10.5 samples demonstrated significantly higher maximum stress when compared to all other gestational time points (E12.5, E14.5, E16.5, and E18.5) but were not statistically different from each other. However, E10.5 samples showed a significantly higher max stress when compared to PP samples that was not observed in NP samples. PP samples only showed a significantly higher max stress when compared to E18.5 samples (Fig. 4(c)). All other gestational time points did not show significant differences in maximum stress.

NP samples only showed a significantly higher modulus when compared to E18.5 samples. However, E10.5 samples showed a significantly higher modulus when compared to additional later time points (E14.5, E16.5, and E18.5). No significant differences were observed in tissue modulus between NP, E10.5, E12.5, and PP samples. Modulus of the other gestational time points after E10.5 were not significantly different when comparing between each other (E12.5, E14.5, E16.5, and E18.5) and were also not significantly different when compared to PP samples (Fig. 4(d)).

Results

Nonpregnant samples were significantly larger compared to other early gestational time points including E12.5 and E14.5. However, later gestational time points (E16.5 and E18.5) showed no significant differences in size when compared to NP samples (Fig. 3(a)). During gestation, cross-sectional area was initially reduced; E10.5 pregnant cervices were significantly smaller than both NP samples as well as later gestational time points (E14.5, E16.5, and E18.5). Interestingly, E12.5 cervices were significantly smaller only when compared to those later gestational time points (E16.5 and E18.5). There were no other differences in area found when comparing the toe region, determined by the last map before the change of slope to the linear region, (3) at 45% of the maximum load, and (4) at 90% of the maximum load (Fig. 2(c)). Re-alignment was defined by the first map after the hold protocol, (1) start of the toe region, (2) at the end of the toe region, 45% of maximum load and 90% of maximum load. (2) Representative load-stretch plots for each gestational time point.

Statistics. One-way analysis of variance were used to compare between groups for mechanical parameters with Bonferroni-corrected post hoc tests when appropriate. Mann–Whitney U tests were used to evaluate differences in fiber re-alignment at each time point. The significance was set at p < 0.05 for all statistical comparisons, while p < 0.1 was defined as a trend.

Cross-sectional area was significantly smaller only when compared to those later gestational time points (E16.5 and E18.5). Interestingly, PP samples were also significantly higher maximum load when compared to all the gestational samples, but was not significantly different when compared to NP samples (Fig. 4(b)). There were no differences found in maximum load between any of the gestational samples tested.

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NP samples only showed a significantly higher modulus when compared to E18.5 samples. However, E10.5 samples showed a significantly higher modulus when compared to additional later time points (E14.5, E16.5, and E18.5). No significant differences were observed in tissue modulus between NP, E10.5, E12.5, and PP samples. Modulus of the other gestational time points after E10.5 were not significantly different when comparing between each other (E12.5, E14.5, E16.5, and E18.5) and were also not significantly different when compared to PP samples (Fig. 4(d)).

Fig. 3 Cross-sectional area of the cervix during gestation. (a) Cross-sectional area is shown for all gestational time points. The white bar on the left represents the NP and during gestation different time points are shown in increasing shades of grey. PP samples are represented on the far right with a checkered pattern. All of the data is presented as means with standard deviations, and significance noted at p < 0.05 with a line. Although no statistical differences were observed between the (b) NP and the (c) E18.5 samples, qualitative visual differences were observed.

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Re-alignment of collagen fibers was observed for all samples between the “toe” and “90% maximum load” time points, except for E12.5 samples (Fig. 5). In both early gestation (E10.5) and later gestation (E16.5), samples experienced a faster response to load, with re-alignment occurring earlier during mechanical testing (Toe-45% and ET-90%; Figs. 5(b) and 5(e)). However, E18.5 samples showed an immediate response to load with collagen fiber re-alignment during all time points examined (Toe-45, Toe-90, ET-45, ET-90, 45–90; Fig. 5(f)).

Discussion

The primary objective of this study was to determine the biomechanical and dynamic collagen fiber structural response of cervical tissue throughout a normal gestational time course. To this end, tissue structural and material properties were determined via a quasi-static tensile load-to-failure test and dynamic collagen fiber re-alignment data were collected simultaneously. Overall, as hypothesized, a pregnant cervix demonstrated significantly reduced structural and material properties as compared to non-pregnant samples. Surprisingly, the majority of the mechanical properties of the cervix decreased at midgestation, while collagen fiber re-alignment occurred earlier in the loading curve for cervi ces at term. The previous research has proposed that the cervix gradually reaches its lowest tensile strength at term, and that this change is a necessary precursor for term delivery [24,44]; however, these factors have not yet been sufficiently studied, and further investigations are warranted [22,24,35,45,47].

