BIAXIAL CONTRACTILITY, PASSIVE BIOMECHANICS, AND MURINE CERVICAL REMODELING

AN ABSTRACT

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BY

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ABSTRACT

Preterm birth (PTB) is a global health concern linked to lifelong health conditions in the mother and child. The etiology of PTB is multifactorial and exact pathways of PTB difficult to elucidate. Cervical insufficiency (CI) is a form of spontaneous PTB in which the cervix dilates in early- to mid-pregnancy without uterine contractions. CI remains difficult to diagnose and treat due to a lack of research into cervical function. During early-pregnancy the cervix must remain stiff to maintain the fetus within the uterus, however, in late-pregnancy the cervix must soften and dilate to allow for the passage of the fetus into the vaginal canal. To accomplish both roles, the cervical extracellular matrix (ECM) remolds during pregnancy. A disruption to the normal remodeling process such as accelerated degeneration of ECM proteins may lead to failure of cervical function. In addition to ECM, cervical smooth muscle cells (cSMCs) work to maintain cervical integrity and assist in physiologic processes such as fertilization and labor. Quantification of microstructural content and mechanical testing permits determination of relationships between ECM, cSMC, and cervical function. Past research quantified microstructural, mechanical, and contractile properties of the cervix; however, mechanical testing and contractility protocols were uniaxial. Uniaxial testing requires disruptive specimen preparation and investigates circumferential and axial properties independently. The cervix, however, is loaded multiaxially in vivo and is anisotropic. Towards this end, biaxial inflation-extension testing of the cervix overcomes these limitations by enabling simultaneous assessment of circumferential and longitudinal mechanical properties and contractility. Determining mechanical properties, contractility, and microstructure of the cervix in the nulliparous and parous state enables the development of computational models of cervical remodeling to better understand the etiology of CI. Therefore, this study seeks to characterize cervical remodeling by determining the evolving biaxial mechanical properties, contractility, and microstructural composition of the nulliparous and parous murine cervix.
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Figure 6.1: In vivo ($n=5$ per genotype) cervical contractility in the WT (white) and HET (grey). (A) In vivo baseline did not significantly change with haplo-insufficient genotype (10.0±2.00 mmHg, WT; 12.0±2.00 mmHg, HET). (B) Additionally, maximum contractile pressure in the WT (39.0±11.0 mmHg) did not significantly differ from the HET (41.0±19.0 mmHg) (C) However, frequency of contractions decreased significantly ($p<0.05^*$) in the HET cervixes (0.02±0.01 Hz) compared to WT (0.05±0.03 Hz)...

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Figure 6.3: (A, B) In vitro circumferential and axial contractility ($n=5$) at the estimated physiologic (EP) length and mean physiologic pressure where the wildtype (WT) is represented by white and the heterozygous (HET) is indicated by gray. Change in diameter (-870±370 µm, WT; -1100±227 µm, HET) and force (14.6±7.30 mN, WT; 28.3±14.1 mN, HET) with maximum contraction did not significantly differ with genotype. (C, D) In vitro wavelength and frequency with maximum contraction at the EP length and mean physiologic pressure. Wavelength (46.1±25.0 s, WT; 75.5±18.6, HET) and frequency (0.03±0.02 Hz, WT; 0.01±0.01 Hz, HET) did not significantly differ with genotype ($p>0.05$)...

Figure 6.4: In vitro axial (A) and circumferential (B) contractility in the wildtype (WT) cervix after 48 hours in culture where white represents the WT control ($n=5$) and black indicates the cultured specimen ($n=1$). Where pressurized culture decreased contractility in the murine cervix with a total change in 0.6 mN of force and 0 µm in diameter. No signs of contamination were observed in the cell media on Day 2...

Figure 7.1: Geometry recorded for the nulliparous (NP; white with closed circles), Day 6 (D6; light gray with squares), Day 12 (D12; dark gray with triangles), Day 15 (D15; white with squares), Day 18 (D18; light gray with triangles), and postpartum (PP; dark gray with circles) time points ($n=5$ per group). (A) Unloaded outer diameter increased significantly at D18 compared to NP, D6, D12, and PP time points. (B) Unloaded length increased...
significantly at D18 compared to NP, D6, and D12. Additionally, length increased at D15 compared to D6. (C) Thickness increased significantly at the PP time point compared to D6, D12, D15, and D18. However, thickness did not change with increasing gestation compared to the NP time point. (D) Volume increased significantly at D18 compared to the NP time point and D6 gestation. Significance values are denoted by: \( p<0.05^*; p<0.01^{**}; p<0.001^{***} \)

**Figure 7.2:** Biaxial maximum contractile behavior of the murine cervix through gestation and postpartum \((n=5\) per group) at the estimated physiologic (EP) length and mean physiologic pressure \((P = 9.0\) mmHg) with significance indicated by: \( p<0.05^*; p<0.01^{**}; p<0.001^{***} \). Where the groups are represented by nulliparous (NP; white with closed circles), Day 6 (D6; light gray with squares), Day 12 (D12; dark gray with triangles), Day 15 (D15; white with squares), Day 18 (D18; light gray with triangles), and postpartum (PP; dark gray with circles). (A) Maximum circumferential contraction induced a significantly greater total change in outer diameter at D15 and D18 compared to NP, D6, D12, and PP. (B) Maximum axial contraction significantly decreased at D6 and D12 compared to NP followed by a significant increase at D15, D18, and PP compared to early and mid-pregnancy time points (D6, D12). (C) The wavelength of contractions decreased from NP to D12 and D15. However, postpartum induced a significant increase in wavelength compared to all time points. (D) Frequency increased from the NP and D6 time points from D12 through D18. However, PP frequency significantly decreased compared to all pregnancy time points.

**Figure 7.3:** Changes in circumferential \((\Delta T_{\theta\theta})\) and axial \((\Delta T_{zz})\) stress representing the active cSMC contribution for each time point wherein nulliparous (NP) is represented by white with close circles, Day 6 (D6) by light gray with squares, Day 12 (D12) by dark gray and triangles, Day 15 (D15) by white with squares, Day 18 (D18) light gray with triangles, and postpartum (PP) by dark gray with circles. (A) Active circumferential stress \((\Delta T_{\theta\theta})\) decreased significantly at D15 compared to NP, early to mid-pregnancy (D6, D12), and PP. Additionally, \( \Delta T_{\theta\theta} \) at D6 increased significantly against D18. (B) Axial stress \((\Delta T_{zz})\) decreased from the NP state throughout pregnancy followed by a significant increase at PP where NP and PP axial stress exceeded D12, D15, and D18 axial stress. Additionally, D15 and D18 decreased significantly compared to D6. Where significance is denoted by: \( p<0.05^*, p<0.01^{**}, \) and \( p<0.001^{***} \)

**Figure 7.4:** Absolute active change in stress with respect to loading direction at the estimated physiologic (EP) length and mean physiologic pressure for each time point \((n=5\) per group). Where circumferential loading direction is represented by white with closed circles and axial direction by gray with squares. Significance values are represented by: \( p<0.05^*; p<0.01^{**}; p<0.001^{***} \). (A) Nulliparous axial stress increased significantly compared to the circumferential stress. (B, C) In D6 and D12 the axial and circumferential stretch did not reach significance. (D, E) However, late pregnancy time points (D15, D18)
resulted in a significant increase in circumferential stress compared to axial. (F) Similar to the nulliparous state, PP axial stress exceeded circumferential stress.

**Figure 7.5:** Passive material stiffness and compliance at the estimated physiologic (EP) length for each group (n=5 per group). Where nulliparous (NP) is represented by white with close circles, Day 6 (D6) by light gray with squares, Day 12 (D12) by dark gray and triangles, Day 15 (D15) by white with squares, Day 18 (D18) light gray with triangles, and postpartum (PP) by dark gray with circles. (A) Passive circumferential material stiffness did not significantly differ with time point. Circumferential stiffness exceeded axial stiffness for all time points. (B) Passive axial material stiffness significantly increased at D18 compared to the NP state. (C) Compliance decreased at D18 compared to PP, however, no other groups achieved significance. Significance values are denoted by: $p<0.05^*$; $p<0.01^{**}$; $p<0.001^{***}$.

**Figure 7.6:** Active and passive circumferential (white) and axial (gray) stress with respect to time point wherein nulliparous is NP, Day 6 (D6), Day 12 (D12), Day 15 (D15), Day 18 (D18), and postpartum (PP). Anisotropy in the NP murine cervix alternated in the active and passive states. Axial stress exceeded ($p<0.01$) circumferential stress during contraction, however, circumferential stress exceeded ($p<0.05$) axial stress in the passive state. Throughout gestation and postpartum the passive circumferential stress increased compared to passive axial stress. However, D6 and D12 active stress did not significantly differ with loading direction. Followed by an increase in active circumferential stress at D15 and D18. Significance denoted by $p<0.05^*$; $p<0.01^{**}$; $p<0.001^{***}$.

**Figure 7.7:** Relative changes (not to scale) in estradiol (blue) and progesterone (pink) compared to the relative changes in compliance (green) and contractile force with maximum contraction (gray) with respect to the nulliparous states (NP), postpartum (PP), and Day 6 (D6), Day 12 (D12), Day 15 (D15), and Day 18 (D18) gestation. Wherein D12 corresponds with the first phase of cervical remodeling, softening, and D18 corresponds to the ripening and dilation phases. Interestingly, compliance and estradiol peak at D18 gestation and contractile force plateaus at D15 gestation correlating with a spike in progesterone. In the PP time point compliance decreased with decreasing levels of steroid hormones, however, contractile force remained elevated compared to the NP state.
Chapter 1: Introduction

1.1 Preterm Birth

Preterm birth (PTB) is a global health condition and the leading cause of childhood morbidity and mortality worldwide [4]. PTB is defined as delivery between 20-37 weeks gestations and results in lifelong psychological and physical consequences to health of the mother and child [4-6]. PTB is a condition that can be separated into categories including: infection mediated PTB, maternal or fetal medically indicated (i.e. preeclampsia, gestational diabetes, intrauterine growth restriction), preterm premature rupture of fetal membranes, and spontaneous PTB [7-9]. The etiology of PTB, particularly spontaneous PTB, remains poorly understood and difficult to prevent, diagnose, or treat [5, 10, 11]. Spontaneous PTB related to cervical insufficiency (CI), a condition in which the cervix prematurely shortens and dilates releasing the uterine contents in the absence of contractions, remains a challenge to diagnose and treat clinically [5, 10, 11]. Clinical signs and risk factors of CI include decreased cervical length and prior history of preterm birth [12]. However, cervical length may be patient dependent and not a reliable indicator of CI [6, 12]. A stiff, short cervix may better endure the dynamic loads of pregnancy as compared to a compliant, long cervix [6, 12]. Additionally, prior research investigated the role of changing extracellular matrix (ECM) constituents and mechanical loading as potential factors of CI [6, 11-13]. Interestingly, elastic fiber integrity/content decreases in cases of CI suggesting altered structural integrity may lead to cervical failure [11]. Therefore, it is critical to improve basic knowledge and understanding of structure and function of the cervix before, during, and after pregnancy.
1.2 Cervix Anatomy and Function

The cervix is a rigid and predominantly collagenous organ located between the vagina and uterine body (Fig. 1) [14, 15]. In humans the cervix is connected to a single bodied uterus, however, in most mammals the cervix is connected to two uterine horns (Fig. 1). Rodents are commonly used to investigate properties of the female reproductive tract because they have a similar anatomy (Fig. 1.1), relatively quick gestation, well-defined cervical remodeling during pregnancy, and decreased cost compared to human samples [16, 17]. Compared to the rat, the mouse is a better model for human anatomy as the murine reproductive system has a single cervical canal (Fig. 1.1) [14]. On the tissue scale, the cervix is composed of three primary layers: epithelium, stroma, and serosa [2, 18, 19]. The ECM and cervical smooth muscle cells (cSMCs) are distributed through the layers to maintain cervical integrity and carry out normal cervical function [20, 21]. The ECM consists of organized collagen, a small percentage of elastic fibers, glycosaminoglycans (GAGs), and a cSMCs [2, 14, 15, 19]. In the non-pregnant state, the cSMCs contract in a regular phasic pattern through the estrous cycle to contribute to processes such as fertilization and the passage of menstrual fluid [22, 23]. The epithelial layer acts to protect the cervix from the external environment.
and secrete mucus to either facilitate or inhibit sperm passage based on hormonal phase of the estrous cycle [18]. The stromal layer primarily consists of collagen, cervical smooth muscle cells (cSMCs), and randomly orientated elastic fibers [13]. The outermost serosal layer contains fibroblast cells, collagen, and elastic fibers [13]. Overall, the non-pregnant cervix plays an important role in protecting the uterine cavity from external factors, facilitate fertilization, and dilate to allow for passage of menstrual contents [22, 23].

During pregnancy, the cervix must remain stiff and closed to maintain the fetus within the uterus, yet, dramatically soften and dilate before delivery to allow for passage of the fetus [16, 24-26]. Cervical remodeling during pregnancy consists of four phases: softening, ripening, dilation, and postpartum repair [16, 24-26]. Cervical softening is a progressive phase of increased tissue compliance during early- to mid-pregnancy [8, 27]. Compared to softening, the ripening and dilation phases progress at an accelerated rate to prepare for delivery of the fetus [8]. During the final phase, postpartum, the cervix stiffens and returns to near nulliparous geometry, microstructure, and mechanical function [21, 28]. Elastic fiber turnover during pregnancy and post-partum healing may be responsible for the dramatic alterations in the mechanical environment of the organ [29]. Previous research by Leppert et al. identified a relationship between degenerative elastic fibers and spontaneous PTB in human cervices from women diagnosed with cervical insufficiency [11]. Increasing compliance in the cervix during pregnancy may be linked to altered collagen deposition resulting in altered collagen crosslink ratios [21]. Hyaluronic acid and glycosaminoglycans (GAGs) provide resistance to compression during pregnancy [30-32]. During postpartum, collagen content increases and cSMC, GAG, and elastic fiber content decreases [28]. While preliminary research into cervical ECM provided critical information
about the evolving function of the cervix, the quantity, phenotype, and active role of cSMCs during pregnancy and postpartum is not known/not fully elucidated. Therefore, a need remains to determine the role of cSMCs in the dual role of maintaining the fetus through pregnancy and facilitating labor.

1.3 Cervical Biomechanics

Mechanical testing in conjunction with quantification of microstructural content enables the determination of relationships between structure (ECM and cSMCs) and cervical function. Previous research quantified biomechanical properties and contractility in the human [11, 13, 19, 33-37], rat [22, 28, 38], and mouse [14, 21, 23, 26] cervix. Uniaxial testing utilizes strips or rings of tissue to independently determine circumferential and longitudinal cervical biomechanical properties and cSMC contractility (Fig. 1.2) [20, 22, 26, 28, 39, 40].

While uniaxial mechanical tests provide important information and lay a groundwork for characterizing mechanical properties, the cervix is anisotropic and loaded multiaxially within the body [2, 25]. Thus, simultaneous assessment of longitudinal and circumferential mechanics and
contractility may better describe cervical function. Further, uniaxial specimen preparation disrupts native ECM-cell interactions and cervical geometry. Biaxial inflation-extension testing overcomes these limitations by simultaneously loading specimens longitudinally via axial-extension and circumferentially by pressurizing the organ within physiologic limits (Fig 1.2) [41]. Previously, biaxial inflation-extension testing quantified mechanical properties of vasculature [42-45], the GI tract [46, 47], and reproductive organs [2, 3, 48-50]. Biaxial inflation-extension testing permits quantification of both passive and contractile function to inform mathematical models [51-54]. Descriptive mathematical models in conjunction with experimental data can predict changes in function over time and to elucidate causes of CI [6, 52].

Therefore, determining relationships between dynamic cervical ECM, cSMC contractility, and passive function will provide critical information to develop diagnostic tools, treatments, and predictive computer models [6, 55].
1.4 Specific Aims

Preterm birth (PTB) is a common condition that affects approximately 1 in 10 pregnancies and can lead to lifelong health risks and complications for the infant and mother. Many cases of preterm birth are linked to infections, yet, a significant percentage of spontaneous PTBs are associated with cervical insufficiency (CI). CI is characterized by premature shortening and dilation of the cervix in the absence of contractions and other normal labor signs. While the etiology of CI remains poorly understood and efficacy of clinical interventions variable, relationships between improper extracellular matrix (ECM) remodeling and corresponding changes in biomechanical properties during pregnancy are linked with CI. Additionally, cervical smooth muscle cells (cSMC) contribute to cervical function and maintenance in conjunction with the ECM. The relationship of cSMC contractility during pregnancy and postpartum with CI is not established. Towards this end, direct quantification of cervical ECM, mechanical properties, and contractility throughout pregnancy and postpartum are limited. Traditional mechanical testing protocols utilizing uniaxial strips or rings of tissue require disruptive tissue preparation steps that alter native geometry and break microstructural connections. Further, the cervix is loaded multiaxially within the body and uniaxial tests do not recapitulate the multiaxial loading environment. Therefore, tools to determine cervical function in multiple loading directions simultaneously are crucial. Biaxial inflation-extension testing provides a unique opportunity to jointly characterize the circumferential and longitudinal passive mechanical properties and SMC contractility, preserve native tissue geometry, and cell-matrix interactions. Quantification of the evolving cervical biaxial mechanical properties and contractility in the nulliparous and parous cervix will advance understanding of cervical
function. Further, such information is necessary to develop computational models of CI to better understand its etiology, as well as to rationally design clinical therapeutics and diagnostic tools.

Therefore, the overall objective of this study was to determine the evolving biaxial mechanical properties and microstructural composition of the nulliparous and parous murine cervix.

**Aim 1: Quantify the microstructural content, biaxial passive mechanical properties, and contractility of the nulliparous murine cervix.**

We hypothesized that the passive cervix would be stiffer in the circumferential direction compared to the axial direction. Additionally, we hypothesized that increased axial and circumferential loading would decrease the maximum amplitude of phasic contractions in the active cervix. This hypothesis was tested by leveraging biaxial inflation-extension testing and quantitative histology.

**Aim 2: Determining the role of elastic fibers on nulliparous cervical passive biomechanics and contractility in vivo and in vitro.**

We hypothesized that decreased elastic fiber content within the murine cervix would result in decreased contractile ability and a stiffer biomechanical response. To accomplish this, we utilized a genetically altered colony of mice with a global knock out of the Fibulin-5 gene. Wildtype and haploinsufficient mice from the colony were used for *in vivo* contractility experiments and biaxial maximum contractility, biaxial basal, and biaxial passive mechanical tests. Additionally, the role of mechanical loading *in vitro* was investigated with a pressurized culture pilot study.
**Aim 3:** Characterize the evolving cervical function and contractility during pregnancy and early post-partum.

We hypothesized that there would be a gradual increase in compliance during pregnancy followed by a rapid return to pre-pregnant mechanical properties postpartum. Further, phasic contractility would decrease through mid-gestation followed by an increase in contractile frequency and amplitude during late pregnancy. These hypotheses were evaluated by quantifying evolving biaxial mechanical properties, contractility, SMC phenotypes, and ECM composition during pregnancy and postpartum healing.

The proposed research increased understanding of the biomechanical and microstructural remodeling of the nulliparous and gravid murine cervix. Such knowledge is expected to inform future research to improve diagnosis criteria for CI and predict potential maladaptive remodeling during pregnancy.
Chapter 2: Protocol Establishment for Passive Biaxial Testing Inflation-Extension of the Murine Cervix and Uterus

2.1 Introduction

The reproductive organs respond to hormones during the estrous cycle, a continuous cycle of communication between the hypothalamus and the sexual organs, to perform normal reproductive function [56-58]. The estrous cycle is comprised of four phases (diestrus, proestrus, metestrus, and estrus) corresponding with increases or decreases of steroid hormones that prepare the reproductive system for ovulation and pregnancy [56]. The diestrus phase is characterized by an increase in progesterone stimulating the thickening of uterine lining to prepare for pregnancy [56, 59]. Nearing the end of the diestrus phase a subsequent decrease in progesterone promotes reabsorption of the uterine lining. In animals that menstruate, the drop in progesterone due to corpus luteum regression signals the uterus to shed the uterine lining resulting in menstruation [56, 59]. Conversely, the estrus is characterized by an upregulation of luteinizing hormone and estrogen resulting in ovulation [56]. During estrus, the hydration of cervical mucus increases facilitating sperm motility through the cervical canal [60]. Additionally, estrogen encourages thickening of the uterine lining thickens in preparation for ovulation and implantation.

In addition to changes in cellular activity, cervical and uterine extracellular matrix (ECM) components remodel in response to hormonal fluctuations which may result in changes to reproductive function. In the ewe cervix within one day of estrus detection, collagen content decreases and matrix metalloproteinase-2 (MMP-2) activity increases suggesting cervical dilation at estrus is a result of collagen degradation [61]. Additionally, glycosaminoglycan (GAG) content in the rat cervix increased significantly at estrus
compared to diestrus [62]. Further, estrogen treatment on ovariectomized rats upregulated GAG production in the uterine horns [63]. Alterations in ECM composition through hormone production may result in changes to the biomechanical properties of the cervix and uterus permitting contrasting function throughout the estrous cycle [64]. However, the mechanical properties of the reproductive system remain relatively understudied and a need remains characterize the mechanical properties of the cervix and uterus within a physiologically-relevant multiaxial loading environment [65-72].

Mechanical testing permits determination of soft tissue mechanical properties \textit{in vitro}. Further, experimental data can be applied to formulate mathematical models that can describe and predict mechanical behavior of tissues in a healthy or pathological state [42, 51, 52]. Prior mechanical testing of the cervix and uterus primarily utilized uniaxial strips or rings of tissue in the mouse [21, 73-75], rat [28, 38], or human [10, 76, 77]. In addition to uniaxial testing, human and porcine studies utilized inflation testing of the cervix [78] and planar biaxial testing of the uterus [65-67]. However, uniaxial and planar biaxial specimen preparation is disruptive to tissue geometry and native ECM-cell interactions [52]. Biaxial inflation-extension testing permits preservation of tissue geometry and can recapitulate increasing intra-abdominal pressure and shifting organ length with normal activity [52].

A need remains to optimize a biaxial inflation-extension protocol for the murine cervix and uterus to better describe physiologically-relevant biomechanical behavior. Further, there is a need to determine an appropriate constitutive strain energy function for the nulliparous murine cervix and uterus. Prior work applied microstructurally-motivated constitutive models to describe arteries [42, 52, 79, 80], veins [45], the gastrointestinal tract
[46, 81, 82], and reproductive tract [48, 78]. The information supplied by the microstructurally motivated model aids in describing normal cervical and uterine function and in developing a model of growth and remodeling over a set time course to describe processes such as pregnancy or pathological conditions such as uterine fibroids or cervical insufficiency [12, 25, 45, 52, 83-85]. Therefore, the objectives of this chapter were to: Adapt and optimize a biaxial testing protocol from vasculature for the nulliparous murine cervix and uterus; Determine the biomechanical properties of the cervix and uterus; Characterize role of estrous cycle phase on reproductive biomechanics; and Identify and apply an appropriate constitutive model for the two organs.

2.2 Methods:

2.2.1 Animal Care

A total of \( n=18 \) C57BL6 female mice aged 4-6 months old housed in a 12-hour light/dark cycle and fed normal chow were utilized for an estrous cycle pilot study \( (n=12; \ n=6 \text{ per group}) \) and constitutive model pilot study \( (n=6) \) with approval from the Tulane University Institutional Animal Care and Use Committee (IACUC). Estrous cycle phase was determined visually utilizing methodology from Byers et al., 2012 for both studies [56]. For the estrous cycle pilot study, mice were randomly assigned to an estrus phase group \( (n=6) \) and diestrus phase group \( (n=6) \). A separate cohort of C57BL6 female mice at estrus aged 4-6 months of age were assigned to the constitutive modeling pilot study \( (n=6) \). After identification of the estrus phase all mice were sacrificed with CO\(_2\) inhalation.

2.2.2 Dissection

Hair removal cream applied on the abdomen removed the fur and exposed the underlying skin. Then dissection scissors were utilized to cut the skin and muscle layer
vertically from pubis to sternum. Next, lateral cuts under the ribs and above the pubis were made to create a “window” into the abdomen. Using blunt forceps, the intestines were gently displaced superiorly, and the reproductive system visualized. Visceral fat around the uterine horns was gently cut away with micro-scissors to better visualize the length of the reproductive system from ovaries to vaginal introitus. The pubic symphysis was then cut with dissection scissors and the immediate fascial connections removed from the anterior vagina and urethra. Using angled forceps and blunt dissection techniques the bladder and urethra was separated from the vaginal wall. Once the entire length of the reproductive tract from vaginal introitus to uterine horn was accessible, India Ink dots were placed along the length of the reproductive tract every 3 mm with land markers placed immediately superior to the vaginal introitus, inferior of the ovaries, and centered on the mid-cervix. Measurements of vaginal, cervical, and uterine length were recorded using digital calipers. Singular cuts were made through the uterine horns immediately below the ovaries and a circular cut around the vaginal introitus released the reproductive system from the body. The reproductive system was placed in a dish filled with 4°C Hank’s Balanced Saline Solution (HBSS) and allowed to rest for 30 minutes. Following release from the internal fascial connections, the reproductive tract retracted axially from the original length. The new length of the vagina, cervix, and uterine horns were recorded and used to calculate a first estimated guess at the estimated physiologic (EP) length during mechanical testing. Reproductive systems were stored in HBSS at -20°C for storage until mechanical testing. Prior research showed that murine cervical passive mechanical properties do not change significantly with one freeze-thaw cycle [26].
2.2.3 Sample Preparation and Cannulation

Reproductive systems were thawed at room temperature prior to mechanical testing. For repeatable identification, the utero-cervical complex was identified as the structure contained within the markings placed superior and inferior to mid-cervical marking for a known in vivo length of 6mm prior to retraction. The utero-cervical complex was isolated from the reproductive system and the vagina fornix carefully dissected away with micro-scissors to expose the external os of the cervix. Following, approximately two silk 6-0 sutures were tied around the base of the randomly assigned contralateral uterine horn to prevent interference on diameter tracking and maintenance of pressure during the biaxial mechanical testing protocols. Additionally, the contralateral uterine horn was cut as close to the sutures as possible to further prevent interference with diameter tracking. The sample was cannulated with 6-0 silk suture within a custom biaxial inflation-extension device (Danish MyoTechnologies, Aarhus, Denmark) on cannulae sized 0.85 mm in diameter. The uterine end was cannulated on the fluid flow inlet and the external os on the outlet to mimic the natural flow of menstrual fluid or childbirth. Samples were maintained in a bath of HBSS kept at 4°C during cannulation.

2.2.4 Biomechanical Testing

The biaxial inflation-extension device (Danish MyoTechnologies, Aarhus, Denmark) consists of a basin with a glass bottom to contain the tissue during testing, a computer-controlled flow meter, inlet and outlet pressure transducers, a force transducer, a temperature probe, a manual axial-micrometer, and a Nikon Eclipse TS100 inverted microscope (Nikon®, Melville, NY, U.S.A.). The components connect with a desktop
computer and custom software (Danish MyoTechnologies, Aarhus, Denmark) to record measurements of pressure, force, temperature, and diameter in real time.

The reproductive organs are loaded multiaxially within the body and biaxial inflation-extension testing permits assessment of mechanical properties within a more physiologically relevant configuration than uniaxial or planar biaxial testing. To best recapitulate the in vivo loading environment in vitro, the pressure range was set to a maximum of 200 mmHg based on experimental reported values of the maximum intra-uterine contractile pressure during menstruation in humans [86, 87]. Pressure gradient and axial-extension were controlled at rates of 2.0 mmHg/s and 0.01 mm/s to prevent dynamic loading of the tissue during experimentation [42].

Post-cannulation and prior to mechanical testing, the axial-micrometer stretched the cannulated utero-cervical complex to an estimated unloaded length. The unloaded length was determined as the length where the organ was not visibly in compression or tension. The unloaded length and unloaded diameters, tracked across the mid-cervix and base of the uterine horn, were measured with digital calipers and a Nikon Eclipse TS100 inverted microscope (Nikon®, Melville, NY, U.S.A.), respectively. Once mounted, the samples were maintained at 37°C in HBSS throughout the mechanical testing protocol.

Soft tissues are nonlinear, viscoelastic materials that exhibit hysteresis, however, cyclic loading diminishes the hysteresis response and results in consistent and repeatable mechanical properties [52, 88]. Therefore, samples were loaded cyclically until the outer diameter and force data followed repeatable cycles prior to testing, known as preconditioning [42, 49, 52]. To ensure repeatable results, samples underwent 5 cycles of
pressure-inflation preconditioning from the minimum to the maximum physiologic pressure (P= 0-200 mmHg) at the unloaded length and 5 cycles of force-elongation preconditioning at a constant pressure P=67 mmHg (1/3 max pressure) from the unloaded length to +10% the unloaded length.

Within arteries there is an assumption that the estimated physiologic length can be determined *in vitro* as the axial length in which the force remains constant with increasing intraluminal pressure [42, 89]. Initially, the sample was axially stretched from the unloaded length to 6.0 mm as a first estimation of the estimate physiologic (EP) length. Next, utilizing the assumption of force preservation over a pressure range, the EP length was found by axially extending and compressing the tissue while increasing pressure. Subsequently, the utero-cervical complex underwent another 5 cycles of pressure-inflation preconditioning (P= 0-200 mmHg) at the EP length and 5 cycles of force-elongation preconditioning (P= 67 mmHg) from ±2% the EP length. Prior work in arteries and the vagina axially-elongated the tissue during biaxial inflation-extension testing to ±5% and ±4%, respectively [42, 49, 50]. However, axial-stretches of ±4% the EP length resulted in plastic deformation of the tissue. After preconditioning, the tissue equilibrated at the EP length and a tare pressure of P=10 mmHg for 10 minutes.

To delineate the mechanical properties of the utero-cervical complex within a physiologically relevant range, the tissue underwent 3 cycles of pressure-inflation testing (P= 0-200 mmHg) at three static lengths (EP length and ±2% EP length) [86, 87]. Following, the tissue underwent 3 force-elongation cycles from -2% EP length to +2% EP length at a tare pressure P=10 mmHg, 1/3 max pressure (P = 67 mmHg), 2/3 max pressure (P= 133 mmHg), and maximum pressure (P= 200 mmHg). The force-elongation protocol
secondarily confirmed the EP length under the assumption that force is preserved at the EP length irrespective of pressure [89, 90].

### 2.2.5 Thickness and Opening Angle Measurements

#### Thickness and Volume Calculations

Following biomechanical testing, the utero-cervical complex was placed within a dish of HBSS and 0.5mm rings were isolated from the mid-cervical and lower uterine region. Rings of cervical and uterine tissue equilibrated after the disruptive isolation for 30 minutes in HBSS. A Moticam 580 HD Digital Camera and Motic Images software (Motic® Richmond, British Columbia) acquired images of the rings and a custom MATLAB code calculated unloaded wall thickness ($H_o$) from the images [42, 51]. Assuming the cervix and uterus geometry can be simplified to a hollow cylinder, the volume ($V$) was calculated using Equation 1:

$$V = \pi L (R_o^2 - R_i^2),$$  \hspace{1cm} (1)

where $L$ is the unloaded length, $R_o$ is the unloaded outer radius, and $R_i = R_o - H_o$ is the unloaded inner radius.

#### Opening Angle

Utilizing the 0.5 mm rings isolated from the mid-cervix and uterus, the tissue was left to rest in 4°C HBSS for 30 minutes after imaging. Following, a full thickness radial cut

**Opening Angle Experiment**

**Posterior**

**Anterior**

**Figure 2.1:** Schematic of opening angle experiment indicating the location of the anterior full thickness radial cut with a dashed line and the measurement of the opening angle ($\Phi$).
was made on the anterior wall of the rings and the tissues equilibrated for an additional 30 minutes. Assuming a residual stress exists within the tissue at the unloaded state, the radial cut releases tension within the wall of the tissue and results in the ring “opening up” [52, 91, 92]. The change in distance between the severed walls caused by the opening of the ring is defined as residual strain which can be experimentally measured as the angle of the opened ring or the opening angle (Fig. 2.1) [52, 91, 92]. Using the Moticam 580 HD Digital Camera and ImageJ the angle from the posterior mid-wall to the tips of the outer wall segments was measured and recorded for the cervix and uterus [42].

2.2.6 Data Analysis

The initial point data from the -2% EP length test was used as the reference configuration to determine circumferential ($\sigma_\theta$) and axial ($\sigma_z$) Cauchy stresses in each organ from pressure-inflation data at each point (Eq. 2, 3) [42].

$$\sigma_\theta = \frac{P_a}{h},$$  \hspace{1cm} (2)

and

$$\sigma_z = \frac{F_t + \pi a^2 P}{\pi h (2a + h)},$$  \hspace{1cm} (3)

Where $P$ is the intraluminal pressure, $a$ is the deformed inner radius, $h$ is the deformed thickness, and $F_t$ is the force from the axial force transducer. Additionally, circumferential ($\lambda_\theta$) and axial ($\lambda_z$) stretch ratios (Eq. 4, 5) which were determined for each point in the pressure-inflation tests via [42]:

$$\lambda_\theta = \frac{r_{mid}}{R_{mid}},$$  \hspace{1cm} (4)
and
\[
\lambda_z = \frac{l}{L},
\]
where \(r_{\text{mid}}\) is the loaded mid-wall radius, \(R_{\text{mid}}\) is the unloaded mid-wall radius, \(l\) is the loaded axial length, and \(L\) represents the unloaded axial length.

Circumferential (\(\sigma_\theta\)) and axial (\(\sigma_z\)) Cauchy stress values were plotted against the circumferential stretch (\(\lambda_\theta\)) to create stress-stretch curves. Bilinear-curve fits, a linear interpolation applied to a non-linear curve, were applied along the toe-region and linear-region of the stress-stretch curve to quantify toe- and elastic-moduli for the pressure-inflation tests (Fig. 2.2) [49, 93].

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig2.png}
\caption{Representative bilinear curve fit of the cervix during the estimated physiological length pressure diameter test showing the experimental data (•) and the fitted curve (-). The toe-region where the collagen fibers are not fully engaged, the transition point (+) that highlights the point where collagen fibers are fully aligned or stretched, and the linear-region representing the phase when the collagen fibers are fully engaged, are indicated.}
\end{figure}

2.2.7 Material Parameters Pilot Study

\textit{Determination of Appropriate Constitutive Model}

A pilot study was performed to determine an appropriate 2D microstructurally-motivated functional form of a stored energy function (SEF) for the cervix and uterus. Constitutive model results were considered via goodness of fit and over-parameterization
Three microstructurally-motivated models were considered: the two-fiber family (2FF), the three-fiber-family (3FF), and the four-fiber-family (4FF) [78, 80, 95-97]. For each microstructurally motivated model, we implemented a quadratic and Neo-Hookean ground matrix term [78, 80, 95-97]. Additionally, the Fung phenomenological model, commonly used for modeling soft-tissues, was considered to describe cervical and uterine biomechanical response [45, 52, 88, 98].

*Microstructurally-Motivated Constitutive Models*

The following microstructurally-motivated SEFs contain an isotropic term Neo-Hookean term to describe the elastin dominated ground matrix or an anisotropic quadratic term to describe the lower stress regime and a collagen dominated anisotropic term which can be set to represent collagen fiber families oriented in 2, 3, or 4 directions [80].

We first started with the 2FF model, initially proposed by Holzapfel *et al.* for artery mechanics [80], with a Neo-Hookean isotropic term (Eq. 6) or an anisotropic quadratic term (Eq. 7) to describe the elastin dominated ground matrix [45, 80, 99]. Additionally, there is an anisotropic term to describe the contribution of collagen that is assumed to be diagonally symmetric about the long axis (Eq. 6, 7):

**Neo-Hookean 2FF Model**

\[
W(C, M^k) = \frac{c}{2}(I_c - 3) + \sum_{k=1}^{2} \frac{c^k}{2c^2_k} \left[ \exp \left( c^k_k \left( \lambda_k^2 - 1 \right)^2 \right) - 1 \right]
\]  

**Quadratic 2FF Model**

\[
W(C, M^k) = q + \sum_{k=1}^{2} \frac{c^k}{2c^2_k} \left[ \exp \left( c^k_k \left( \lambda_k^2 - 1 \right)^2 \right) - 1 \right]
\]

where \( c \) is a material parameter with units of stress corresponding with the Neo-Hookean component, \( c_1 \) is a material parameter with units of stress related to the collagen
contribution, $c_2$ is a dimensionless material parameter, $C = F^T F$ is the right Cauchy-Green tensor, $M^k = [0, \sin\alpha_0^k, \cos\alpha_0^k]$ is the fiber orientation unit vector, $I_C$ is the first invariant of $C$, $I_C = trC = \lambda_\theta^2 + \lambda_z^2 + \lambda_r^2$, and $\lambda_k = \sqrt{M^k \cdot C M^k}$ is the fourth invariant which defines the stretch experienced by the $k^{th}$ fiber family. Lastly, $q$ represents the quadratic component to account for low stress anisotropy described by Eq. 8 [45, 99]:

$$q = b_\theta^2 E_\theta^2 + b_z E_z^2 + b_\theta E_\theta E_z$$ (8)

Following, we utilized the 3FF model (Eq. 9, 10), an extension of the Holzapfel 2FF model, where the collagen-dominated term assumes the microstructure consists of two collagen families diagonally symetric along the long axis and one collagen family aligned with either the circumferential or longitudinal axis [78]. Assuming the cervix and uterus contained a majority of circumferentially aligned collagen, we set the third fiber family to the circumferential orientation for the pilot study [100-102]. To describe the ground matrix of the tissues, we utilized a Neo-Hookean isotropic term (Eq. 11) or an anisotropic quadratic term (Eq. 12) [45, 80, 99]:

*Neo-Hookean 3FF Model*

$$W(C, M^k) = \frac{c_2}{2} (I_C - 3) + \sum_{k=1}^{3} \frac{c_k}{4c_2^k} \left[ \exp \left( \frac{c_k}{c_2^k} \left( \lambda_k^2 - 1 \right)^2 \right) - 1 \right]$$ (11)

*Quadratic 3FF Model*

$$W(C, M^k) = q + \sum_{k=1}^{3} \frac{c_k}{4c_2} \left[ \exp \left( \frac{c_k}{c_2} \left( \lambda_k^2 - 1 \right)^2 \right) - 1 \right]$$ (12)

where $k = 1, 2, 3$ and $M^k = [0, \sin\alpha_0^k, \cos\alpha_0^k]$ is the fiber orientation unit vector where $\alpha_0^k$ was set for circumferential alignment and diagonally symmetric alignment and with respect to the long axis.
Lastly, we utilized the 4FF model (Eq. 13, 14), another extension of the Holzapfel 2FF model, where the collagen is assumed to be aligned diagonally symmetric along the long axis, along the longitudinal axis, and along the circumferential axis [42, 45]. Similarly to the 2FF and 3FF models, the isotropic Neo-Hookean component (Eq. 13) and the anisotropic quadratic component (Eq. 14) were utilized to describe the ground matrix [42, 45].

**Neo-Hookean 4FF Model**

\[
W(C, M^k) = \frac{c}{2} (I_C - 3) + \sum_{k=1}^{4} \frac{c^k_1}{4c_2^k} \left[ \exp \left( \frac{c^k_2}{2} \left( \frac{\lambda^2_k}{2} - 1 \right)^2 \right) - 1 \right] \tag{13}
\]

**Quadratic 4FF Model**

\[
W(C, M^k) = q + \sum_{k=1}^{4} \frac{c^k_1}{4c_2^k} \left[ \exp \left( \frac{c^k_2}{2} \left( \frac{\lambda^2_k}{2} - 1 \right)^2 \right) - 1 \right] \tag{14}
\]

where \( k = 1, 2, 3, 4 \) and fiber angle, \( \alpha_0^k \), was set for \( 0^\circ \) for axial alignment, circumferential alignment, and diagonally symmetric with respect to the long axis.

**Fung Phenomenological Model**: 

Lastly a phenomenological model, a model comprised of empirically determined material parameters, was employed to describe the behavior of the cervix and uterus. Specifically, we utilized the Fung model due to the prevalence of the model in describing soft tissues (Eq. 15) [45, 52, 88, 98, 103]:

\[
W = \frac{1}{2} c_0 (\exp(Q) - 1) \tag{15}
\]
where \( Q = c_1E_{zz}^2 + c_2E_{\theta\theta}^2 + 2c_3E_{zz}E_{\theta\theta} \), \( c_0 \) is a stress-like material parameter with units of stress, \( c_1, c_2, \) and \( c_3 \) are non-dimensional material parameters, and \( E_{zz} \) and \( E_{\theta\theta} \) are the axial and circumferential components of Green strain \( E = \frac{1}{2} (F^T \cdot F - I) \) respectively.

Assuming incompressibility, theoretical Cauchy stresses were determined from Equation 16 [52]:

\[
t = -pI + \frac{2F}{J} \frac{\partial W}{\partial \varepsilon} F^T
\]

(16)

where \( p \) is the Lagrange multiplier, \( F \) is the deformation tensor, and \( J = \text{det}F \).

To calculate best fit model parameters that are unique and to minimize error between theoretical and experimental data, a non-linear regression was utilized via MATLAB using the following objective function [42]:

\[
e = \sum_{i=1}^{N} \left[ \left( \frac{P_{th} - P_{exp}}{P_{exp}} \right)^2_i + \left( \frac{F_{th} - F_{exp}}{F_{exp}} \right)^2_i \right],
\]

(17)

where \( P_{th} \) and \( P_{exp} \) are the respective theoretical and experimental intraluminal pressures, \( P_{exp} \) is the mean experimental pressure, \( F_{th} \) is the theoretical force, \( F_{exp} \) represents experimental forces, and \( F_{exp} \) is the mean experimental force [42].

2.2.8 Linearized Stiffness

A total of \( n=6 \) mice were utilized for a pilot study to calculate linearized stiffness within a physiologic pressure range utilizing the theory of small deformations on large deformations [79]. Considering the overall goodness of fit and over-parameterization results from the constitutive model pilot study, linearized stiffness was calculated for each sample utilizing the 2D Neo-Hookean 2FF and the Fung phenomenological model. Prior
research reported a non-pregnant in vivo luminal pressure of 37mmHg within the utero-cervical complex, therefore, stiffness measurements are taken at a point on the stress-stretch curve corresponding with 37mmHg [104].

Circumferential and axial linearized material stiffness at the in vivo deformations (P= 37mmHg) were calculated using the theory of small deformations on large deformations [79]. Cauchy stress was calculated as:

\[ \mathbf{t} = \mathbf{t}^o + \mathbf{t}^* \]  

(18)

\[ \mathbf{t}^o = -p^o \mathbf{I} + \frac{2}{J} \mathbf{F} \frac{\partial W}{\partial \mathbf{C}} \mathbf{F}^T \]  

(19)

\[ \mathbf{t}^* = -p^* \delta_{ij} \delta + \mathbf{C}_{ijkl} \epsilon_{kl}. \]  

(20)

where \( \mathbf{t}^o \) is the stress due to the large deformation, \( \mathbf{t}^* \) is the stress due to the linearized small deformation, \( \mathbf{C}_{ijkl} = \delta_{ik} t^o_{lj} + t^o_{kj} \delta_{jk} + 4 F_{lA} t^o_{jB} F_{kP} t^o_{iQ} \frac{\partial W}{\partial \epsilon_{AB} \epsilon_{PQ}} \) is the linearized material stiffness, \( \delta \) is the Kronecker delta, \( \epsilon \) is infinitesimal strain, and the two sets of indices \( i,j,k,l \) and \( A,B,P,Q \) vary from 1 to 3 [79].

### 2.2.9 3D Constitutive Modeling

While 2D constitutive models provide valuable insights into the mechanical behavior of soft tissues, 3D modeling contributes a more robust description of tissues. Therefore, we completed a 3D constitutive modeling pilot study on the cervix and uterus from \( n=6 \) female C57BL6 mice at estrus. The Neo-Hookean 2FF microstructurally motivated strain energy function was utilized due to the overall goodness of fit in the 2D configuration. Opening angle experiments (Chapter 2.2.5) contributed information about the residual stress and
strain within the tissue. Due to the inclusion of new information the circumferential stretch (Eq. 21) was calculated as:

\[
\lambda_\theta = \frac{\pi}{\pi - \Phi} * \frac{r}{R}
\]  

(21)

where \(\Phi\) is the opening angle in radians, \(r\) is the loaded mid-wall radius, \(R\) is the unloaded mid-wall radius.