Fig. 4 Cervix structural and material mechanical properties. (a) Stiffness, (b) Max Load, (c) Max Stress, and (d) Modulus for all samples. The white bar on the left represent the NP samples and during gestation different time points are shown in different shades of gray. PP samples are represented on the far right with a checkered pattern. All of the data is presented as means with standard deviations, and significance is noted at $p<0.05$ with lines.

A significant reduction in tissue stiffness was observed after the E10.5 time point. Similar observations have been made in...
previous studies where a significant drop of cervical tissue stiffness was observed after embryonic day 11 [22]. Interestingly, some of the values reported in this previous study were an order of magnitude lower than the current study [22], which could potentially be due to differences in mechanical testing protocols. Stiffness calculations can vary significantly based on the region selected in the force-displacement curve. Since the previous study reported much lower stiffness values, they might have been obtained from the toe region of the cervix tensile response curve. The maximum stress values in our study compare very well to the maximum stress values reported in another recently published study examining mechanical properties of the normal cervix during pregnancy [32]. In our study, the stiffness of PP samples was comparable to those of NP samples indicating a rapid return to baseline within 24 h after delivery. This outcome was not observed in a previous study, where significant differences in tissue stiffness were observed between PP and NP samples [32].

Our study indicated that the pregnant cervical tissue fails at a significantly lower maximum load when compared to NP or PP samples. This was partially expected, given the observed reductions in cervix CSA when compared to the PP samples. However, the result is not fully explained by area alone, since the NP samples and later gestational ages showed no differences in CSA. Overall, these structural data support the cervical-softening theory proposed in the early gestation. However, if the structural parameters measured are an indication of whether the cervix is “primed” for parturition, our studies suggest this occurs much earlier in gestation than proposed in previous mouse molecular studies [21–24,35,48].

The chosen mechanical testing regime in the current study allowed for the evaluation of cervix material properties, specifically, Young’s modulus and ultimate stress. The ultimate stress values generally followed similar trends as observed with the stiffness data. Interestingly, however, E10.5 samples had significantly higher ultimate stress than PP samples, which might indicate a protective mechanism during early gestation. Further, tissue modulus was only observed to change significantly at the E18.5 time point when compared to NP samples. This combination of structural and material property changes at the E18.5 point might be the necessary mechanical precursor to a timely term delivery.

Given the primary load bearing role of collagen fibers in biological tissue and specifically the cervix, collagen fiber re-alignment during tensile loading was examined for cervical tissue at all gestational time points (except PP). Some level of early re-alignment was observed at the E10.5 time point, which might be a precursor to the significant drop in mechanical properties observed at the E12.5 time point. Further, significant and early collagen fiber re-alignment was observed for the E16.5 and E18.5 samples, which...
The data presented in the current study are consistent with previous studies showing that the mouse cervix remodels structurally and mechanically during gestation [22, 23, 32, 35]. However, this study showed that modulus changed significantly from the nonpregnant state just before delivery (E18.5). In contrast to the previous work, this study revealed a prompt recovery of most mechanical properties at the PP time point [32]. Understanding how the cervix “regains” function will be essential toward uncovering the processes that cause the cervix to “lose” function as is proposed to occur in spontaneous PTB. Knowledge of the regulation behind this phenomenon could improve potential therapeutic strategies of spontaneous PTB. This study demonstrated novel findings concerning the biomechanics of the pregnant cervix yet, further work is necessary to (1) correlate our findings of biomechanical changes with reported molecular changes that differentiate these time points during mouse gestation and (2) more thoroughly explain how the cervix maintains load during pregnancy. Further, correlation of these findings with human pregnancy will be necessary.

In conclusion, we demonstrated that the pregnant cervix has significantly reduced structural and material properties, which decreased at midgestation, as compared to nonpregnant samples, and not just at term. These decreased properties were accompanied by changes in collagen fiber re-alignment occurring earlier in the loading curve for cervices at term.

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References