Additionally, utilizing the compressibility assumption \((\lambda_\theta \lambda_z \lambda_r = 1)\), the inner radius (Eq. 22) and radial stretch (Eq. 23) were calculated as:

\[
r_i = \sqrt{r_o^2 - (R_o^2 - R_i^2) \frac{\pi - \Phi}{\lambda_z \pi}}
\]  

(22)

and

\[
\lambda_r = \frac{\partial r}{\partial R}
\]  

(23)

where \(r_i\) is the loaded inner radius, \(r_o\)the loaded outer radius, \(R_i\) the unloaded inner radius, \(R_o\) the unloaded outer radius, and \(\Phi\) is the opening angle in radians.

Lastly, the radial Cauchy stresses \((\sigma_r)\) derived from the experimental pressure-inflation data utilizing Equation 24:

\[
\sigma_r = \frac{-p r_i}{r_o + r_i}
\]  

(24)

2.2.9 Statistics

A two-way ANOVA (Axial-Extension, Estrous Phase) was applied to bilinear fit parameters for both cervical and uterine data to determine difference with hormones and axial-extension. Posthoc t-tests with Bonferroni corrections were applied when
appropriate. Paired t-tests determined differences in cervical and uterine linearized stiffness calculations.

2.3 Results

2.3.1 Role of Hormones on Biomechanics of the Cervix and Uterus

Two-way ANOVA analysis of bilinear fit parameters did not reveal significant differences between estrus and diestrous phases, axial-extension, or interactions between axial-extension and hormones in the cervix and uterus ($p > 0.05$).

2.3.2 Identification of an Appropriate Constitutive Model

Overall, the Neo-Hookean 2FF model (Table 2.1) described the cervical and uterine data with the with comparable RMSE to the “gold-standard” Fung Model (Table 2.7) without over-parameterization ($\text{detR} > 10^{-5}$) [94]. The quadratic 2FF model (Table 2.2) described the data with similar RMSE to the Neo-Hookean 2FF, however, uterine samples 1-4 and sample 6 were overparameterized ($\text{detR} < 10^{-5}$) [94]. In addition, $\text{detR}$ values less than $10^{-5}$ determined overparameterization for cervical Samples 3 and 6 [94]. The Neo-Hookean 3FF model (Table 2.3) resulted in over-parameterized uterine and cervical data except for cervical Sample 4 [94]. The inclusion of the quadratic term in the 3FF (Table 2.4) resulted in over-parameterization ($\text{detR} < 10^{-5}$) of all samples and difficulties in describing uterine Sample 1 ($\text{RMSE} = 6.5*10^6$) [94]. Similar to the quadratic 3FF (Table 2.4), the Neo-Hookean 4FF (Table 2.5) and quadratic 4FF (Table 2.6) resulted in over-parameterized samples ($\text{detR} < 10^{-5}$) and large RMSE for uterine Samples 1, 3, and 6 [94].
### Table 2.1: 2D Neo-Hookean 2FF

<table>
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<tr>
<th></th>
<th>Cervix</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( c ) (kPa)</td>
<td>( c_1 ) (kPa)</td>
</tr>
<tr>
<td>Sample 1</td>
<td>3.7E-11</td>
<td>64.8</td>
</tr>
<tr>
<td>Sample 2</td>
<td>5.8E-14</td>
<td>173</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.35</td>
<td>10.2</td>
</tr>
<tr>
<td>Sample 4</td>
<td>0.45</td>
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<td>Sample 5</td>
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<td>27.3</td>
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<td>Sample 6</td>
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### Table 2.2: 2D Quadratic 2FF

<table>
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</tr>
</thead>
<tbody>
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<td></td>
<td>( b_{\theta\theta} ) (kPa)</td>
<td>( b_{zz} ) (kPa)</td>
</tr>
<tr>
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<tr>
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<td>Sample 4</td>
<td>18.6</td>
<td>5.4E-10</td>
</tr>
<tr>
<td>Sample 5</td>
<td>1.2E-12</td>
<td>6.3E-14</td>
</tr>
<tr>
<td>Sample 6</td>
<td>2.53</td>
<td>44.3</td>
</tr>
</tbody>
</table>

**Note:** RMSE and detR values are indicative of model fit accuracy.
Table 2.3: 2D Neo-Hookean 3FF

<table>
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<th>$c$</th>
<th>$c_1^{(1,2)}$</th>
<th>$c_2^{(1,2)}$</th>
<th>$c_1^{(3)}$</th>
<th>$c_2^{(3)}$</th>
<th>$\alpha^\circ$</th>
<th>RMSE</th>
<th>detR</th>
</tr>
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<td></td>
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<tr>
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Table 2.6: 2D Quadratic 4FF

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2.3.3 Linearized Stiffness

Circumferential linearized material stiffness determined for the 2FF SEF at the in vivo configuration (P = 37 mmHg) significantly increased ($p < 0.05$) at the cervix (150±40 kPa) compared to the uterus (53±13 kPa). Additionally, the axial material stiffness significantly increased ($p < 0.05$) for the cervix (40±33 kPa) compared to the uterus (50±8.7 kPa).

For the Fung SEF, circumferential material stiffness increased ($p < 0.05$) at the cervix (170±46 kPa), compared to the uterus (54±13 kPa). The axial stiffness for the cervix (140±48 kPa) did not significantly differ from the uterus (37±6.7 kPa).

2.3.4 3D Constitutive Modeling

To optimize the minimum time needed to achieve the repeatable best fits, we ran the nonlinear regression at 10, 50, 100, 500, and 1000 guesses. Compared to 100 runs, 10 runs and 50 runs resulted in overall decreased fits and greater parameter variability between
runs. However, 100 through 1000 runs resulted in stable material parameters and overall consistent fits. Therefore, the MATLAB code underwent 100 guesses within the linear regression to optimize the best fit material parameters and conserve time (Fig. 2.3).

Overall, the 3D Neo-Hookean 2FF model did not describe the uterus well as shown by the RMSE in Samples 4 and 5 in Table 2.8. Further, compared to the 2D Neo-Hookean 2FF model, the 3D Neo-Hookean 2FF model resulted in greater RMSE (0.29±0.06 vs 0.34±0.06, respectively) and over-parameterized samples (Fig. 2.4) [94].

Figure 2.3: Representative cervical sample of the radial stresses (A), circumferential stress (B), and axial stresses (C) plotted against the pressure in kPa and the normalized radius for the 3D Neo-Hookean 2FF.

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<th>c_1 (kPa)</th>
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<th>RMSE</th>
<th>detR</th>
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2.4. Discussion

This chapter presented methods development of biaxial inflation-extension protocols for the murine cervix and uterus, characterized the biomechanical properties of the murine cervix and uterus at estrus and diestrus, and identified an appropriate constitutive model to describe the biomechanical behavior of the reproductive organs.

Prior work focused on the function of the cervix and uterus throughout pregnancy [10, 26, 65, 67, 73]. However, the role of the reproductive organs in the non-pregnant state is crucial and complex. Depending on the cycle phase and normal hormonal fluctuations

Figure 2.4: Experimental data for the cervix (black circles) and uterus (gray circles) and the theoretical fits for the cervix (black line) and uterus (gray line). Compared to the 2D Neo-Hookean 2FF model, the 3D Neo-Hookean 2FF theoretical fits did not accurately describe cervical and uterine biomechanical behavior.
the function of the cervix and uterus alternates between preparing for ovulation or pregnancy [56, 59]. However, we identified no significant differences in biomechanical properties in the murine cervix and uterus at estrus and diestrus. Power analysis identified a minimum of $n=38$ and $n=36$ samples required to determine significance with respect to estrous phase for the murine cervix and uterus, respectively. Potentially, remodeling may occur primarily in the non-load bearing epithelial layer due to the sensitivity of the epithelial cells to hormones. Preliminary ultrasound imaging studies performed by Hamna Qureshi and Craig Goergen at Purdue University identified variability and significant differences ($p<0.05$) in the outer diameter of the murine cervix in the same mouse at two separate estrus cycles (Methods further expanded in Chapter 3). In the ewe cervix, estrus phase induced upregulation of hyaluronic acid and GAGs in the epithelial layer of the ewe cervix [18]. Additionally, Rodríguez-Piñón et al. identified upregulation of MMP-2 and collagen content in the ewe cervix suggesting increased turnover of collagen throughout the estrus cycle [61]. Potentially, increases in collagen content and MMP-2 activity may be exacerbated in larger animal models. Further, the mouse estrus cycle lasts for approximately 4-5 days compared to approximately 17 days in the ewe [56, 61]. This increased time frame may allow for a greater extent of collagen turnover within the reproductive organs.

The Neo-Hookean 2FF (Table 2.1) described the cervical and uterine data relatively well compared to the “gold standard” Fung model (Table 2.7) and no samples were over-parameterized as set by the guidelines in Yin et al. [94]. The quadratic 2FF (Table 2.2) model described the data with comparable RMSE to the Fung model, however, the addition of the anisotropic quadratic term over-parameterized the model. Prior work
utilized a 3D 3FF model to describe the uterine cervix, however, the 2D Neo-Hookean 3FF (Table 2.3) culminated in overparameterized results. Further, the inclusion of the quadratic term in the 3FF and 4FF model resulted in large variations in the RMSE for the uterine samples and high variability amongst the parameters (Tables 2.4, 2.6). Utilizing the theory of “small on large” with the Neo-Hookean 2FF and Fung models, we calculated the linearized stiffness at an estimated physiologic point. Wherein, we determined the physiologic point on the stress-stretch curve as the circumferential stretch corresponding to P = 37 mmHg, an in vivo intrauterine pressure measurement in the non-menstruating human [104]. Linearized stiffness confirmed anisotropy in the murine cervix and uterus wherein the circumferential linearized stiffness significantly increased compared to the axial linearized stiffness in both organs. A decrease in axial stiffness in addition to maladaptive collagen and elastic fiber remodeling in the murine cervix may provide rationale for the development of cervical insufficiency, a condition clinically characterized by the shortening of the cervix during early- to mid-pregnancy resulting in preterm birth [11, 13, 17, 21]. While 2D modeling provides crucial fundamental knowledge, 3D modeling provides robust descriptions of soft tissue mechanics. To bridge the gap between 2D and 3D modeling, we adapted the Neo-Hookean 2FF for 3D analysis utilizing the mean opening angle results to gather radial information. However, the 3D model resulted in overparameterized material parameters for the cervix and large variations in RMSE for the uterus. The model assumed incompressibility, yet, estrogen upregulates GAGs and hyaluronic acid in the murine cervix and uterus. Including compressibility and the Neo-Hookean 2FF strain energy function into a 3D finite element could potentially result in more robust 3D results [6, 12, 76].
In conclusion, this grouping of pilot studies presents adaption and optimization of biaxial inflation-extension testing methods from vasculature and identified a 2D microstructurally-motivated constitutive model for the cervix and uterus. Implementation of the biaxial inflation-extension methodology and Neo-Hookean 2FF model for the murine cervix and uterus may provide fundamental information on the function of the organs in the nulliparous state and promote investigation into remodeling paradigms such as pregnancy and postpartum.

Acknowledgements and Funding

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Chapter 3: Biaxial Biomechanical Properties of the Nonpregnant Murine Cervix and Uterus


3.1 Introduction

Recent work on the female pelvic floor emphasized the importance of quantifying biaxial properties to improve the basic understanding of physiological function and pathologies of the female reproductive tract [25, 54, 65, 68]. Conditions such as cervical insufficiency or pelvic organ prolapse have unknown etiologies, however, remodeling of the microstructure and dynamic loading environment play a significant role [11, 21, 105]. Fluctuations in geometry and microstructural composition dictate changes in biomechanical properties and organ function in response to physiologic changes such as the cyclical hormones, pregnancy, and post-partum recovery [21, 27, 106].

Two organs of interest, the cervix and uterus, have complex functions, geometry, and microstructure. Previous work quantified the microstructure and mechanical response of the cervix and uterus in human [13, 33, 34, 77, 107], rat [38, 108, 109], and mouse [14, 74, 110]. To date, cervical and uterine biomechanical properties are primarily determined uniaxially [21, 28, 39, 77, 107, 111]. The cervix and uterus, however, are loaded multiaxially within the body, resulting in changes in luminal diameter and length with the menstrual cycle and alterations in intra-abdominal pressure [38, 104].
Common testing techniques, such as planar uniaxial or biaxial testing, require sample preparation that alters the shape of the tissue and does not preserve the matrix-cell interactions. Biaxial inflation-extension protocols, contrastingly, preserve tissue structure, ECM interactions, and permit evaluation of tissue anisotropy within an estimated physiologic axial extension over a physiologic range of pressures [41, 51, 89, 90]. This technique, to date, has not been used for the murine cervix or uterus.

A need remains to investigate the biomechanical properties of the organs to better understand the axial-circumferential interactions within the organs and to investigate the organs as a continuous structure. Maintaining the organs’ native geometry and matrix interactions enables quantification of the gradient of mechanical properties through the reproductive tract. Quantifying the geometry, microstructure, and biomechanics of each organ is necessary to develop accurate models to determine the causes of reproductive pathologies [6, 12, 112]. Therefore, the objective of this study was to quantify the regional biaxial biomechanical properties, geometry, and microstructure of the murine cervix and uterus.

3.2 Methods

3.2.1 Animal Care

The care and use of animals were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Tulane University and Purdue University. A total of \( n=22 \) (\( n=10 \) from Purdue University and \( n=12 \) from Tulane University) virgin female C57BL/6J mice at 4-6 months of age were utilized for the study (Jackson Laboratory, Bar Harbor, ME). Mice were housed in a 12 hour light/dark cycle on a normal
chow diet. Estrous cycle phase was monitored and determined visually and all experiments were performed on female mice at estrus [113].

3.2.2 Ultrasound imaging of in vivo geometry

Reproductive tracts from \( n = 10 \) female C57BL/6 mice between 4-6 months of age were imaged using a Vevo2100 ultrasound system (FUJIFILM VisualSonics, Inc, Toronto, ON, Canada) (Fig. 3.1). Utilizing a 22-55MHz transducer with a central frequency of 40MHz (MS550D) and a linear step motor, 2D short axis B-mode slices were taken with a step size of 0.19mm along the length of the reproductive system and compiled into 3D representations (Fig. 3.2).

Measurements of thickness and diameter were taken at the base of the uterine horn and the mid-cervix (Fig. 3.3A, 3.3B). Animals were euthanized at estrus via \( \text{CO}_2 \)
inhalation, frozen at -20°C, shipped to Tulane University, and stored at -20°C. Noting that prior research determined there are no significant effects of a freeze-thaw cycle on the passive mechanical behavior of the cervix [26].

**Figure 3.2:** Ultrasound images compiled into 3D segmentations of the reproductive tract exploring the regions of interest (ROI). (A) Coronal view of the murine reproductive tract that extends into the uterine horns and the external os which is located at a transitional region between the cervix and vagina. Length of the ROI was measured from the split of the uterine horns to the edge of vaginal/cervical border region based on gross anatomical descriptions from past work in rodent models c.f. Leppi, 1964 [10]. (B) Sagittal view along the length of the murine reproductive system depicting the cervical/vaginal border region transitioning into the right uterine horn. (C) Axial view of murine cervix showing a cross-section at the region above the opening into the vaginal canal. (D) Compiled 3D view along the length of the murine cervix and lower uterine segment.

### 3.2.3 Sample collection and preparation

Mouse reproductive systems were exposed and stain markings were placed every 3mm along the reproductive tract with India Ink (Dick Blick Art Materials, Galesburg, IL) [49]. Markings were placed immediately inferior to the ovary, centered on the cervix, and immediately superior to the vaginal introitus. During excision, the reproductive organs retracted from their original positions following isolation from supporting structures. For
repeatability, the uterocervical complex was identified as the structure within three markings guided by the central cervical marking. Singular cuts were made superior to the mark above the central cervix mark and inferior to the mark below to the central mark for a known in situ length of 6mm as an initial approximation of the physiologic length (Fig. 3.4A) [49].

**Figure 3.3:** (A), (B) Representative measurements of outer horizontal diameter (OD), anterior thickness (AT), and posterior thickness (PT) taken in the region of the cervix (A) and the uterus (B), where the canal for both uterine horns acts as a location marker for geometric analysis. (C) The horizontal diameter for the cervix was significantly larger (p < 0.01**) than the uterus in which cervical diameter was 3.59±0.12mm and uterine diameter 1.95±0.06mm (D) Calculated wall thickness, from averaged anterior and posterior measurements at both locations, was significantly larger within the cervix at 0.63±0.04mm compared to 0.54±0.05mm in the uterus (p < 0.05*).

Samples were bathed in Hank’s balanced saline solution (HBSS) at 4°C, excess vaginal tissue removed from the exterior of the external os of the cervix, and one uterine horn was randomly selected for mechanical assessment while the secondary uterine horn
was ligated with 6-0 silk suture (Fig. 3.4B,3.4C). Specimens were cannulated at the uterine horn and external os within a myograph inflation-extension device (Danish MyoTechnologies, Aarhus, Denmark; Fig. 3.4C) with 6-0 silk suture.

**Figure 3.4:** (A) Schematic of the murine female reproductive system with black dots representing the marks made to measure the length of the reproductive tract and dashed lines representing the locations of the singular cuts. (B) Graphic of the utero-cervical complex in preparation for cannulation. The randomly chosen, contralateral uterine horn is ligated and the tissue within dotted outline represents the excess vaginal tissue to be removed to expose the external os. (C) A schematic testing setup of the custom myograph inflation-extension device (Danish MyoTechnologies, Aarhus, Denmark) with utero-cervical complex cannulated in a view from the side. The cervix (CER) and uterine (UTE) body are positioned within the system so that the inlet is connected to the uterine horn and the outlet to the vaginal cervix to mimic the flow from the uterus into the cervix during menstruation or birth.

Cannulated specimens were stretched to an estimated unloaded length visually determined as the length wherein the organ was not in compression or tension. A Nikon Eclipse TS100 inverted microscope (Nikon®, Melville, NY, U.S.A.) optically measured unloaded *ex situ* diameters at the uterine and cervical regions and digital calipers measured *ex situ* lengths. Samples were maintained at 37°C in HBSS.
3.2.4 Biomechanical testing

Utero-cervical complexes from Purdue (n=10) and Tulane (n=6) were utilized for extension-inflation testing. Extension-inflation testing permits evaluation of changes in organ diameter in response to variations in intraluminal pressure (pressure-inflation tests) and a controlled length to simulate multiaxial loading within the body [38, 104]. Additionally, extension-inflation testing permits measuring changes in axial force and organ diameter while increasing the organ’s length and maintaining a constant intraluminal pressure (force-elongation tests).

Prior studies show that the measurement of organ mechanical properties is more consistent, repeatable, and mathematically interpretable following preconditioning [88]. Preconditioning provides organs with a “memory” of applied loads and minimizes any permanent (plastic) deformations or loss of energy during mechanical testing [88]. The pressure-inflation preconditioning consisted of five cycles performed over a physiologic range of pressures (P=0 to 200mmHg) (Fig. 3.5B) [52, 86, 87]. This was followed by five cycles of force-elongation preconditioning at a constant pressure of 67mmHg (1/3 of maximum physiologic pressure) up to 10% above the unloaded length.

An estimated physiologic length was approximated utilizing the assumption that minimal changes in force occur over a range of pressures at the in vivo length to conserve energy [89]. The specimen was stretched above the unloaded length to the known in situ length (6mm). The organs were subsequently stretched or compressed around the estimated value until the force remained near constant over a range of pressures (Fig. 3.5).
Subsequently, the organs are subjected three cycles of increasing pressure (P=0-200 mmHg) at static lengths ±2% estimated physiologic lengths (Fig. 3.5) within a physiologic range above and below the physiologic length determined previously. Cyclically pressurizing the organs at set lengths permits quantification of the mechanical response of the organ throughout a range of physiologic changes.

**Figure 3.5:** (A) Schematic of the testing protocol demonstrating the changes in pressure and force over time for the different segments of the protocol. The protocol is divided as follows: (I) five cycles of pressure-inflation preconditioning at the unloaded length over the range of physiologic pressures; (II) five force-elongation preconditioning cycles at a constant 1/3 of maximum pressure over a range of stretches up to 10% above the unloaded length; (III) shows three cycles of pressure-inflation at 2% below the estimated physiological (EP) length (a), at the EP length (b), and 2% above the EP length (c); (IV) force-elongation tests with changing stretches from 2% below to 2% above the EP length at constant pressures of 10 mmHg (d), 67 mmHg (e), 133 mmHg (f), and 200 mmHg (g). (B) To confirm estimated physiologic (EP) axial stretch, pressure-inflation test data was plotted with axial force against increasing pressure (P=0-200 mmHg) from a representative sample at three different stretches (2% below estimated physiologic, estimated physiologic, and 2% above estimated physiologic length).

Force-elongation cycles are performed in which pressure (P = 10, 67, 133, and 200 mmHg) is held constant and the length of the organs is cycled through the physiologic range of organ lengths (±2% estimated physiologic lengths). Assuming that organs have a preferred length to conserve force over a range of pressures, the force-elongation protocol
confirms the estimated physiologic length at the intersection point of the force to axial stretch ratio, $\lambda_z$ [89, 90].

3.2.5 Elastase Digestion Pilot

Each component of the ECM contributes to the overall mechanical properties of soft tissues; and connective tissue disorders with mutations in ECM production result in many pathologies of organ systems including the reproductive system [97, 114-116]. Further, prior work observed a decreased incidence of elastic fibers and desmosine in cases of cervical insufficiency in human women [11]. Therefore, we sought to determine the role of elastic fibers with a pilot study of $n=6$ samples. After completion of the mechanical testing protocol, the tissue was returned to the EP length and 3.75 U/mL of pancreatic porcine elastin was introduced to the tissue intraluminally for 15 minutes. The elastase was washed out with HBSS and the mechanical testing protocol was repeated.

3.2.6 Thickness and Volume Calculations

Following biomechanical testing, 0.5mm thick rings were isolated from the mid-region of the cervix and uterus and imaged with a Moticam 580 HD Digital Camera and Motic Images software (Motic® Richmond, British Columbia) to quantify unloaded organs thickness for 12 samples. For data analysis of the remaining 4 samples, an averaged data set of thickness values (626±22.9 μm for the cervix and 690 ±19.2 μm for the uterus) was utilized. A custom MATLAB code calculated unloaded wall thickness from the ring images [42, 51].

Assuming a simplified geometry (hollow cylinder), the unloaded cervical and uterine volumes ($V$) were determined using equation: 
\[ V = \pi L (R_o^2 - R_i^2) \tag{1} \]

where \( L \) is the unloaded length and \( R_o \) and \( R_i \) are the undeformed outer and inner radii, respectively.

### 3.2.7 Histology

Cervical and uterine rings 0.5mm thick \((n=10)\) were isolated, fixed in 10\% formalin solution for 24 hours, embedded in paraffin wax, and cut into 4\% serial sections. The sections were then stained with Hart’s elastic stain, Picrosirius Red (PSR), and Masson’s Trichrome (MT). Images were subsequently taken using an Olympus BX51 microscope, an Olympus DP27 Digital Camera, and cellSens\textsuperscript{TM} software (Olympus Corporation, Center Valley, PA, U.S.A.) at 4x, 10x, 20x, and 40x magnification.

All samples were analyzed at 4X magnification. A custom MATLAB code determined ratios comparing large diameter collagen fibers against small diameter collagen fibers assuming that red and orange pixel area fractions associated to large diameter collagen fibers and yellow and green pixel area fractions associated to small diameter collagen fibers [117]. Color deconvolution, an open source plug-in for ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.) [118, 119], and an open-source GNU image manipulation program (GIMP), were leveraged to determine smooth muscle cell and collagen area fractions [120-122]. Within GIMP the outer and inner ring were selected to diminish background noise. Using a histogram feature within the software, pixels over a range of intensities were chosen to represent the ECM contents and divided out from the total pixels for a calculation of area fraction. For quantifying elastic fiber area fractions, the GIMP select by color tool was utilized to isolate elastic fibers from the section.
3.2.8 Data analysis

Although the cervix and uterus are thick-walled organs and 3D modeling is a necessary step to completely describe organ response, there is limited basic science information on the heterogeneity of the organs’ walls. Thus, cervical and uterine geometry was simplified to a thin-walled hollow cylinder to be initially assessed in 2D. Additionally, the cervix and uterus were assumed to demonstrate a nonlinear and pseudoelastic biomechanical behavior [39, 78].

Using the initial point data from the -2% estimated physiologic length test as the reference configuration, pressure-diameter data was used to calculate circumferential ($\sigma_\theta$) and axial ($\sigma_z$) Cauchy stresses [42] at each location using equations:

$$\sigma_\theta = \frac{P a}{h},$$

and

$$\sigma_z = \frac{F_t + \pi a^2 P}{\pi h (2a + h)},$$

where $P$ is intraluminal pressure, $a$ is the deformed inner radius, $h$ is the deformed thickness, and $F_t$ is the force from the axial force transducer.

Pressure-diameter data was used to calculate the circumferential and axial stretch ratios which were determined via [42]:

$$\lambda_\theta = \frac{r_{mid}}{R_{mid}},$$

and

$$\lambda_z = \frac{l}{L},$$
where $r_{mid}$ and $R_{mid}$ represent the loaded and unloaded mid-wall radii and $l$ and $L$ represent the loaded and unloaded axial lengths, respectively.

Bilinear curve fits, an application of linear interpolation, were applied along the two major components of the stress-stretch curve: the toe-region (the lower non-linear region) and the linear region of the stress-stretch data to quantify moduli for each pressure-diameter test [49, 93].

### 3.2.9 Constitutive modeling

A pilot study was performed to determine an appropriate microstructurally-motivated functional form of a stored energy function (SEF) for the cervix and uterus (Chapter 2). The 2D two-fiber family model with a neo-Hookean term (2D 2FF+NH) was employed. The 3D three-fiber family model, previously used to describe human cervical material properties [78], was not used as it was over-parameterized for the murine data [94]. Yin et al. described over-parameterization as the calculated determinant of the correlation matrix, $R < 10^{-4}$. Data from the 2% below estimated physiologic stretch were excluded from data analysis as the axial force measured indicated imminent bending or buckling of the organs as described previously [123].

The SEF, $W$, of the 2D 2FF+NH microstructural model utilizes an isotropic term that is assumed to represent the elastin-dominated ground matrix with a neo-Hookean behavior and an anisotropic collagen-dominated term representing two diagonal fiber directions, measured relative to the axial direction and symmetric about the long axis with an assumed Fung-type exponential [80]:

$$W(C, M^k) = \frac{c}{2} (I_C - 3) + \sum_k \frac{c^k}{2e^2} \times \exp \left( c^k \left( \lambda_{kk}^2 \right)^2 - 1 \right)^2 - 1 \right)$$

(6)
where \( c \) and \( c_1 \) are material parameters that have units of kPa and \( c_2 \) is a dimensionless material parameter, \( \mathbf{C} = \mathbf{F}^T \cdot \mathbf{F} \) is the right Cauchy-Green tensor, \( M^k = [0, \sin \alpha^k_0, \cos \alpha^k_0] \) is the fiber orientation unit vector where \( \alpha^k_0 \) was set at \( \pm \alpha_0 \) for diagonal, symmetric alignment, \( I_C \) is the first invariant of the right Cauchy Green tensor, \( I_C = tr \mathbf{C} = \lambda^2_\theta + \lambda^2_z + \lambda^2_r \), where \( \lambda_r = 1/ \lambda_\theta \lambda_z \), and \( \lambda^2_k = M^k \cdot \mathbf{C} M^k \) is the fourth invariant which defines the stretch experienced by the \( k^{th} \) fiber family. Theoretical Cauchy stresses were determined from [52]:

\[
\mathbf{t} = -p \mathbf{I} + \frac{2}{J} \mathbf{F} \cdot \frac{\partial \mathbf{W}}{\partial \mathbf{C}} \cdot \mathbf{F}^T, \tag{7}
\]

where \( p \) is the Lagrange multiplier, \( \mathbf{F} \) is the deformation tensor, and \( J = det \mathbf{F} \).

**Equation 7** and non-linear regression were utilized via MATLAB to minimize error between the theoretical and experimental data, determine best-fit parameters, and ensure unique parameters for the 2D 2FF+NH model using the following objective function [42]:

\[
e = \sum_{i=1}^{N} \left[ \left( \frac{P_{th} - P_{exp}}{P_{exp}} \right) _i^2 + \left( \frac{F_{th} - F_{exp}}{F_{exp}} \right) _i^2 \right], \tag{8}
\]

where \( P_{th} \) and \( F_{exp} \) are theoretical and experimental forces, \( P_{th} \) and \( P_{exp} \) are the theoretical and experimental intraluminal pressures, \( P_{exp} \) is the mean experimental pressure, and \( F_{exp} \) is the mean experimental force [42].

### 3.2.10 Statistical analysis

All data within the study are presented as mean±standard error of the mean (SEM). One sample was removed due to a disruption in tracking the outer diameter resulting in a total of 15 samples compared for mechanical testing. Biomechanical parameters from
bilinear curve fits were analyzed using a two-way repeated measures ANOVA (location, axial coupling), followed by paired Student’s t-tests with Bonferroni corrections when appropriate ($p<0.05$). A paired t-test was used to compare the material parameters, geometries, and area fractions of ECM constituents at the cervix and uterine horn. A Pearson’s correlation analysis was performed directly comparing histological area fractions and material parameters from the same sample for both organs.

3.3. Results

3.3.1 Bilinear Fits

All parameters for the bilinear fit response are reported in Table 3.1. The two-way repeated measures ANOVA for $\lambda_\theta$ vs $\sigma_\theta$ revealed a significantly larger toe-region ($p<0.005$) and linear-region ($p<0.001$) moduli for the cervix, as compared to the uterus (Table 3.1, Fig. 3.6). The uterus demonstrated increased circumferential stretch ($p<0.001$) at the transition point between the toe- and linear-regions, as compared to the cervix (Table 3.1, Fig. 3.6). For $\lambda_\theta$ vs $\sigma_z$, the circumferential stretch at the transition point increased significantly ($p<0.001$) for the uterus compared to the cervix.

The cervix, however, exhibited significantly higher toe ($p<0.01$) and linear-region ($p<0.001$) elastic moduli compared to the uterus (Table 3.1). Comparisons of $\sigma_\theta$ against and $\sigma_z$ at the same $\lambda_\theta$ indicated a significantly larger ($p<0.001$) stress value and greater ($p<0.001$) toe- and linear-regions elastic moduli within the circumferential direction within the cervix. The uterus demonstrated a significantly larger circumferential stress ($p<0.05$), toe-region modulus ($p<0.005$), and greater linear-region modulus ($p<0.001$) in the circumferential direction (Table 3.1, Fig. 3.6).
Table 3.1: Bilinear Fit Parameters

<table>
<thead>
<tr>
<th></th>
<th>$\sigma_\theta$ vs $\lambda_\theta$</th>
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<th>$\sigma_z$ vs $\lambda_\theta$</th>
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<td>Toe-Modulus [kPa]</td>
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</table>

Figure 3.6: (A) Outer diameter changes with increasing pressure in the cervix (•) and uterus (+) during the pressure-diameter tests. Data shown as mean±SEM (n=12). Representative circumferential (B) and axial (C) Cauchy stress-stretch curves for the cervix (•) and uterus (+) demonstrate the cervix is significantly (p<0.001) less distensible than the uterus with reported circumferential transition stretches of 1.15±0.02 and 1.39±0.05, respectively. Furthermore, both organs are stiffer in the circumferential direction compared to the axial with significantly greater circumferential toe- and linear-moduli (Table 3.1).
3.3.2 Material Parameters

![Graph showing pressure-diameter tests for cervix and uterus with model fits](image)

**Figure 3.7:** Pressure-diameter tests for cervix (*) and the uterus (○) with model fits (—) (mean±SEM) for the 2D two-fiber family with a neo-Hookean term (2D 2FF+NH) model Cauchy circumferential stress (A) and Cauchy axial stress (B). The neo-Hookean term representing the isotropic ground matrix was significantly larger (p<0.05) in the cervix. Further, both the $c_1$ stress-like parameter (p<0.01) and the $c_2$ parameter (p<0.05) representing the symmetric diagonal collagen fibers was significantly higher than the uterus, suggesting that the cervix is less distensible.

The constitutive model described the biomechanical response of the cervix (RMSE=0.27±0.02) and uterus (RMSE=0.28±0.02) reasonably well ([Fig. 3.7, Table 3.2, 3.3](#)[80]). The neo-Hookean parameter $c$, the $c_1$ stress-like parameter, and the $c_2$ parameter were significantly larger ($p<0.05$) for the cervix compared to the uterus ([Table 3.2, 3.3](#)).

Fiber angles between the two locations were significantly different ($p<0.001$), with the cervix and uterus ([Table 3.2, 3.3](#)). Fiber angles between the two locations were significantly different ($p<0.001$), with the cervix and uterus having average fiber angles of 52.3±2.18° and 45.6±2.17°, respectively ([Table 2, 3](#)).
Table 3.2: Material Parameters obtained through 2D 2FF+NH model

<table>
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<tr>
<th>Uterus</th>
<th>( c ) [-]</th>
<th>( c_1 ) [kPa]</th>
<th>( c_2 ) [-]</th>
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<td>9.7E-12</td>
<td>7.5</td>
<td>2.9</td>
<td>51</td>
</tr>
</tbody>
</table>

Table 3.3: Material Parameters obtained through 2D 2FF+NH model

<table>
<thead>
<tr>
<th>Cervix</th>
<th>( c ) [-]</th>
<th>( c_1 ) [kPa]</th>
<th>( c_2 ) [-]</th>
<th>RMSE</th>
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</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>3.0E-10</td>
<td>77</td>
<td>29</td>
<td>58</td>
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<tr>
<td>Sample 2</td>
<td>3</td>
<td>180</td>
<td>140</td>
<td>63</td>
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<tr>
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<tr>
<td>Sample 4</td>
<td>8.8</td>
<td>75</td>
<td>30</td>
<td>55</td>
</tr>
<tr>
<td>Sample 5</td>
<td>2.7E-14</td>
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<td>11</td>
<td>54</td>
</tr>
<tr>
<td>Sample 6</td>
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<td>3.7</td>
<td>120</td>
<td>39</td>
</tr>
<tr>
<td>Sample 7</td>
<td>7.3E-14</td>
<td>79</td>
<td>20</td>
<td>58</td>
</tr>
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<td>Sample 8</td>
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<td>3.0</td>
<td>42</td>
</tr>
<tr>
<td>Sample 9</td>
<td>3</td>
<td>44</td>
<td>11</td>
<td>55</td>
</tr>
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<td>Sample 10</td>
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<td>14</td>
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<td>Sample 11</td>
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<td>Sample 15</td>
<td>27</td>
<td>0.02</td>
<td>26</td>
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</tr>
</tbody>
</table>
3.3.3 Structure

Paired t-tests performed between the cervical and uterine ultrasound measurements of the \textit{in vivo} thickness and diameter resulted in a significant increase ($p<0.05$) in both geometric parameters at the cervix (Fig. 3.5). Histology demonstrated a significant ($p<0.005$) increase in collagen content for the cervix (75.6±4.49\%) in comparison to the uterus (69.6±5.23\%) (Fig. 8). PSR analysis revealed a significantly ($p<0.001$) greater amount of large diameter collagen fibers in comparison to the amount of small diameter fibers at both locations. Further, analysis detected a greater content of larger diameter fibers within the cervix with an average large diameter to small diameter ratio of 7.47±2.94\% compared to 6.12±1.17\% in the uterus. The area fraction of the smooth muscle within the uterus was significantly greater ($p<0.001$) within the uterus (uterus: 18.4±1.55\% vs. cervix: 15.7±3.90\%), however, elastic fiber was not significantly different (uterus: 3.63±1.16\% vs. cervix: 3.32±0.57\%) (Fig. 3.8). The Pearson’s correlation identified no correlation between the collagen content within the cervix and uterus and the material parameters $c_1$ and $c_2$. 
Figure 3.8: Picrosirius red (PSR) (A, D), Masson’s trichrome (MT) (B, E), and Hart’s Elastic stain (C, F) images taken at 10x magnification of the cervix (top row) and uterus (bottom row) with labeling for the layers of each organ. The cervix collagen fiber ratio of large diameter to small diameter was approximately 8:1 (A), collagen and smooth muscle content was 75.6±4.49% and 15.7±3.90%, respectively (B), and elastic fiber content was 3.32±0.57% (C). The uterus presented a collagen fiber ratio of approximately 6:1 (D) and collagen (E), smooth muscle (E), and elastic fiber (F) area fractions of 69.6±5.23%, 18.4±1.55%, and 3.63±1.16%, respectively. A significantly greater collagen content (p < 0.005) and significantly decreased SMC content (p <0.001) was identified for the cervix compared to the uterus. No significant differences were identified for elastic fiber content between organs.
3.3.4 Elastase Digestion Pilot

Elastase treatment with 3.75 U/mL did not result in significant differences in the biomechanical properties of the murine cervix (Fig. 3.9A, 3.9B). Circumferential and axial linear-modulus significantly increased ($p<0.025; p<0.005$) in the elastase treated uterus compared to the control (Fig. 3.9C, 3.9D). However, no significant differences with respect to elastase treatment were detected for the material parameters. Power analysis calculated a minimum sample size of $n=76$ and $n=99$ to determine significant differences in material parameters for the cervix and uterus, respectively.

![Figure 3.9: Cauchy stress-stretch curves for the cervix (A,B) and uterus (C,D) where controls are represented by (•) and elastase treated are symbolized by (·). (A,B) Circumferential and axial mechanical properties of the murine cervix were not significant ($p > 0.05$). (C,D) Circumferential and axial linear-modulus significantly increased in the elastase treated group for the murine uterus. Where significance is indicated by $p < 0.05$. 0.02<50*; 0.00 5 * *](image-url)
3.4 Discussion

In this study, the biaxial material parameters of the cervix and uterus were quantified while preserving native organ structure, matrix interactions, and geometry [41, 42, 90]. The constitutive model used herein identified significantly larger $c_1$ and $c_2$ parameters in the cervix compared to the uterus. Additionally, the bilinear fits performed on the stress-stretch curves demonstrated larger linear modulus in both the axial and circumferential directions at the cervix compared to the uterus (Fig. 3.6). A higher collagen content was identified in the cervix suggesting that the higher collagen content contributes to the increased $c_1$ and $c_2$ parameters and decreased distensibility noted in the cervix.

In addition to greater collagen content, differences in collagen fiber diameter, type I:III composition, organization, and undulation may contribute to the observed regional differences in mechanical properties along the length of the reproductive tract [122]. Towards this end, a significantly greater amount of large diameter collagen fibrils identified in the cervix may also contribute to the increased collagen model parameters and decreased distensibility. A greater circumferential bias in the collagen fiber alignment in the cervix compared to the uterus, indicated by the larger fiber angle parameter, $\alpha$, confirms increased resistance to load in the circumferential direction compared to the axial direction.

Clinically, greater resistance to circumferential loading of the cervix and reduced axial resistance correspond with shortening of the cervix in cases of cervical insufficiency [11, 39]. Interestingly, a uniaxial study found a stiffer longitudinal direction within the vagina as compared to the circumferential direction within the cervix and uterus [124]. However, the uniaxial experiments were performed at greater loads and the organs may have a different response at high loads in comparison to low loads [49].
Collagen cross-links may also contribute to differences in stiffness between the cervix and uterus [21]. Increased cross-link density and maturity are associated with increasing organ stiffness, therefore, may contribute to greater stiffness identified in the cervix compared to the uterus [21, 125, 126]. Transition stretch was significantly larger in the uterus in both the circumferential and axial directions (Fig. 3.6, p<0.05) which may be related to collagen undulation. Prior work showed that collagen undulation in tendons was associated with the toe-region of the stress-stretch curve [127-129]. Therefore, increased transition stretch for the uterus may be due to higher undulation of collagen fibers in the uterus as compared to the cervix. However, differences in collagen undulation between the cervix and uterus in the mouse are currently unknown.

Interestingly, an increase in the c parameter in the cervix indicates a less distensible material. This term is assumed to represent the isotropic ground matrix. As expected from studies of the elastin content in the non-pregnant uterus and cervix in mouse [75], rat [108, 109], and human models [34, 130], elastin content was not significantly different between locations.

This implies that other components of the ECM, such as proteoglycans or glycosaminoglycans (GAG), may also contribute to the differences in the ground matrix response. GAG content within the uterus is greater in comparison to the cervix in rats [10, 63, 76]. Variability within the c parameter for the cervix may be related to the ground matrix constituents (Table 3.3). Decreased microstructural content of the ground matrix within the cervix may alter the accuracy of describing the non-linear response as the neo-Hookean term in research on the vein and carotid artery did not describe the forces within the low strain regime accurately [98, 131].
Previous studies employed mechanical testing and constitutive modeling on the cervix with varying results [25, 26, 78]. Liao et al., described mechanical responses in the human cervix utilizing the three fiber family (3FF) model with a neo-Hookean component [78]. Our present study utilized a neo-Hookean term is assumed to describe the elastic fiber isotropic matrix term, however, the 3FF model for the murine cervix was over-parameterized [94]. Past studies investigated the uniaxial cervical response through pregnancy utilizing a fibrous composite model for murine and human organs [25, 26]. Differences between model results may be linked to comparing uniaxial experiments in previous studies to the biaxial experiments performed within this study. Applying the material properties and the 2FF+NH model to a finite element model may provide insights into the interface of the cervix and uterus leading to improved understanding of pathologies such as cervical insufficiency [6, 12].

Differences in mechanical properties within the estrous cycle were not considered for this study. A small pilot study (n=6) comparing mechanical properties in estrus and diestrus mice revealed no significant differences in transition stretch (p>0.05), transition stress (p>0.05), toe-modulus (p>0.05), and linear-modulus (p>0.05) for the cervix and uterus, respectively, which is consistent with results from the rat vagina (Chapter 2) [132]. Additionally, several studies chose a single point in the estrous cycle for consistency and as a representative of the non-pregnant cervix [74, 133]. Thickness values for 4 control samples were a calculated average from the cervix and uterus. The use of an averaged value for the four samples may cause minute changes in the calculation of mechanical properties, however, prior research used averaged thickness values within calculations [134]. Elastase treatment did not result in significant differences in mechanical properties of the murine
cervix. This may be due, in part, to the dose (3.75 U/mL) where a prior study in the vagina used 15 u/mL [48]. *In vivo* intracervical catheter experiments in the murine cervix performed in Chapter 4 present a mean physiologic pressure of \( P = 10.0 \pm 2.0 \) mmHg and maximum contractile pressure of \( P = 39.0 \pm 8.0 \) mmHg. Comparatively, loading the murine cervix and uterus to the human intrauterine maximum pressure of \( P = 200 \) mmHg may result in tissue rupture with small disruptions to the ECM integrity.

While 2D frameworks are valuable tools for modeling experimental data [42, 44, 52], they remain approximations and make assumptions such as homogenous residual stress through the wall and a thin-walled geometry. However, more work in the field is needed to characterize physiologic relevant pressures, the multiaxial loading on the uterocervical complex, and investigate the collagen undulation and orientation of the murine reproductive system to improve accuracy of computational models.

In summary, the biaxial mechanical properties of the non-pregnant murine cervix and uterus were determined with inflation-extension biaxial testing. The cervix was significantly stiffer than the uterus in the axial and circumferential directions. Furthermore, *in vivo* ultrasound measured significant differences in geometry and histological analysis identified microstructural differences with location. Increased stiffness and a larger cervical outer diameter may inform future research modeling the uterocervical complex. The techniques developed herein may be beneficial for future studies examining the changing structure and region-dependent mechanical environment of the female urogenital system such as finite element models for modeling (patho)physiologic processes.
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Chapter 4: Protocol Development for Determination of the \textit{In Vivo} Contractility and Active Biomechanical Properties of the Nulliparous Murine Cervix

4.1 Introduction

Historically, the cervix was considered primarily a stiff, collagenous organ with a small percentage of smooth muscle that played a secondary role to overall cervical function [33, 135, 136]. Outside of pregnancy, the cervix was considered a reservoir of sperm for fertilization and a primarily passive barrier between the internal uterine lining and the external environment [136, 137]. However, over the past 50 years research proposed a distinct active cervical behavior in processes such as fertilization, menstruation, and pregnancy [19, 34, 40, 106, 113, 138]. Yet, the pharmacology and role of cSMC in reproductive function remains understudied [22, 35, 40, 135]. Attempting to bridge the knowledge gap, in vitro studies of cSMC behavior present phasic contractions through the estrous cycle and pregnancy with distinct patterns separate from the uterus and vagina [22, 40]. Further, Vink \textit{et al.} hypothesizes that the cervix acts as a sphincter and maladaptive remodeling of extracellular matrix and cSMC during pregnancy may lead to premature cervical funneling [19, 135]. However, the relationship between cSMC and native ECM in the nulliparous state remains undetermined. Therefore, a need remains to characterize the relationship between cSMC and ECM constituents in the nulliparous cervix.

Combining active and passive mechanical testing provides an opportunity to subtract the active function (cSMCs) from the passive function (ECM). However, prior work independently assessed active or passive mechanical properties of the cervix. Additionally, the traditional uniaxial testing does not preserve tissue geometry and independently investigates the function of longitudinal and circumferential ECM and
cSMCs [21, 22, 26, 40, 52, 106]. Further, contractility experiments principally induce maximum contractility with agonists. While maximum contractile tone can provide insights into the function of the organ, the resting or basal tone of the organ may further inform our understanding of the mechanical properties of the cervix [3, 44, 50]. To overcome the limitations of uniaxial testing, an inflation-extension system can be used to assess multiaxial cSMC contractility, basal tone, and passive mechanics while preserving native tissue geometry and cell-matrix interactions [3, 42, 48-50, 52].

Therefore, the objectives of this study were to develop protocols that quantify: 1) the optimal dose of the agonist potassium chloride (KCl) to induce maximum contraction in the murine cervix, 2) basal and passive mechanical properties in the same cervical sample for direct comparisons, and 3) in vivo cervical contractile behavior.

4.2 Methods and Protocol Optimization

4.2.1 Animal Care

A total of (n=13) C57BL6 female mice at estrus aged 4-6 months were utilized for protocol optimization. All experiments were performed with approval from the Tulane Institutional Animal Care and Use Committee (Tulane IACUC). Tulane university housed and maintained the animals in a 12 hour light/dark cycle and fed normal chow. Estrus cycle staging was determined via methods from Byer et al., 2012 [56].

4.2.2 Specimen Preparation

Reproductive systems were freshly isolated immediately post-euthanasia via guillotine to preserve SMC contractility from (n=10) C57BL6 female mice at estrus. Post-explant the tissue was bathed in 4°C Hanks Balanced Saline Solution (HBSS) and
connective tissue was fine dissected from the organs. Cuts immediately posterior of the bifurcation of the uterine horns and superior to the vaginal introitus were made. Contrastingly to the methods in Chapters 2 and 3, the lower uterine segment was removed. The uterine horns fuse into a single canal beyond the bifurcation point and prior to the cervical canal. The fused segment is classified as the uterine body that transforms into the cervical os [2, 15]. The cervical canal is characterized by a “dog bone” shape that is distinct from the oval uterine canal (Fig. 4.1) [2, 15]. However, the removal of the vaginal fornix and uterine body resulted in a cervical structure too short to cannulate and possible stress concentrations near the tracking location due to the effects of Saint-Venant’s principle. Therefore, the uterine horns were cut down as close to the uterine body as possible and the wall between the remainder of the uterine horns was cut to allow for appropriate cannulation length (Fig. 4.2). Additionally, the upper vagina was kept intact to expand organ length for cannulation and prevent interference from suture induced stress concentrations on the external os of the vagina (Fig. 4.2). Cervical complexes were cannulated within an inflation-extension device.

![Figure 4.1: Schematic of cervical internal canal (A) compared to the uterine body canal (B).](image-url)
using 6-0 silk suture and bathed in aerated Krebs Ringer Buffer at 37°C. Unloaded diameter was recorded via a digital camera and unloaded length through a digital micrometer.

Figure 4.2: Schematic of dissection procedure to prepare for cannulation. Singular cuts, indicated by the dashed lines, were made inferior to the uterine bifurcation and superior the vaginal introitus (A). Illustration of approximate geometry after isolation from the remaining reproductive tract (B). To ensure successful cannulation, the wall between the uterine canals superior to the uterine body was cut (indicated by dashed lines) and the external os within the vaginal canal was visualized to confirm that the vaginal cut did not damage the cervical body (C).

4.2.3 Preconditioning

Initially, pressure ranges were adapted from Chapter 3 [2, 86, 87, 104], however, the range of pressures from the human intrauterine measurements resulted in a loss of contractility in the cervical samples. Therefore, as a first step, we adapted pressures from biaxial inflation-extension vaginal experiments (P=0-15mmHg) to preserve SMC contractility [3]. Samples were preconditioned circumferentially (P=0-15mmHg) at the unloaded length for five cycles. Force-extension preconditioning was excluded as cSMC exhibited a loss in contractility in response to potassium chloride (KCl) following the force-extension protocol. Next, an estimated physiological (EP) length was determined based on the assumption that soft tissues seek to maintain force over a range of pressures at an approximate in vivo length [42, 89]. Samples were subjected to secondary circumferential
preconditioning at the EP length (P = 0-15 mmHg). To acclimate cSMC to KCl, the cervix was pressurized to the mean pressure (P = 7.0 mmHg) and axially stretched the tissue until the force transducer read F = 0mN. The tissue was dosed with 40mM of KCl for 5 minutes based on vaginal KCl dosing for maximum contractility studies [3]. Following preconditioning, the unloaded length was re-determined as the length in which there was minimal changes in force with decreasing axial length [3].

4.2.4 Dose Response

To determine the optimal dose of KCl to induce contraction of the cervix, an isobaric isometric dose response protocol of KCl was performed at the EP length and mean pressure (P = 7 mmHg) on n=5 cervical samples [139]. The cervix extended to the EP length and inflated to the mean pressure (P = 7 mmHg) and dosed with increasing concentrations of potassium chloride (4.7-100mM) sequentially. KCl induced contractions were recorded for 5 minutes and between each dosing the tissue rested for 5 minutes after the basin was replenished with fresh 37°C Kreb’s Buffer. Utilizing a Vevo 2100 (FUJIFILM VisualSonics Inc., Toronto, ON, Canada), B-mode ultrasound images (40MHz transducer) of cervical thickness were made at the unloaded geometry.

4.2.5 Basal Mechanical Testing

A separate cohort of n=5 female C57BL6 mice at estrus were utilized for basal and passive mechanical testing. After preconditioning, the cervix was subjected to three cycles of the pressure-inflation protocol (P=0-15mmHg) at static axial extensions of -2% EP, EP, 1% EP, and 2% EP length. Due to the sensitivity of the cSMC to axial-stretch, we included an additional axial-extension (1% EP length) compared to the passive studies in Chapters 2 and 3 and force-elongation protocols were excluded [2]. Samples were treated with
20mM KCl (based on optimal KCl dose from the dose response experiments) after the basal experiment to incite phasic contractions to confirm cSMC activity.

4.2.6 Passive Mechanical Testing

Samples were treated with 2mM EGTA and immersed in a solution of calcium free Krebs Buffer for 30 minutes to de-activate cSMCs [3]. EGTA was washed out of the bath with fresh calcium free Krebs Buffer prior to passive testing. Three cycles of the pressure-inflation experimental protocol (P=0-15mmHg) at the four axial-extensions (-2% EP, EP, 1% EP, and 2% EP length) was repeated for the passive state. Cervices were returned to the unloaded length and ultrasound B-mode images of the tissue were taken after the pressure-inflation cycles for both the basal and passive experiments.

4.2.7 In Vivo Pressure Measurements

To optimize a protocol to characterize in vivo cervical contractility and determine a proper loading environment for the murine cSMC in future studies (Chapter 5, 6, and 7), transcervical pressure measurements were recorded in (n=3) C57BL6 mice [140]. Prior to starting the experiment, a 2F Millar Mikro-tip® Catheter (ADInstruments, Colorado, USA) was connected to the ADInstruments Bridge Amplifier and PowerLab (ADInstruments, Colorado, USA) and then placed in a water bath of physiologic saline at 37°C to equilibrate for 30 minutes. The size of the catheter was chosen based inner diameter measurements from Chapter 3 [2]. Following the 30 minute equilibration, the catheter was inserted into a Y connector tube attached to a pressure gauge and placed back in the water bath. Utilizing the AD Instruments LabChart Pro software (ADInstruments, Colorado, USA) and the pressure gauge, the voltage values were converted to pressure and the calibration of the catheter completed. The catheter was found to be sensitive to light, temperature, static
discharge, and fluid medium. Care was taken to ground the user and avoid material with a propensity to build static charge. When calibrated in room temperature air under fluorescent light, pressure readings would drift dramatically during the experiment. Additionally, using too much force to insert the catheter resulted in an off set of the zero pressure point and drifting pressure measurements. Engulfing the catheter in sterile water-based surgical lubricant warmed in the water bath to 37°C decreased the amount of force needed to place the catheter through the cervical os and insulated the catheter against heat loss during the transfer from the water bath to the animal. Catheter calibration and steady pressure reading relied on recapitulation of the \textit{in vivo} environment (dark and physiologic saline at 37°C as the medium).

Female C57BL6 mice at estrus \((n=3)\) were anesthetized with 1%-1.5% isoflurane mixed with 100% oxygen via nose cone and placed on a heating pad for the duration of the experiment. Blunt forceps and a disposable plastic tube gently spread the vaginal opening to visualize the cervix. The 2F catheter was placed transcervically to approximately 6mm from the vaginal opening based on prior studies of vaginal length [3, 49]. Secondary confirmation included an increase in pressure [140] and phasic contractions [23]. Subsequently, the catheter equilibrated for 5 minutes followed by 5 minutes of recording baseline pressure, contractile pressure, and contraction frequency measurements [140]. Mice were placed in a recovery cage for 5-10 minutes before returning to the housing facility.

\subsection*{4.2.8 Data Analysis}

\textit{Thickness and Volume Calculation}
Utilizing Image-J (National Institutes of Health, Bethesda, MD, U.S.A.), the inner canal and outer circumference excluding the vaginal fornix were traced in the ultrasound images of the unloaded active and passive cervix (Fig. 4.3). Next, lines were drawn between the inner and outer perimeters and the lengths used to calculate average unloaded thickness ($H_o$) of the tissue.

Assuming conservation of volume (incompressibility) and a simplified hollowed cylinder geometry, Equations 1 and 2 calculated the cross-sectional area ($A$) and volume ($V$) from the unloaded geometry:

$$A = \pi (R_o^2 - R_i^2)$$  \hspace{1cm} (1)

$$V = \pi L (R_o^2 - R_i^2)$$, \hspace{1cm} (2)

where $R_o$ is the unloaded outer radius, and $R_i = R_o - H_o$ is the unloaded inner radius, and $L$ is the unloaded length.

**In Vitro Contractility**

Circumferential and axial contractions were measured via changes in mid-cervical diameter and axial force with contraction, respectively. Contractile response was
normalized with respect to cross sectional area \((A)\) to calculate contractile circumferential \((\sigma_\theta)\) and axial \((\sigma_z)\) Cauchy stresses (Eq. 3, 4).

\[
\sigma_\theta = \frac{Pr_i}{h},
\]

(3)

and

\[
\sigma_z = \frac{F_t + \pi r_i^2 P}{\pi h (2a + h)},
\]

(4)

where \(P\) is the mean pressure, \(r_i\) is the loaded inner radius, \(h\) is the loaded thickness, and \(F_t\) is the axial force measured.

**Basal and Passive Mechanics**

The initial point data from the first test (-2% EP length) in the basal or passive state was used as the reference configuration. From the pressure-inflation data, circumferential \((\sigma_\theta)\) and axial \((\sigma_z)\) Cauchy stresses were calculated at each point using Equations 3 and 4, respectively [42]. Circumferential \((\lambda_\theta)\) and axial \((\lambda_z)\) stretch (Eq. 5, 6) were determined for each point in the basal and passive pressure-inflation tests via [42]:

\[
\lambda_\theta = \frac{r_{mid}}{R_{mid}},
\]

(5)

and

\[
\lambda_z = \frac{l}{L},
\]

(6)

where \(r_{mid}\) is the deformed mid-wall radius, \(R_{mid}\) is the unloaded mid-wall radius, \(l\) is the deformed axial length, and \(L\) represents the unloaded axial length.
Utilizing the circumferential ($\sigma_\theta$) and axial ($\sigma_z$) Cauchy stress values and circumferential stretch ($\lambda_\theta$) from the pressure diameter tests, stress-stretch plots were created for circumferential and axial stresses. Bilinear curve fits were applied to the basal and passive stress-stretch curves to describe parameters such as toe- and linear-modulus [2, 49, 93].

4.2.9 Statistics

One-way ANOVA (Dose) with post hoc t-tests determined differences in contractile circumferential stress and axial stress. Paired t-tests were used to compare geometry between the passive and basal state. Two-way ANOVAs (axial extension, state) determined differences in bilinear fit parameters followed by post hoc paired t-tests when applicable.

4.3 Results

4.3.1 Dose Response

Optimal dose to induce maximum contraction was identified at 20mM KCl (Fig. 4.4). At this concentration, phasic activity was maintained and there was a significant increase in axial stress ($p<0.001$) and an inverse effect on the circumferential stress ($p<0.05$) compared to all doses.

**Figure 4.4:** Change in axial stress (gray circles) and change in circumferential stress (black circles) with contraction induced by increasing dose of KCl. Axial stress increased significantly ($p<0.001$)*** and circumferential stress decreased significantly ($p<0.05$) with contraction induced by 20mM KCl compared to all doses.
4.3.2 Active and Passive Geometry

All samples exhibited spontaneous contractility, contractions without KCl induction, in the active state. Unloaded volume and thickness did not significantly differ between the basal and passive states \((p > 0.05)\) (Fig. 4.5). However, physiologic length significantly increased \((p < 0.05)\) in the passive state and passive outer diameter significantly increased \((p < 0.05)\) compared to the basal state (Fig 4.5).

![Figure 4.5](image)

**Figure 4.5:** Unloaded thickness (A) and volume (D) did not change \((p > 0.05)\) from the basal (white) to passive state (gray). However, physiologic length (B) and physiologic diameter (C) increased significantly \((p < 0.05^*)\) in the passive state compared to the basal state.

4.3.3 Active and Passive Biomechanical Properties

Two-way ANOVA of bilinear curve fit parameters revealed no significant differences \((p > 0.05)\) in mechanical properties between the basal and passive state or axial-extension. Although mechanical properties did not significantly differ, the transition-
stretch in the passive state increased empirically (Fig. 4.6). Additionally, circumferential linear-modulus in the basal and passive state significantly exceeded \((p<0.05)\) the axial linear modulus (Fig. 4.6).

![Figure 4.6: Circumferential (A) and axial (B) stress-stretch curves of the basal and passive response at the estimated physiological (EP) length. Two-way ANOVA did not detect significant differences between basal and passive mechanical properties. However, a non-significant increase in circumferential stretch was observed \((p<0.01)\) indicated by the arrows. Circumferential linear modulus in the basal and passive state exceeded the axial linear modulus \((p<0.05)\).](image)

**4.3.4 In Vivo Pressure**

*In vivo* cervical contractile behavior at in the C57BL6 murine cervix at estrus presented as phasic. On average the cervix contacted once every 36.5±6.17 seconds or 0.03±0.004 Hz. Intracervical *in vivo* baseline pressure measured \(P=10.0±2.00\) mmHg and contractile pressure measured \(P=38.0±6.00\) mmHg.

**4.4 Discussion**

The role of cSMC in non-pregnant and pregnant cervical function remains debated [19, 33, 40, 106, 135, 136]. However, recent advances in research investigating cSMC contractile behavior proposes a fundamental relationship between the ECM stiffness and cSMC contractile ability [135]. Therefore, understanding the passive mechanical
properties, represented by the ECM and passive cSMC contribution, and the active mechanical properties may improve understanding of how the cervix functions in normal reproductive function and provide insights into remodeling during pathological conditions and pregnancy. Thus, this chapter optimized protocols and presented pilot data in the C57BL6 murine cervix that characterize 1) a KCl dose response curve for the optimal dose to induce maximum contraction, 2) the basal and passive structural and mechanical properties, and 3) the behavior of in vivo cervical contractility.

The dose response protocol depicted a phasic contractile pattern in the murine cervix at the optimal dose of 20mM KCl followed by a tonic contractile behavior at doses 30mM and above. Dose response results mirrored uniaxial in vitro experiments of cervical contractility in which the cervix presented phasic patterns with spontaneous contractions and KCl induced contractions [22, 40]. The tonic behavior observed at higher doses may result from the remaining portion of vaginal tissue. However, the optimal dose of KCl for maximum vaginal contractile response in the mouse requires 40mM [3]. Therefore, we expect minimal contribution of vaginal contractility to the cervical results in future studies.

Interestingly, the passive diameter and length increased compared to the basal diameter (Fig. 4.5). However, thickness and volume did not significantly increase with passivation of the tissue (Fig. 4.5). Potentially, the relaxation of the circumferentially and axially aligned cSMC resulted in the observed increase in diameter and length, respectively. Further, the mechanical properties between the basal and passive state did not significantly differ. Observationally, transition-stretch in the passive increased non-significantly (p<0.1) which can be visualized as the rightward shift from the basal to
passive state in Figure 4.6. This suggests an increase in distensibility with loss of basal tone. Contrastingly, basal tone resulted in decreased material stiffness and a rightward shift on the stress-stretch curve in the murine vagina [3]. The absence of change in mechanical properties between the basal and passive state may be related to the phasic nature of the cervix. Basal tone may play a minimal role in the function of the non-pregnant cervix. Potentially the non-pregnant cervix relies on the regular phasic contractions identified in vivo during the pressure catheter experiment to contribute to processes such as fertilization post-coitus [40]. Additionally, the non-pregnant cervix is a dense fibrous tissue that is composed of approximately 80-90% collagen and the basal tone of SMC within the tissue may contribute minimally to maintain rigidity [19, 39]. During early- to mid-pregnancy the cervix must remained close and maintain the fetus and a phenotypic change activates the cSMCs during late pregnancy and labor [22, 40]. Combined with an increase in cSMC and a decrease in collagen crosslinking during pregnancy the contribution of the basal tone may increase in the pregnant state contributing to the cervical sphincter hypothesis [21, 35, 106, 135].

Further work is needed to elucidate the role of basal and max contractile tone in comparison to the passive state to inform mathematical models of the non-pregnant cervix. Adapting these protocols further may improve our understanding the role of SMC in the non-pregnant and pregnant cervix.

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Chapter 5: The Role of Biaxial Loading on Smooth Muscle Contractility in the Nulliparous Murine Cervix

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5.1 Introduction

The coordination of smooth muscle cells (SMC) and extracellular matrix (ECM) components impart function to the female reproductive system [40]. Altered contractility and passive mechanical properties, in response to mechanical loading and hormonal signaling, bestow the reproductive system with the ability to undergo dramatic changes in geometry and function during physiological processes, such as pregnancy [20, 22, 28, 38, 108, 141-144]. Determining contractile and passive biomechanical properties of the female reproductive organs could provide crucial information about physiologic reproductive function. Specifically, the cervix functions to protect the uterine cavity from external factors, facilitate fertilization, and dilate to allow for the passage of menstrual contents in the non-pregnant state through regular phasic contractions [22, 40]. However, the role and behavior of cervical SMC (cSMC) in reproductive function is contradictory and remains poorly understood [19, 33, 135].

While uterine contractility and passive mechanical properties during the estrous/menstrual cycle and pregnancy garnered focus in prior research, the demonstration that SMCs are present in the human and rodent cervix warrants further understanding of their role in the non-pregnant state [2, 65, 77, 87, 104, 140, 145-149]. In 1947, Danforth described the human cervix as a primarily collagenous organ with limited SMC and
contractile ability [33]. The research proposed a significant active SMC contribution from the uterus and a passive role of the cervix during pregnancy and labor [33]. However, recent research emphasizes the independent role of cervical contractile function and mechanical behavior in the non-pregnant and pregnant states [2, 19, 20, 22, 40, 135]. Prior work, to a limited extent, investigated in vitro cervical contractility utilizing strips, rings, or individual cSMCs in human [19, 135] and rodent models [20, 22, 40]. Although the experimental methods provided fundamental information about the contractile behavior of the cervix, the experiments require disruptive specimen preparation techniques and assess circumferential and axial properties independently [20, 22, 26, 28, 39, 40]. The cervix is anisotropic and loaded multiaxially within the body, thus, there is a need to utilize testing methods that retain cervical geometry, native ECM-cSMC interactions, and simultaneously quantify circumferential and axial contractility within a physiologic loading environment [2, 3, 42, 50].

Biaxial inflation-extension testing overcomes limitations imposed by uniaxial configurations by simultaneously loading tissues circumferentially via pressurizing the organ within a physiologic range and longitudinally by axially extending the organ [41]. Previously, biaxial inflation-extension testing quantified mechanical properties of vasculature [41, 42, 51, 97, 131], the GI tract [46, 47], and reproductive organs [2, 3, 48-50]. Additionally, research determined contractile behavior in vasculature [53, 150, 151] and the vagina [3] utilizing inflation-extension testing. However, there is a need to describe the in vivo cervical loading environment to permit assessment of contractility and passive biomechanics within a physiologically relevant range ex vivo. Therefore, this study seeks to introduce methods to determine the in vivo cervical pressure environment and utilize the
information to design a protocol to determine the biaxial maximum contractility and passive mechanics of the murine cervix.

5.2 Materials and Methods

5.2.1 Animal Care

Mechanical testing experiments herein were conducted on a total of $n=10$ nulliparous CD-1 female mice aged 8-12 weeks (Charles River, Houston, TX). The Tulane University Institutional Animal Care and Use Committee (IACUC) approved of care and the conducted experiments. Immunofluorescence experiments were conducted on a total of $n=2$ nulliparous C57Bl6/SvEv female mice aged 12-24 weeks with approval from the University of Texas Southwestern IACUC. These mice were bred and maintained within a breeder colony at The University of Texas Southwestern Medical Center (Dallas, TX). Mice were provided a normal chow diet and housed in a 12-hour light/dark cycle. All mice were cycle matched at estrus via visual determination [113].

5.2.2 In Vivo Pressure Measurements

Attempting to recapitulate the key aspects of the in vivo loading environment during mechanical testing can provide important information about the mechanical behavior and contractility at an estimated physiologic state. Therefore, transcervical pressure measurements were taken from ($n = 5$) mice at estrus to determine the estimated loading environment for the murine cervix during mechanical testing (Fig. 5.1) [140]. A 2F Millar Mikro-tip® Catheter (ADInstruments, Colorado, USA) was connected to ADInstruments Bridge Amplifier and PowerLab (ADInstruments, Colorado, USA) and allowed to equilibrate in a water bath filled with physiologic saline at 37°C for 30 minutes. Utilizing
a Y connection tube, the catheter was calibrated with a pressure gauge after the 30-minute equilibration.

Mice were anesthetized with 1%-1.5% isoflurane mixed with 100% O₂ and placed on a heating pad [140]. Using blunt forceps and a disposable plastic tube acting as a speculum, the vagina was gently spread laterally. Next, a directed light source aligned down the plastic tube allowed for the cervical centered within the vaginal fornix to be visualized. Following, the pressure catheter was inserted into the cervical canal. Appropriate placement of the catheter was confirmed with a marking approximately 6 mm from the probe based on the length of the vagina from prior studies [3, 49], an increase in pressure [140], and phasic contractions [22, 40]. Upon confirmation of appropriate placement, the catheter equilibrated for 5 minutes followed by 5 minutes of recorded data on LabChart Pro software (ADInstruments, Colorado, USA). Recorded data measured baseline

**Figure 5.1:** (A) Representative recording of in vivo pressure measurements utilizing ADInstruments Labchart software. In vivo cervical smooth muscle behavior exhibited a phasic response characterized by regular contractions throughout the recording. Average pressure between contractions, at the troughs of the waves, determined baseline pressure (black arrows and box). Contractile pressure (gray arrow and open circle) were taken at the peaks to determine average contracted pressure. Time from peak to peak determined the frequency (black closed circles) of contractions for each data set. (B) Average in vivo baseline (black) and maximum contractile (gray) pressure measurements in nulliparous mice at estrus (n=5). ADInstruments Labchart software determined the average baseline (P= 9.00±3.00 mmHg) and maximum (P= 22.0±4.00 mmHg) pressures. Pressure increased significantly (p<0.01; **) from the baseline to maximum amplitude during contraction. (C) Graphic representing the catheter placement within the cervix in vivo during data collection.
pressure, pressure with contraction, and frequency of contraction (Fig. 5.1). After data recording, the catheter was removed and soaked in Terg-A-Zyme®, an enzymatic cleaning solution, for 15 minutes. Between each procedure the catheter was cleaned with the enzymatic cleaning solution, equilibrated for 30 minutes, and calibrated. Mice were monitored and allowed to recover in a separate clean cage before returned to littermates.

5.2.3 Specimen Preparation
A total of \((n=10)\) CD-1 female mice cycle matched at estrus were utilized for dose response and contractility protocols. Wherein, recovered mice from the \textit{in vivo} pressure procedure \((n=5)\) were assigned to the dose response study at the following estrus phase. Additionally, a separate cohort of CD-1 mice \((n=5)\) were utilized for the maximum contractility and passive mechanics protocol [113]. The following sample preparation techniques applied to both protocols listed below. All mice were euthanatized via guillotine to preserve SMC viability [3]. The reproductive systems were excised from mice immediately and submersed in \(4^\circ C\) Hanks Balanced Saline Solution (HBSS). The cervical complex was isolated from the reproductive tract by singular cuts superior to the distal vaginal and inferior to the uterine body (Fig. 5.2). Next, the cervix was cannulated with 6-0 silk suture within a biaxial inflation-extension device (Danish MyoTechnologies, Aarhus, Denmark). HBSS was replaced with \(37^\circ C\) Kreb’s Ringer Buffer (Kreb’s Buffer) aerated with 95% \(O_2\) and 5% \(CO_2\).
During excision, the reproductive organs retract from the original position following dislocation of the pubic synthesis and subsequent removal of fascial tetherings [2, 49, 50]. Due to the retraction, the cervix was extended to an estimated unloaded length in which the organ was neither in tension nor buckled and pressurized with a tare pressure.
of $P= 3.0$ mmHg to prevent collapse of the organ at $P= 0$ mmHg [2, 48, 49]. Unloaded length and unloaded outer diameter at the tare pressure was recorded with digital calipers and a Nikon Eclipse TS100 inverted microscope (Nikon®, Melville, NY, U.S.A.), respectively. Utilizing the mean *in vivo* baseline pressure measurements from the *in vivo* pressure measurement experiments (Fig. 5.1), the mean physiological pressure for mechanical testing protocols was determined as $P= 9.0$ mmHg. To maintain SMC viability, the maximum pressure extended to one standard deviation below the mean maximum contractile pressure ($P= 22.0\pm4.0$ mmHg). Cervices were preconditioned circumferentially at the unloaded length for 5 cycles of increasing and decreasing pressure ($P= 0$-$18$ mmHg). Following, an estimated physiological (EP) length was determined first by using the measured retraction following dissection, followed by leveraging the theoretical assumption that axial force will be maintained with increasing pressure over the physiologic range to preserve energy as described previously [89]. Additional circumferential preconditioning ($P= 0$-$18$ mmHg) was performed at the EP length for 5 cycles and axial preconditioning performed by cyclically stretching cervices axially $\pm1\%$ the EP length at $1/3$ max pressure ($P= 6.0$ mmHg) [2, 51]. Following preconditioning, the unloaded length was re-determined [3]. To acclimate cSMCs to potassium chloride (KCl), the cervical complex was pressurized to the mean physiologic pressure ($P= 9.0$ mmHg) at the unloaded length, axially extended until the axial force held constant at 0mN, and dosed with 20mM KCl for 5 minutes [3, 53]. Following, the bath was replaced with fresh Kreb’s buffer and the cervix elongated to the EP length. The cervix at the EP length and mean physiologic pressure equilibrated for 10 minutes [2, 49].
5.2.4 Dose Response

To determine the optimal dose of the agonist potassium chloride (KCl) to induce maximum contraction, an isometric-isobaric dose response protocol was performed at the EP length and the mean physiologic pressure (P = 9.0 mmHg) for the first cohort of animals.

**Figure 5.3:** KCl dose response circumferential (A) and axial (B) contractility results with respect to the estimated physiologic (EP) length and mean physiologic pressure. (A) Change in diameter with contraction decreased from 4.7-20 mM KCl ($p<0.05$; 4.7 circle; 10 square) compared to 20mM and plateaued from 20-100mM. However, total change in diameter with contraction at 60mM increased significantly ($p<0.05$; ¥) compared to 20mM. (B) Change in force with contraction increased significantly at 20mM compared to all doses ($p<0.05$; *). Further with increasing dose, contractile behavior altered from a phasic (C) to tonic (D) pattern as shown in the representative sample for 20mM (C) and 60 mM (D). Phasic contractile behavior persisted through 20mM KCl (C), and doses 40-100mM exhibited tonic contractile behavior resulting in plateaued force and outer diameter. Frequency of contractions increased non-significantly from the baseline concentration (4.7mM; 0.03±0.02Hz) up to 0.05±0.02 Hz at 20mM. Following 40 mM of KCl, the concentration frequency decreased to 0Hz with tonic contraction.
(n=5). Following the equilibration period, tissues were subjected to increasing concentrations of KCl (4.7-100mM) at the EP length and mean physiologic pressure [139]. Between each dosing, the cervix was washed and submerged in fresh Kreb’s solution and equilibrated for 5 minutes. Circumferential and axial contractions were measured via diameter changes tracked at the mid-cervix and changes in measured axial force with a camera and force transducer, respectively (Fig. 5.3).

5.2.5 Biaxial Contractility and Passive Mechanics

Maximum Contractility

Utilizing the second cohort of mice (n=5), cervices underwent a contractility protocol with nine combinations of physiologic lengths and pressures to assess the role of circumferential and axial loading on contractility following the equilibration period (Fig. 5.4) [53]. Each combination randomized the length (the EP length and ±1% EP length) and pressure (mean ± standard deviation of the physiologic pressure; P= 9.0±3.0 mmHg). Data was recorded for 5 minutes after 20mM KCl dosing followed by a resting period for 5 minutes after buffer replacement or after pressure and axial length change [53]. Then, the cervices were returned to the unloaded geometry and B-mode ultrasound images (Vevo2100; 40MHz transducer) of cervical thickness were taken at the unloaded state [2, 3].

Passive Mechanics
 Returned to the EP length of the maximum contractility experiment, cervices were bathed in calcium-free Kreb’s and dosed with 2mM egtazic acid (EGTA) for 30 minutes to remove active SMC contribution. Abiding by the steps outlined in section 5.2.3 Specimen Preparation, passive unloaded geometry measurements, unloaded circumferential preconditioning, determination of the passive EP length, and circumferential and axial preconditioning were performed followed by 10 minutes of equilibration. Three cycles of a pressure-inflation protocol were performed at the EP length and ±1% the EP length from P=0-18 mmHg [2, 49, 50]. Following, axial force-elongation protocols ±1% EP length over

Figure 5.4: Maximum contractility testing protocol schematic for set axial-extension and alternating pressures. Cervices equilibrated after each test for five minutes followed by five minutes of equilibration with each change in pressure or axial-extension. (A) Times and concentrations of KCl within the tissue bath. Wherein 20 mM induced maximum contraction and 4.7 mM acted as the baseline content in Kreb’s solution. (B) Pressure throughout maximum contractility testing for one axial-extension. Maximum contractility was induced at the mean physiologic pressure (P=9.0±3.0 mmHg). Dashed lines represent the pressure change throughout the protocol. (C, D) Change in force and outer diameter with maximum contraction and during equilibration periods.
a range of pressures (P= 3, 6, 12, and 18 mmHg) were performed [2, 49, 50]. The cervix was returned to the unloaded geometry and the thickness recorded with ultrasound.

### 5.2.6 Immunofluorescence

Transverse and longitudinal sections (5µm) of the cervix at estrus (n=2) were deparaffinized, blocked, and co-stained with primary and secondary antibodies. Markers targeted included αSMA (Anti-Mouse 1:300, Monoclonal Anti-Actin, α-Smooth Muscle, A2547, Sigma-Aldrich) and Vimentin (Anti-Rabbit 1:250, Recombinant Anti-Vimentin antibody [EPR3776]-Cytoskeleton Marker, ab92547, Abcam). After overnight primary antibody incubation at 4ºC, the slides were washed and incubated with secondary antibody (Goat anti-Rabbit 1:500 (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488.A-11008, and Goat anti-Mouse 1:500 (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546, A-11030, Invitrogen-ThermoFisher Scientific) for 30 minutes at room temperature. After PBS washes, slides were mounted with ProLong™ Gold Antifade Mountant with DAPI (P36935, ThermoFisher Scientific) and viewed on a Zeiss LSM-880 Confocal Microscope (Zeiss International, New York, USA) at 20X magnification. For each transverse and longitudinal tissue section, both the sub-epithelial and mid-stromal regions were imaged in at least four locations at 20X and 40X.

αSMA and Vim positive area fractions for the mid-stroma and subepithelium in the transverse and longitudinal sections were calculated utilizing ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.) and GIMP, an open source image manipulation program. Images from the red (αSMA) and green (Vim) color channels were inverted and converted to gray scale in ImageJ. The inverted images were opened in GIMP and a histogram tool within the software was adjusted to represent the range of intensities of the positive cell
staining. The area fraction was determined as the number of pixels within the boundaries of the threshold divided by the total number of pixels in the image.

5.2.7 Data Analysis

Thickness, Area, and Volume Calculation

Utilizing ultrasound images and ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.) software, the inner canal and outer circumference excluding the vaginal fornix were traced and lines drawn between the inner and outer perimeters were used to measure thickness. To determine cross sectional area and volume, cervical geometry was simplified to a hollow cylinder. Applying unloaded geometry (length, diameter, and thickness), cross-sectional area \( A \) and volume \( V \) were determined using the following equations (Eq. 1, 2):

\[
A = \pi (R_o^2 - R_i^2) \quad (1)
\]

\[
V = \pi (R_o^2 - R_i^2)L \quad , \quad (2)
\]

where \( R_o \) is the undeformed outer radii, \( R_i \) the undeformed inner radii, and \( L \) the unloaded length.

Circumferential stretch \( (\lambda_\theta) \) and axial stretch \( (\lambda_z) \) were determined by (Eq. 3, 4) [42]:

\[
\lambda_\theta = r_{mid}/R_{mid} \quad (3)
\]

and

\[
\lambda_z = l/L \quad (4)
\]
wherein $r_{mid} = r_o - r_i/2$ is the deformed mid-wall radius, $R_{mid} = R_o - R_i/2$ is the unloaded mid-wall radius, and $l$ is the deformed length.

**Contractility**

Circumferential and axial contraction were determined via changes in diameter and axial force, respectively (Fig. 5.5). Additionally, circumferential ($T_{\theta\theta}$) and axial ($T_{zz}$) 1st Piola-Kirchhoff stress with maximum contraction were determined (Eq. 5, 6) [52, 150].

$$T_{\theta\theta} = \frac{P r_i}{\lambda_\theta (r_o - r_i)},$$  \hspace{1cm} (5)

$$T_{zz} = \frac{F_t}{\lambda_2 \pi (r_o^2 - r_i^2)} + \frac{P r_i^2}{\lambda_2 (r_o - r_i)(r_o + r_i)},$$  \hspace{1cm} (6)

Wherein $P$ is intraluminal pressure, $r_i = \sqrt{r_o^2 - \frac{A}{\pi \lambda_2}}$ is the
deformed inner radius, $r_o$ is the deformed outer radius, and $F_t$ is the force from the axial force transducer [2, 42, 150]. Contribution of the active SMC to the change in stress with contraction ($\Delta T$) was calculated by subtracting the relaxed or passive state stress ($T_{\text{passive}}$) from the contracted stress ($T_{\text{contracted}}$) at matching axial-extensions and pressures (Eq. 7, 8) [3, 53, 150-152]:

$$\Delta T_{\theta\theta} = T_{\theta\theta}^{\text{contracted}} - T_{\theta\theta}^{\text{passive}}$$

(6)

$$\Delta T_{zz} = T_{zz}^{\text{contracted}} - T_{zz}^{\text{passive}}$$

(7)

Material Stiffness

Material stiffness in the circumferential and axial loading directions were determined by calculating the slope of the stress-stretch curves at a physiologic range of pressures ($P= 9.0\pm3.0$ mmHg).

5.2.8 Statistics

Paired t-tests were utilized to determine differences between in vivo baseline and maximum pressure and differences in passive and active geometry. One-way ANOVA with respect to dose determined changes in circumferential and axial contractility. Two-way ANOVAs (axial-stretch, pressure) were utilized to determine differences in circumferential and axial contractility. Further, a two-way ANOVA with respect to axial-stretch and loading direction was used to determine differences in circumferential and axial material stiffness. Posthoc t-tests with Bonferroni corrections were utilized when appropriate ($p<0.05/2$).
5.3 Results

All results presented herein are represented as mean±SEM apart from the *in vivo* data which is presented as the mean±sd. *In vivo* pressure data are represented as mean±sd to provide a larger margin of error to better capture the contractile response within the *in vivo* pressure range.

5.3.1 *In vivo* Pressure and Dose Response

*In vivo* transcervical pressure measurements at estrus revealed an average *in vivo* baseline pressure of 9.00±3.00 mmHg (mean±sd), maximum contractile pressure of 22.0±4.00 mmHg (mean±sd), and a frequency of one contraction per 23.5±5.64 seconds (mean±sd) (**Fig. 5.1**). Paired t-tests confirmed a significant increase (*p*<0.01) in cervical pressure from baseline to maximum amplitude with contraction *in vivo*.

Dose response curves of KCl at the EP length and mean physiologic pressure identified 20mM as the optimal dose to induce maximum contraction (**Fig. 5.3**). Axial force with contraction with dose increased significantly (*p*<0.05) at 20mM compared to all doses followed by a plateau of axial force during contraction at higher doses (30mM-100mM) of KCl. Change in diameter with contraction decreased maximally at 20mM-100mM compared to 4.7mM, 10 mM KCl, and 60mM dosing. Further, phasic behavior abated with increasing dose (30-100 mM) of KCl and the axial force and outer diameter transitioned into a tonic contractile behavior (**Fig. 5.3**).
5.3.2 Geometry

Passive physiologic diameter \((p<0.05)\), EP length \((p<0.01)\), and volume \((p<0.005)\) significantly increased compared to active geometry. However, thickness did not differ significantly between the active and passive state (Fig. 5.6).

5.3.3 Biaxial Contractility

Induction of maximum contraction with 20mM KCl induced phasic contractions that resulted in a decrease in diameter and increase in axial force (Fig. 5.4, 5.7). Additionally, all samples contracted spontaneously without an agonist introduced to the bath as seen previously in nulliparous mice at

![Figure 5.6](image_url)

**Figure 5.6:** Geometry recorded during mechanical testing at the active (black) and passive (gray) states revealed a significant increase in passive geometry. (A) Physiologic diameter, the diameter at the physiologic length and mean physiologic pressure, increased significantly \((p<0.05; \star)\) from 4.67±0.25 mm to 5.59±0.53 mm. (B) Physiologic length significantly increased \((p<0.01; \star\star)\) from 6.96±0.54 mm to 7.59±0.66 mm between active and passive protocols. (C) Thickness did not significantly increase in the passive state \((0.62±0.04 \text{ mm vs } 0.64±0.03 \text{ mm})\). (D) Volume of the unloaded cervix increased significantly \((p<0.005; \star\star\star)\) after smooth muscle relaxation where the active volume measured 44.2±7.22 mm\(^3\) and the passive measured at 64.3±11.0 mm\(^3\).
estrus [40]. Two-Way ANOVA (axial-stretch, pressure) did not detect differences with axial-extension \((p=0.60)\) or between interactions \((p=0.90)\). However, ANOVA detected significance \((p<0.001)\) with respect to pressure for circumferential contractility. Posthoc t-tests with Bonferroni corrections \((p<0.05/2)\) determined significant effects on change in outer diameter with increased pressure \((p<0.001)\). Specifically, total change in diameter at high pressure \((P=12 \text{ mmHg})\) diminished significantly \((p<0.001)\) compared to total change in diameter at low pressure \((P=6 \text{ mmHg})\) (Fig. 5.7). However, Two-Way ANOVAs (axial-stretch and pressure) determined no significant differences with axial-extension \((p=0.40)\), pressure \((p=1.00)\), or interactions \((p=1.00)\) with the change in axial force with maximum contraction. Additionally, frequency and wavelength of contractions during the maximum contractility protocol did not differ with alterations to pressure \((p=0.90)\), axial-stretch \((p=0.20)\), or interactions \((p=0.95)\) (Fig. 5.7).

The contracted SMCs and passive ECM contribute to the overall cervical biomechanical properties, including wall stress (force over oriented area). Subtracting the passive stresses from the active contractile stresses at matching pressures and axial-stretches quantifies the contribution of the active SMC during contraction [53, 150]. Maximum contraction induced a decrease in the change in circumferential stress \((\Delta T_{\theta\theta})\) and increase in axial stress \((\Delta T_{zz})\) at all pressures and axial-stretches (Fig. 5.8). Two-Way ANOVA (axial-stretch, pressure) did not detect significant differences for \(\Delta T_{\theta\theta}\) or \(\Delta T_{zz}\) with change of pressure or axial-stretch. Total \(\Delta T_{zz}\) increased significantly with maximum contraction compared to \(\Delta T_{\theta\theta}\) \((p<0.05)\) at the EP length and pressure (Fig. 5.8).
5.3.4 Biaxial Passive Mechanics

Passive material stiffness calculated from the slope of stress-stretch curves over a range of physiologic pressures (P=9.0±3.0 mmHg) was significantly greater (p<0.01) in the circumferential direction compared to the axial direction (Fig. 5.8) [3, 153].

Figure 5.7: (A) Circumferential contraction measured by change in diameter during maximum contractility protocol at 9 combinations of pressures (P=9.0±3.0 mmHg) and axial-extensions ±1% EPL (n=5). Where 6 mmHg is represented by gray closed circles, 9 mmHg by dark gray open circles, and 12 mmHg as black closed circles. Total change in diameter during contraction significantly decreased (p<0.001; ****) at the high pressure (P=12 mmHg; black closed) compared to the low pressure (P= 6 mmHg; gray closed) loading for all axial-stretches. (B) Axial contraction measured by change in force did not significantly differ with axial-stretch or pressure. (C, D) Wavelength and frequency of contractions induced by 20 mM KCl did not significantly differ with increase pressure or axial-stretch.
Circumferential stiffness at the physiologic length measured 327±142 kPa and axial stiffness measured 136±51.2 kPa (Fig. 5.8). Further, no significant differences in circumferential or axial stress were identified with axial-extension (Fig 5.9).

Figure 5.8: (A) Isolated active mechanical contribution in circumferential stress against circumferential stretch during at the estimated physiologic length (EP L; dark gray open circles), -1% EP L (closed gray circles), and +1% EPL (closed black circles) for all pressures (n=5). Stresses within a pressure grouping are separated by dashed lines. (B) Isolated active contribution with respect to the axial stress plotted against axial stretch for all axial stretches and pressures. Where dashed lines separate the mean physiologic pressure (P=9.0 mmHg) from the above (P=12 mmHg) and below (P=6.0 mmHg) pressure groupings. (C) Absolute change in circumferential (black) and axial (gray) stress at the EP length and pressure where axial stress (3.40±0.70 kPa) significantly (p<0.001; **) increased compared to circumferential stress (2.44±0.28 kPa) during maximum contraction. (D) Circumferential (black) and (E) axial (gray) stress-stretch curves with respect to the circumferential stretch, respectively. The physiologic range of pressures (P=9.0±3.0 mmHg) during the passive mechanical test were mapped to corresponding circumferential stretches and stresses. The lines denote the area of the stress-stretch curves in which the slope was determined for material stiffness calculations. (F) Circumferential (black) and axial (gray) material stiffness from the EP length. Two-way ANOVA (axial-stretch, direction) and post-hoc t-tests confirmed a significant increase (p<0.01) in circumferential stiffness (327±142 kPa) compared to axial stiffness (136±51.2 kPa).
5.3.5 Immunofluorescence

Immunofluorescence of transverse and longitudinal cervical sections identified fibroblast (αSMA−, Vim+) and cSMC (αSMA+, Vim−) cell types within the stroma and the stromal region adjacent to the epithelia, termed the sub-epithelial layer. Within the mid-stroma, cSMC (αSMA+, Vim−) and fibroblast (αSMA−, Vim+) populations are identified (Fig. 5.10). Within the transverse sections, αSMA+ cells comprised 9.49±3.69% of the area and Vim+ cells filled 14.2±0.94% of the area. Longitudinal section area fractions calculated 11.2±3.30% αSMA+ cells and 17.7±3.80% Vim+ cells. In contrast, the sub-epithelial layer contains only fibroblast (αSMA−, Vim+) cells in the estrus cervix with an area fractions of 16.45±3.45% and 13.4±2.07% in the circumferential and axial directions, respectively (Fig. 5.10). Interestingly, a subpopulation of (αSMA+, Vim+) cells reside within the mid-stroma.
5.4 Discussion

This study, for the first time, presented methods to determine biaxial maximum contractility within a physiologic loading environment in the murine cervix utilizing inflation-extension techniques. Additionally, transcervical pressure catheter experiments
described the *in vivo* baseline pressure and contractile amplitude and frequency in the murine cervix.

Historically, research considered the cervix as an extension of the uterus with minimal or no independent contractile ability [33]. However, recent studies emphasized the importance and individual nature of the cSMC microstructure and behavior compared to the other reproductive organs [19, 20, 22, 40, 135]. Further, Vink *et al.* described an altered contractile response of cSMC with respect to ECM stiffness suggesting that mechanical loading may dictate cervical contractile behavior [135]. While prior work investigated the uniaxial contractile behavior of the cervix, the methods did not preserve the native ECM-cell interactions and did not account for multiaxial loading of the cervix *in vivo* [20, 22, 40]. Biaxial inflation-extension active and passive mechanical testing methods well-established in vasculature [42, 52, 53, 139, 150, 151] provide a blueprint for determining protocols to assess biaxial contractility in hollow organs, such as the cervix. Through adapting maximum contractility protocols from vasculature [53] and the vagina [3], this study fulfills a need to determine experimental procedures to describe the multiaxial contractility of the cervix within a physiologically relevant mechanical loading environment.

Herein, we described biaxial maximum contractility and passive biomechanics within a physiologic loading environment motivated by *in vivo* pressure measurements of the murine cervix. Interestingly, axial contractility increased \((p<0.001)\) compared to circumferential contractility for all axial-extensions and pressures (Fig. 5.8C). However, in the passive state, circumferential stress and stiffness increased \((p<0.01)\) compared to axial stress and stiffness at matching pressures and axial-extensions (Fig. 5.8F). Further,
circumferential contractility decreased with increasing pressure, yet, axial force with contraction did not change with increasing pressure (Fig. 5.7). Moreover, axial active stress contributed 54.7±9.19% to the total axial stress while circumferential active stress only contributed 35.0±6.09% to the total circumferential stress. This may suggest a predominant role of axial SMC to resist loading in the axial direction whereas circumferentially aligned collagen within the cervix may function to resist circumferential loading [2, 21, 36]. Supplementing contractility and passive biomechanical data, immunofluorescent imaging demonstrated populations of cSMCs (αSMA+ cells) within the mid-stroma in both the circumferential and axial planes (Fig. 5.10). cSMC populations existed only within the mid-stroma suggesting the stroma to be the primary active component of the cervix. Remodeling of ECM and cSMCs within this region may be critical to maintain normal function during pregnancy [20, 21, 154]. Further, analysis identified a larger area fraction of cSMCs within the longitudinal mid-stroma compared to the transverse sections. A larger population of axially aligned cSMCs may prescribe the increased axial contractility within the murine cervix. However, due to the small change in percentage between transverse and longitudinal sections there may be additional cellular mechanisms contributing to axial contractility. Furthermore, rat vaginal tissue under biaxial loading exhibited a stronger axial contraction in the presence of KCl, however, electrical field stimulation induced increased circumferential contractility [155]. The exact biological mechanisms driving contractility and normal cSMC pacemaker activity within the cervix remain relatively understudied and further investigation into the cholinergic nervous stimulation for cSMC with respect to direction is needed [40].
Cervical insufficiency (CI), a condition in pregnancy in which the cervix prematurely shortens and dilates releasing the uterine contents in the absence of contractions, remains a challenge to diagnose and treat clinically [5, 10, 11]. Prior CI research investigated the role of changing extracellular matrix (ECM) constituents and mechanical loading as potential factors of CI [6, 11-13]. Interestingly, elastic fiber integrity and content decreases in cases of CI suggesting altered ECM integrity during pregnancy may lead to cervical failure [11]. However, the role of cervical SMC and SMC-ECM interactions remains unknown. Potentially, a loss of axial SMC contractility in response to maladaptive ECM remodeling may result in cervical shortening and dilation characteristic of cervical insufficiency [11, 135]. Future research in pregnancy to describe the dynamic passive biomechanical and contractile behavior of the cervix is needed. The customized methodology described in the current study sets a foundation for future studies in which to explore contraction potential in the cervix through normal pregnancy.

Prior research on the mechanical properties of the murine cervix in uniaxial tension reported similar values of circumferential stiffness (229.74±133.20 kPa/mm) to the values calculated herein (307±133 kPa) [21]. Compared to biaxial active and passive data from the murine vagina, the cervix exhibited similar anisotropic behavior and increased axial contractility. Cervical circumferential stiffness and axial contractility increased compared to the murine vagina in the biaxial inflation-extension configuration [3]. Interestingly, while cervical circumferential stiffness exceeded vaginal measurements, vaginal and cervical axial stiffness were similar in magnitude. Similarly, increased axial contractility with KCl dose was observed in the rat and mouse vagina [3, 155]. Further, the anisotropic
behavior of the passive murine cervix with a preference for the circumferential loading
direction was observed in the rat and murine vagina and the murine uterus [2, 3, 155].

The study did not evaluate potential changes in cervical contractility and passive
biomechanics throughout the estrus cycle. However, samples were evaluated at a single
stage of the 5-day cycle (estrous) to prevent variability in results. Further, Gravina et al.
determined the highest contractile potential of the murine cervix at estrus and metestrus
[40]. Additionally, pilot studies of the passive biomechanics of the murine cervix and
uterus revealed no significant differences with estrous phase [2]. The inclusion of the lower
uterine and upper vaginal segments may introduce variability into the results. To reduce
variability, we tied silk suture over the vaginal and uterine segments during cannulation to
prevent contribution to cervical contractility. The dosage of 20 mM diminished
contribution of the vaginal segment as the rodent vagina responds to doses greater than 30
mM and optimally at 40 mM with tonic contractions [3, 72]. This study assumed
conservation of volume, incompressibility, of the cervix in the active and passive protocols,
respectively. The estrus cervix may be compressible and the tissue swelled throughout the
experiment as glycosaminoglycans sequestered water resulting in a larger passive diameter
[62]. However, cervical thickness could not be tracked real-time throughout the experiment
due the thickness of the cervical wall preventing light penetration through the tissue and
prolonged compression from the ultrasound transducer resulted in a loss of contractility.
Further investigation on isochoric motion during biaxial mechanical testing of the
reproductive organs is needed [3, 53].

In summary, the study introduced methods to determine in vivo cervical pressure
loading environment and apply the in vivo pressure to design a protocol to assess biaxial
maximum contractility and passive biomechanics of the murine cervix. Determining relationships between cervical SMC contractility and passive function will provide critical information to develop fundamental understanding of physiological cervical function. Further, applying the methods herein throughout pregnancy may provide crucial data about normal remodeling and provide clinically relevant insights into potential mechanisms by which the cervix fails in premature birth induced by cervical insufficiency.

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Chapter 6: Determining the Role of Elastic Fibers on Cervical Contractility *In Vivo* and *In Vitro*

6.1 Introduction

Preterm birth (PTB) is a global health conditioning effecting 1 in 10 pregnancies worldwide [4]. While infection mediated PTB leads in percentage of cases, spontaneous PTB remains difficult to diagnose and treat effectively [5, 10, 11]. Specifically cervical insufficiency (CI), a form of spontaneous PTB that results in early dilation and shortening of the cervix during early- to mid-pregnancy, remains a challenge to diagnose and treatments such as progesterone supplementation or cerclage have low efficacy [5, 10, 11]. The etiology of CI remains unknown, improper extracellular matrix (ECM) and cervical smooth muscle cell (cSMC) remodeling is implicated as a factor [19, 21, 135]. Further, Leppert *et al.* reported decreased elastic fibers and desmosine in human cervixes affected by CI [11]. Therefore, elastic fiber deficiency may result in maladaptive cervical remodeling during pregnancy and postpartum [11, 13]. In other soft tissues such as the arteries, elastic fiber deficiency increases stiffness, decreases contractility, and simulates vascular aging [53, 97]. Further, connective tissue disorders associated with elastic fiber deficiency result in pathological conditions of the pelvic organs such as pelvic organ prolapse [114, 115, 156, 157]. Further, ECM stiffness and elastic fibers potentially play a role in cSMC homeostasis and contractile potential [135, 158]. Therefore, elastic fiber deficiency and a subsequent loss in cSMC contractile ability during pregnancy may induce maladaptive remodeling of the cervix resulting in CI.
Prior work utilized the fibulin-5 (Fbln5) global knockout mouse as a model of elastic fiber deficiency for vascular and pelvic organ pathologies [97, 156]. Utilizing the Fbln5 mouse model in conjunction with the biaxial inflation-extension methods developed for the murine cervix provides an opportunity to elucidate the role of elastic fibers in normal cervical function [43, 156]. Further, biaxial inflation-extension methods maintain native tissue geometry, cell-ECM connections, and better recapitulates multiaxial loading [52, 159]. Therefore, this study seeks to utilize Fibulin-5 haplo-insufficient mouse model to determine the role of elastic fibers in cSMC function.

6.2 Methods

6.2.1 Animal Care

A total of (n =21) female mice aged 3-6 months at estrus from a C67BL6x129SvEv lineage were utilized for in vivo contractility, in vitro biaxial maximum contractility, biaxial basal and passive mechanical testing, and pressurized culture protocols. Mice were divided into groups depending on genotype where n=11 mice were wildtype controls with both copies of the Fibulin-5 gene (WT; Fbln5+/+) and n=10 were haploinsufficient mice with one copy of the Fibulin-5 gene (HET; Fbln5+/-). All experiments were performed with approval from the Tulane Institutional Animal Care and Use Committee (Tulane IACUC). Tulane University housed and maintained the mouse colony in a 12 hour light/dark cycle and fed normal chow. Estrus cycle staging was determined via visual methods from Byer et al., 2012 [56].

6.2.1 In Vivo Pressure Measurements

To determine in vivo contractile behavior with respect to genotype, transcervical pressure measurements were taken in the WT and HET (n=5/group) mice at estrus [140,
Firstly, the tip of the 2F Millar Mikro-tip® Catheter (ADInstruments, Colorado, USA) was placed in a water bath of physiologic saline at 37°C to equilibrate for 30 minutes. The condensation of the lid of the water bath created a dark environment for the catheter and prevented offsets to the calibration due to fluorescent light interference. The catheter was connected to the ADInstruments Bridge Amplifier and PowerLab device (ADInstruments, Colorado, USA) with care to keep the connector away from wet surfaces or static discharge. Following equilibration, the catheter was inserted into a Y connector tube filled with saline and attached to a pressure gauge. The connector tube and catheter were placed back in the water bath for pressure calibration utilizing the AD Instruments software.

WT and HET (n=5/group) female mice at estrus were anesthetized with 1%-1.5% isoflurane mixed with 100% oxygen in an anesthesia chamber [140]. Depth of anesthesia was determined via toe pinch and the animal was transferred to a nose cone and placed on a heating pad for the duration of the experiment. Blunt forceps and a disposable plastic tube were coated with warmed (37°C) sterile lubricating gel and inserted into vaginal opening to visualize the cervix. The 2F catheter was inserted approximately 5-6mm from the vaginal introitus based on the insertion in the C57BL6 and CD-1 mouse in Chapters 4 and 5, respectively [160]. Additionally, prior studies of vaginal length recorded murine vaginal measurements in the C57BL6 mouse to be approximately 6 mm [3, 49]. An increase in pressure [140] and recorded phasic contractions [23, 160] indicated proper placement within the cervix. However, baseline pressure dropping after insertion could indicate the catheter placement within the uterus [140]. Once placement was confirmed the catheter equilibrated for 5 minutes in the cervix followed by 5 minutes of recording.
baseline pressure, contractile pressure, and contraction frequency measurements [140, 160]. Mice were monitored for recovery before returning to the housing facility.

### 6.2.2 Specimen Preparation

A separate cohort of nulliparous female mice at estrus \((n=5\) per genotype) were sacrificed via guillotine to preserve smooth muscle cell viability. The reproductive system was isolated from the body and placed in a dish of 4°C Hank’s Balanced Saline Solution (HBSS). Blunt dissection removed connective tissue and fat from the reproductive tract to ensure clear and repeatable tracking during testing. Utilizing the preparation methods from Chapters 4 and 5: the cervix was isolated from the reproductive tract with a cut inferior to the uterine horn bifurcation and a cut inferior to the external os [160]. The remaining uterine horn tissue was trimmed and the septum between the uterine horns cut to allow for proper cannulation. Following, the cervix was cannulated within the basin of a biaxial inflation-extension device (Danish MyoTechnologies, Aarhus, Denmark) with 6-0 silk suture. Care was taken to tie the sutures down on the remaining segments of uterine and vaginal tissue to prevent contribution during active and passive mechanical testing. The estimated unloaded length was visually determined as the length in which the tissue was neither in compression nor tension [2, 160]. The basin was filled with Kreb’s Ringer Buffer at 37°C aerated with 95% \(O_2\) and 5% \(CO_2\) to maintain pH.

### 6.2.3 Preconditioning

Unloaded diameter and length were recorded with a Nikon Eclipse TS100 inverted microscope (Nikon®, Melville, NY, U.S.A.) and digital calipers, respectively. At the unloaded length tissues underwent 5 cycles of pressure inflation preconditioning \(P = 0-28.0\) mmHg based on the results from the catheter pressure measurements (Fig. 6.1). The
maximum pressure for *in vitro* was determined as the maximum *in vivo* pressure minus one standard deviation to ensure cSMC viability [160]. Utilizing the assumption from vasculature that there is an energy preserving length in soft tissues, the estimated physiologic (EP) length was determined as the length in which force remained constant with increasing pressures [89]. Subsequently, pressure-inflation preconditioning (P= 0-28.0 mmHg) was repeated for at the EP length and 5 cycles of force-elongation preconditioning were performed at 1/3 maximum pressure (P = 9.0 mmHg) from -1% EP length to +1% EP length [42, 160]. Then the unloaded length was redetermined as the compressed length in which the tissue is no longer in tension and the force readings remained constant [3]. The tissue was pressurized to the mean physiologic pressure for the WT (P = 10.0 mmHg) or HET (P = 12.0 mmHg), respectively. The tissue was then axially extended until the transducer force read 0 mN [3, 53]. To acclimate cSMC to potassium chloride (KCl), the tissue was dosed with 20mM KCl, the optimal dose to induce maximum contraction in the murine cervix (Chapters 4 and 5), and the contractions were recorded for 5 minutes [3, 53, 160]. Following, the basin was filled with fresh Kreb’s buffer and the tissue equilibrated.
for 5 minutes. Subsequently, the tissue was returned to the EP length and pressurized to the mean physiologic pressure and equilibrated for 10 minutes.

6.2.4 Biaxial Maximum Contractility

Following preconditioning, the cervices underwent a maximum contractility protocol at the EP length. Prior work in Chapter 5 presented no significant differences in contractility with axial length [160]. The cervices were dosed with 20 mM of KCl at three pressures around the mean physiologic pressure where P = 10.0 ± 2.0 mmHg for the WT and P = 12.0 ± 2.0 mmHg in the HET. Between each dosing, the Kreb’s buffer within the basin was replenished and the tissue equilibrated for 5 minutes.

6.2.5 Basal and Passive Mechanical Testing

Following the maximum contractility protocol, cervical samples underwent three cycles of the pressure-inflation testing (P= 0-28.0 mmHg) at static axial extensions of -1% EP, EP, and 1% EP length. Next, samples were subjected to 3 cycles of force-elongation testing from -1% to +1% EP length at static pressures corresponding with a tare pressure, 1/3 maximum, 2/3 maximum, and maximum pressure (P = 3.0, 9.0, 18.0, and 28.0 mmHg). Following, a Vevo2100 ultrasound recorded images of cervical at the unloaded length.

To deactivate cSMCS, samples were treated with 2mM EGTA and immersed in a solution of calcium free Krebs Buffer for 30 minutes [3]. The basin was washed out with fresh calcium-free Krebs Buffer and the new unloaded geometry recorded. The preconditioning, pressure-inflation, and force-elongation protocols were repeated. Cervices were returned to the unloaded length and ultrasound B-mode images of the tissue were taken with a 40Mhz transducer.
6.2.6 Pressurized Culture Pilot

A pilot study \((n=1)\) on the effect of mechanical loading in culture was performed. To begin, the cervix was explanted from the reproductive system using sterile technique. The sample was cannulated within a sterile 3D printed device and submerged in cell culture media (Dulbecco’s Modified Eagle Medium and 20% Fetal Bovine Serum). Cell culture media was pushed through the lumen and pressurized to the mean physiologic pressure (Fig. 6.2). The device was placed in a cell culture incubator at 37°C and 5% CO\(_2\) for 48 hours. After 48 hours, the sample was removed from the device and cannulated within the biaxial inflation-extension device. The sample underwent the maximum contractility protocol to determine cSMC viability.

6.2.7 Data Analysis

*Thickness, Cross-sectional Area, and Volume Calculations*

Ultrasound images were exported into ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.) where the inner canal and outer circumference were traced. Lines were drawn between the inner and outer tracings to gather an average unloaded thickness \((H)\) value for the tissue in the basal and passive state.

Next, assuming the geometry of the cervix is a hollow cylinder and utilizing the unloaded geometry we calculated the cross-sectional area \((A)\), and volume \((V)\):
where $R_o$ is the undeformed outer radii, $R_i$ the undeformed inner radii, and $L$ the unloaded length.

**Biaxial Maximum Contractility**

Circumferential maximum contraction was determined via the change in diameter from the basal to the maximally contracted state. Conversely, axial maximum contraction was calculated as difference in force between baseline force and maximally contracted force measured via force transducer.

**Basal and Passive Mechanics**

Data from the basal and passive pressure-inflation tests was utilized to created stress-stretch curves. Wherein circumferential stretch ($\lambda_\theta$) and axial stretch ($\lambda_z$) were determined by **Equations 3 and 4** [42]:

$$\lambda_\theta = \frac{r_{mid}}{R_{mid}}$$

(3)

and

$$\lambda_z = \frac{1}{L}$$

(4)

where $r_{mid}$ is the deformed mid-wall radius, $R_{mid}$ is the unloaded mid-wall radius, and $l$ is the deformed length.

Circumferential ($T_{\theta\theta}$) and axial ($T_{zz}$) 1st Piola-Kirchhoff stresses for each state were determined via (**Eq. 5, 6**) [52, 150].
\[ T_{\theta\theta} = \frac{Pr_i}{\lambda_{\theta\theta}(r_0 - r_i)} \]

\[ T_{zz} = \frac{F_t}{\lambda_{zz}(r_0 - r_i)} + \frac{Pr_i^2}{\lambda_{zz}(r_0 - r_i)(r_0 + r_i)} \]

wherein \( P \) is intraluminal pressure, \( r_i = \sqrt{r_0^2 - \frac{A}{\pi \lambda_z}} \) is the deformed inner radius, \( r_o \) is the deformed outer radius, and \( F_t \) is the force from the axial force transducer [2, 42, 150].

**Material Stiffness**

Material stiffness was determined for the circumferential and axial directions by calculating the slope of the stress-stretch curves at a physiologic range of pressures (\( P = 10.0 \pm 2.0 \) mmHg) [3, 160].

### 6.2.8 Statistics

T-tests compared *in vivo* pressure and frequency measurements between genotypes. Additionally, t-tests were utilized to compared unloaded geometry between genotypes. Two-way ANOVA (Genotype, Pressure) compared changes in outer diameter and force with maximum contraction. Further, material stiffness was analyzed for differences with Three-Way ANOVA (Genotype, Tone, Axial-Extension). Posthoc t-tests with Bonferroni correction were utilized where appropriate.

### 6.3 Results

#### 6.3.1 In Vivo Pressure

*In vivo* contractile frequency increased significantly \((p<0.05)\) in the WT compared to the HET, however, baseline and maximum pressure with contraction did not significantly change with genotype (Fig. 6.1).
6.3.2 Geometry

T-tests detected no significant differences in unloaded cervical geometry with genotype.

6.3.3 Active and Passive Biomechanical Properties

Two-way ANOVA did not detect significant differences \((p>0.05)\) with genotype with circumferential contractility, axial contractility, wavelength, or frequency (Fig. 6.3).

![Graphs showing active and passive biomechanical properties](image)

**Figure 6.3:** (A, B) *In vitro* circumferential and axial contractility \((n=5)\) at the estimated physiologic (EP) length and mean physiologic pressure where the wildtype (WT) is represented by white and the heterozygous (HET) is indicated by gray. Change in diameter \((-870±370 \mu m, WT; -1100±227 \mu m, HET)\) and force \((14.6±7.30 \, mN, WT; 28.3±14.1 \, mN, HET)\) with maximum contraction did not significantly differ with genotype. (C, D) *In vitro* wavelength and frequency with maximum contraction at the EP length and mean physiologic pressure. Wavelength \((46.1±25.0 \, s, WT; 75.5±18.6, HET)\) and frequency \((0.03±0.02 \, Hz, WT; 0.01±0.01 \, Hz, HET)\) did not significantly differ with genotype \((p>0.05)\).

Power analysis calculated at least \(n=10\) samples to determine differences in wavelength, \(n=21\) for frequency, \(n=12\) for axial contractility, and \(n=25\) for circumferential contractility.

Further, three-way ANOVA did not detect differences \((p>0.05)\) in material stiffness between axial-extension, state, or genotype. Power analysis revealed a sample size of \(n=8\) needed to determine...
statistical differences between the basal and passive circumferential stiffness in the WT. Additionally, \(n=13\) samples are required to determine differences between the basal and passive axial material stiffness in the WT. Comparatively \(n=59\) and \(n=42\) samples are required to determine significant differences in HET circumferential and axial material stiffness with state, respectively. A minimum of \(n=12\) samples are needed to determine significant differences between the WT and HET basal state and \(n=29\) samples are needed to determine differences in the passive circumferential stiffness. However, circumferential stiffness exceeded \((p<0.05)\) axial stiffness for both genotypes and states.

6.3.4 Pressurized Culture Pilot

Cervical contractility 48 hours after pressurized culture resulted in diminished cSMC response to KCl. Maximum contraction at the EP length and mean physiologic pressure resulted in a change in force of 0.6mN in the WT cervix. Further, diameter did not change with contraction (Fig. 6.4).

![Figure 6.4](image_url)

**Figure 6.4:** *In vitro* axial (A) and circumferential (B) contractility in the wildtype (WT) cervix after 48 hours in culture where white represents the WT control \((n=5)\) and black indicates the cultured specimen \((n=1)\). Where pressurized culture decreased contractility in the murine cervix with a total change in 0.6 mN of force and 0 \(\mu\)m in diameter. No signs of contamination were observed in the cell media on Day 2.
6.4 Discussion

This Chapter sought to identify differences in in vivo contractility, biaxial maximum contractility, and basal and passive mechanical properties between WT and HET mice with a Fibulin 5 haploinsufficiency.

In vivo, Fibulin-5 haplo-insufficiency induced a significant decrease in the frequency of contractions, however, baseline and maximum contractile pressures did not significantly differ (Fig 6.1). Further, in vitro maximum contractility and passive mechanical properties did not significantly differ with genotype (Fig 6.3). Unlike the knockout (KO, Fbln5−/−) animals, the single Fibulin-5 gene in the HET may provide compensation. The KO phenotype develops prolapse by 2-3 months of age [161], however, the HET mouse does not present with prolapse. Additionally, there are multiple elastic fiber proteins and crosslinking components that may play a larger role in elastic fiber regulation in the murine cervix. Nallasamy et al. reported a decrease in Fibulin-5 expression in the murine cervix throughout pregnancy with corresponding increases in elastin (Eln) and fibrillin-2 (Fbn2) [64]. Power analysis of wavelength and axial contraction suggest a larger sample size is needed to determine if significance between WT and HET exist.

Fibulin-5 global knockout animals are a beneficial tool to characterize the role of Fibulin-5 and elastogenesis within the soft tissues [157, 161, 162]. However, global knockout of Fibulin-5 results in amplified progression female reproductive conditions such as pelvic organ prolapse [156]. Wherein, the KO phenotype typically develop prolapse by 2-3 months of age and 90% develop prolapse by 6 months of age irrespective of delivery status [157, 161]. Contrastingly, human development of prolapse occurs as a function of age, hormone status, and parity [157, 163-165]. To further understand the role of elastic
fibers and inflammatory cytokines in vaginal pathologies, Chin et al. developed a tissue-specific conditional knockout (cKO) mouse for the Fibulin-5 gene within smooth muscle cells and myofibroblasts [157]. Similar to the global Fibulin-5 HET mouse, the cKO induced upregulation of MMP-9 and normal elastic fiber morphology [157, 166]. However, cKO developed preclinical signs of pelvic organ prolapse postpartum and observable prolapse with elastase injection [157]. Therefore, future research on the Fibulin-5 cKO mouse may yield insights into the role of elastic fibers on cSMC function with greater efficacy than the global Fibulin-5 haploinsufficient mouse [157].

Interestingly, basal and passive mechanical properties did not differ significantly in either the WT or HET. Potentially, the high collagen content in the nulliparous cervix predominately provides biomechanical function to the tissue with minimal basal contribution and the organ relies on patterned phasic contractions to contribute to processes such as fertilization and menstruation [2, 22, 40]. Although significant differences in passive mechanical properties were not identified, the cervix dilates at estrus and produces cervical mucus with low viscosity in preparation for fertilization [18, 60, 167, 168]. Considering the sphincter hypothesis from Vink et al., the action of the cSMC responsible for maintaining basal tone and keeping the cervix closed tight might be inactivated or quiescent as a result of hormonal status [35, 135].

Pressurized organ culture for 48 hours resulted in decreased contractility and cSMC response to KCl. Preliminary studies in the vagina resulted in a similar loss of contractility at the EP length and mean physiologic pressure. Further pilot studies are needed to determine appropriate media composition to preserve cSMC contractility in culture.
In conclusion, this chapter characterized the *in vivo* contractile behavior, *in vitro* maximum contractility, and basal and passive biomechanical properties of the WT (*Fbln5^{+/+}*) and HET (*Fbln5^{+/−}*) murine cervix. Further studies examining the interplay between elastic fiber remodeling and cSMC function could inform normal and pathological function of the cervix.

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Chapter 7: Cervical Biaxial Contractility and Passive Mechanics During Pregnancy

7.1 Introduction

Preterm birth (PTB) is a global health condition and the leading cause of childhood morbidity and mortality worldwide [4]. Spontaneous PTB related to cervical insufficiency (CI), a condition in which the cervix prematurely shortens and dilates releasing the uterine contents in the absence of contractions, remains a challenge to diagnose and treat clinically [5, 10, 11]. Clinically, CI is characterized by decreased cervical length and cervical effacement in early- to mid-pregnancy [6, 12]. Cervical length may not be a reliable indicator of CI as a stiff short cervix may endure the loading of pregnancy better than a long compliant cervix [6, 12]. The pathophysiology of CI is multifactorial and poorly understood, however, extracellular matrix (ECM) remodeling, cellular remodeling, and mechanical loading are contributing factors [11, 12, 21, 26]. Previously, cervical research hypothesized the role of cervical smooth muscle cells (cSMCs) as secondary to uterine contractions with respect to contributing to reproductive function [33, 135]. Presently, the cSMCs are proposed to act in conjunction with the ECM throughout pregnancy to maintain to accomplish the dual role of maintaining the fetus within the uterus and assisting labor and safe passage of the fetus at delivery [20, 23]. Recent research further reports a relationship between ECM stiffness and cSMC contractile potential [135]. While cervical ECM remodeling throughout pregnancy is relatively well defined, cervical contractility remains relatively understudied and primarily investigated in mid- to late-pregnancy [20, 22, 23, 27, 64, 111, 169]. However, a need remains to investigate the contractile behavior of the cervix during throughout the entire time course of pregnancy to better understand cervical function at critical phases of remodeling [64].
Cervical contractile properties and passive mechanical properties during pregnancy were determined primarily uniaxially [20, 21, 23, 26]. However, the cervix is anisotropic and loaded multiaxially within the body. To overcome these limitations, biaxial testing provides necessary information on evolving active and passive tissue anisotropy by simultaneously assessing circumferential and axial properties. Further, biaxial inflation-extension is a useful tool implemented within vascular [53, 139, 151], vaginal [2, 3, 49], and nulliparous cervical [2, 50, 160] studies to assess biaxial contractility and passive mechanical properties.

Therefore, this study seeks to characterize biaxial maximum contraction and passive biomechanical changes during pregnancy and early postpartum in the murine cervix. Improved understanding of biaxial contractility and mechanics during pregnancy is expected to inform fundamental knowledge on cervical contractility and biaxial passive mechanics throughout gestation.

7.2 Methods:

7.2.1 Animal Care

A total of $n=30$ ($n=5$ per group) female CD-1 mice aged 8-12 weeks (Charles River, Houston, TX) were utilized for maximum biaxial contractility and passive mechanical testing protocols with Tulane University Institutional Animal Care and Use Committee (IACUC) approval. Nulliparous mice and mice at gestation days 12 (D12), 15 (D15), 18 (D18), and 24-36 hours postpartum (PP) were ordered from Charles River. NP mice at estrus were observed visually for estrus prior to mechanical testing [56]. For Day 6 (D6) gestation, nulliparous females were bred and housed overnight with male CD-1 mice and checked twice daily (07:00 and 19:00) for vaginal mucus plugs. Confirmation of
vaginal plug indicated Day 0 of gestation and the male removed from the breeding cage. Starting at D15 gestation animals were observed every 12 hours (07:00 and 19:00) for the presence of pups. Postpartum mice were collected 24 hours after confirmation of pups within the cage. Animals were housed in a 12 hour light/dark cycle and fed a normal chow diet.

7.2.2 Specimen Preparation

Mice were sacrificed (n=5 per group) via guillotine to conserve cSMC viability and function. Neonatal pups from the PP time point were decapitated with sharp scissors prior to dam euthanasia. The parous uterus was immediately removed with cuts inferior to the ovary and inferior to the lowest pup. The uterus was placed in 4°C Hank’s Balanced Saline Solution (HBSS) and cut vertically to expose the fetal pups. Fetal pups were decapitated with sharp scissors as a secondary form of euthanasia. Following, the cervix and vagina were isolated from the pelvic cavity and placed in a dish with 4°C HBSS. Utilizing methods from Chapter 4 and 5, the remaining uterine horn tissue was trimmed down below the bifurcation and the vagina shortened to inferior the external os [160]. Blunted instruments were used to dissect away connective and adipose tissue to ensure acceptable diameter video tracking. The use of non-blunted tools in dissections for D12-D18 created micro-tears in the tissue wall resulting in holes forming during mechanical testing. Blunted tools allowed for repeatable successful dissections and mechanical testing procedures. Connective tissues during pregnancy became highly vascularized and the pubic symphysis elongated with increasing gestation. The cervix was mounted within the biaxial inflation device (Danish MyoTechnologies, Aarhus, Denmark) on cannula with 6-0 silk suture. The
basin of the device was filled with 37°C Kreb’s Ringer Buffer (Kreb’s Buffer) and aerated with 95% O₂ and 5% CO₂ to maintain physiologic pH.

### 7.2.3 Biaxial Contractility and Passive Mechanics

**Preconditioning**

Removal of the fascial tetherings, uterine horns, and pubic symphysis during dissection resulted in retraction of the reproductive tissues. As a first estimation of the unloaded configuration, the tissue was stretched to a length in which the tissue did not appear in compression or tension and the organ pressurized to a tare pressure of 3.0 mmHg to prevent collapse of the organ at P = 0 mmHg [49, 50, 160]. A Nikon Eclipse TS100 inverted microscope (Nikon®, Melville, NY, U.S.A.) and digital calipers recorded the unloaded diameter at the tare pressure (P= 3.0 mmHg) and unloaded length, respectively.

In a prior study (Chapter 5), we determined the *in vivo* baseline (P= 9.0±3.0 mmHg) and maximum contractile pressure (P= 22.0±4.0 mmHg) of the nulliparous murine cervix [160]. Adapting those measurements and the protocol from Chapter 5, we utilized the baseline pressure as the mean physiologic pressure (P= 9.0 mmHg) and maximum *in vitro* pressure (P= 18.0 mmHg) as one standard deviation below the maximum contractile pressure to preserve cSMC viability [160]. Cervices were circumferentially preconditioned for 5 cycles (P= 0-18.0 mmHg) at the estimated unloaded length. Next, utilizing the assumption that there is a preferred homeostatic axial length of soft tissues, we determined an estimated physiologic (EP) length wherein the tissue preserved force over an increasing range of pressures (P= 0-18.0 mmHg) [2, 3, 42, 49, 50, 89]. At the EP length circumferential preconditioning cycles were repeated (P= 0-18.0 mmHg). Axial
preconditioning was performed by axially extending the tissue from -1% below EP length to 1% above EP length at 1/3 max pressure \((P = 6.0 \text{ mmHg})\) for 5 cycles \([2, 51]\). The unloaded length was redetermined and unloaded geometry recorded \([3]\). To acclimate or precondition the cSMCs to potassium chloride (KCl), cervixes were pressurized to the mean physiologic pressure \((P = 9.0 \text{ mmHg})\), axially extended until the force transducer reported \(F = 0 \text{ mN}\), and dosed with the optimal nulliparous KCl dose \((20 \text{ mM})\) for 5 minutes \([160]\). Following cSMC preconditioning to KCl, the Kreb’s Buffer was replaced and the tissue was equilibrated for an additional 5 minutes \([3, 53]\). Following, the tissue was extended to the EP length and equilibrated at the EP length and mean physiologic pressure for 10 minutes prior to the maximum contractility protocol \([2, 49]\).

**Biaxial Maximum Contractility**

To determine the role of pressures and axial loading on the parous murine cervix, samples underwent a maximum contractility protocol with nine combinations of physiologic lengths and pressures (Table 7.1) \([53, 160]\). Utilizing a random number generator \((1-9)\), the combination of length \((-1\% \text{ EP length, EP length, } +1\% \text{ EP length})\) and pressure \((\text{mean } \pm \text{ standard deviation of the physiologic pressure; } P = 9.0 \pm 3.0 \text{ mmHg})\) was selected. At the specified length and pressure, 20mM of KCl injected into the bath induced contractility. Changes in diameter and force with contraction were measured for 5 minutes with a camera-based tracking software and an axial force transducer, respectively. Following the 5 minutes of data collection, the Kreb’s Buffer in the basin was replaced with fresh buffer and the tissue rested for an additional 5 minutes. Additionally, 5 minute resting periods were implemented with each change in pressure or axial-length \([53]\). Upon completion of the contractility protocol, the cervix was returned to the unloaded geometry
and ultrasound B-mode images (Vevo2100; 40MHz transducer) of cervical thickness were recorded [2, 3]. The cervix was returned to the EP length and tare pressure following imaging.

Table 7.1: Length-Pressure Maximum Contractility Combinations

<table>
<thead>
<tr>
<th>Axial-Extension</th>
<th>Mean Non-pregnant Physiologic Pressures (P= 9.00±3.00 mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Below EP Length</td>
<td>Below P= 6.0 mmHg</td>
</tr>
<tr>
<td></td>
<td>Mean P= 9.0 mmHg</td>
</tr>
<tr>
<td></td>
<td>Above P= 12.0 mmHg</td>
</tr>
<tr>
<td>EP Length</td>
<td>Below P= 6.0 mmHg</td>
</tr>
<tr>
<td></td>
<td>Mean P= 9.0 mmHg</td>
</tr>
<tr>
<td></td>
<td>Above P= 12.0 mmHg</td>
</tr>
<tr>
<td>1% Above EP Length</td>
<td>Below P= 6.0 mmHg</td>
</tr>
<tr>
<td></td>
<td>Mean P= 9.0 mmHg</td>
</tr>
<tr>
<td></td>
<td>Above P= 12.0 mmHg</td>
</tr>
</tbody>
</table>

Biaxial Passive Mechanics

To deactivate the cSMC contractile mechanism, the sample was bathed in 37°C calcium-free Kreb’s Buffer and 2mM egtazic acid (EGTA) mixed into the basin for 30 minutes to remove calcium from the cells. Calcium-free Kreb’s Buffer was replenished and the EP length was considered the estimated unloaded length for the passive experiment. Unloaded diameter and length were recorded and the tissue underwent circumferential preconditioning (P= 0-18.0 mmHg). The passive EP length was determined utilizing the methods in the Preconditioning section [89]. Following, pressure-inflation (P= 0-18.0 mmHg) at the EP length and force-elongation (±1% EP length) at 1/3 max pressure (P= 6.0 mmHg) were performed [2, 49, 50]. The unloaded geometry was redetermined for the passive state and the sample equilibrated at the EP length and mean physiologic pressure (P= 9.0 mmHg) for 10 minutes. The cervices underwent three cycles of pressure-inflation.
tests at static lengths (−1% EP length, EP length, and +1% EP length) over the physiologic pressure range (P = 0-18.0 mmHg). Next, 3 cycles of force-elongation protocols were performed where the sample was axially stretched ±1% EP length at four static pressures corresponding with a tare pressure, 1/3 max pressure, 2/3 max pressure, and max pressure (P= 3.0, 6.0, 12.0, 18.0 mmHg) [2, 49, 50, 160]. The cervix was returned to the passive unloaded geometry and transverse 2D ultrasound B-mode images taken for thickness analysis.

7.2.4 Data Analysis

**Thickness, Cross-Sectional Area, and Volume Calculations**

The inner canal and outer circumference of the murine cervix were traced from ultrasound images imported into ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.). Unloaded thickness (H) for the cervical samples was calculated as the mean length between the lumen and outer wall. The unloaded cross-sectional area (A) and volume (V) were calculated using the unloaded geometry in **Equations 1 and 2**:  

\[ A = \pi (R^2_o - R^2_i) \]  

\[ V = \pi (R^2_o - R^2_i) L \],

where \( R_o \) is the undeformed outer radii, \( R_i \) the undeformed inner radii, and \( L \) the unloaded length.

**Contractility**

Biaxial maximum contractility was measured by changes in force and diameter for axial and circumferential contractions, respectively. Wavelength was measured as the
time from trough to trough and frequency measured as the inverse of time from peak to peak.

**Active and Passive Stress**

Unloaded geometry used for the reference configuration of the active and passive state. The deformed mid-wall radius \( r_{\text{mid}} \) and unloaded mid-wall radius \( R_{\text{mid}} \) were utilized to calculate circumferential stretch \( (\lambda_{\theta}) \) (Eq. 3):

\[
\lambda_{\theta} = \frac{r_{\text{mid}}}{R_{\text{mid}}}.
\]  

(3)

Utilizing the deformed length \( l \) and undeformed length \( L \) the axial stretch \( (\lambda_{z}) \) was calculated using **Equation 4:**

\[
\lambda_{z} = \frac{l}{L}.
\]  

(4)

1\textsuperscript{st} Piola-Kirchhoff circumferential \( (T_{\theta\theta}) \) and axial \( (T_{zz}) \) stresses were determined for the contracted and passive state \((\text{Eq. 5, 6})\) \([52, 150]\).

\[
T_{\theta\theta} = \frac{P r_{i}'}{\lambda_{\theta}(r_{o}-r_{i})'},
\]  

(5)

\[
T_{zz} = \frac{F_{r}}{\lambda_{z}(r_{o}-r_{i})(r_{o}+r_{i})'} + \frac{P r_{i}^2}{\lambda_{z}(r_{o}-r_{i})(r_{o}+r_{i})'},
\]  

(6)

wherein \( P \) is intraluminal pressure, \( r_{i} = \sqrt{r_{o}^2 - \frac{A}{\pi \lambda_{z}}} \) is the deformed inner radius, \( r_{o} \) is the deformed outer radius, and \( F_{r} \) is the force from the axial force transducer \([2, 42, 150]\). Active contractile stress contribution was calculated as the change in stress \((\Delta T)\) between the contracted \((T_{\text{contracted}})\) and passive states \((T_{\text{passive}})\) at matching pressures and axial-extensions \((\text{Eq. 7, 8})\) \([3, 53, 150-152]\).
\[ \Delta T_{\theta\theta} = T_{\theta\theta}^{\text{contracted}} - T_{\theta\theta}^{\text{passive}} \]  

(6)

\[ \Delta T_{zz} = T_{zz}^{\text{contracted}} - T_{zz}^{\text{passive}} \]  

(7)

where \( \Delta T_{\theta\theta} \) and \( \Delta T_{zz} \) are the active circumferential and axial stresses, respectively.

**Material Stiffness and Compliance**

Circumferential and axial material stiffness values were calculated as the slope of the stress-stretch curves at circumferential stretches corresponding with the range of the mean physiologic pressure (\( P = 9.0 \pm 3.0 \) mmHg). Compliance of the cervix was determined as the change in diameter over the change in pressure over the range of the mean physiologic pressures (\( P = 9.0 \pm 3.0 \) mmHg) (Eq. 8)

\[ C = \frac{OD_{12 \text{mmHg}} - OD_{6 \text{mmHg}}}{P_{12 \text{mmHg}} - P_{6 \text{mmHg}}} \]  

(8)

where OD is the outer diameter at the corresponding pressures ±SD.

**7.2.5 Statistics**

Two-way ANOVA (Time, State) were utilized to determine differences in geometry with respect to time point and state. Three-way ANOVAs (Time, Axial-Stretch, Pressure) were utilized to determine differences in circumferential contractility, axial contractility, wavelength, and frequency. Tukey posthoc tests were performed when appropriate. Passive mechanical properties (material stiffness and compliance) were determined not to be normally distributed. Therefore, Friedman Rank Sum Tests (Time, Axial-Extension) were utilized to determine differences with Nemenyi posthoc tests utilized where appropriate.
7.3 Results

Results reported herein are represented as mean ± standard error of the mean.

7.3.1 Geometry

Figure 7.1: Geometry recorded for the nulliparous (NP; white with closed circles), Day 6 (D6; light gray with squares), Day 12 (D12; dark gray with triangles), Day 15 (D15; white with squares), Day 18 (D18; light gray with triangles), and postpartum (PP; dark gray with circles) time points (n=5 per group). (A) Unloaded outer diameter increased significantly at D18 compared to NP, D6, D12, and PP time points. (B) Unloaded length increased significantly at D18 compared to NP, D6, and D12. Additionally, length increased at D15 compared to D6. (C) Thickness increased significantly at the PP time point compared to D6, D12, D15, and D18. However, thickness did not change with increasing gestation compared to the NP time point. (D) Volume increased significantly at D18 compared to the NP time point and D6 gestation. Significance values are denoted by: *p<0.05; **p<0.01; ***p<0.001.
Two-way ANOVA revealed significant differences with time ($p<0.001$) and state ($p<0.001$). Tukey posthoc reported a significant increase ($p<0.05$) in passive diameter. Additionally, D18 diameter increased significantly compared to NP, D6, D12, and PP (Fig. 7.1A). Two-way ANOVA reported significant differences ($p<0.001$) in thickness and length with respect to time. PP thickness increased significantly ($p<0.01$) compared to D6, D12, D15, and D18 gestation (Fig. 7.1B). Unloaded length significantly increased ($p<0.05$) at D18 compared to NP, D6, and D12. Additionally, length increased ($p<0.05$) at D15 compared to D6 (Fig. 7.1C). Lastly, two-way ANOVA reported significant differences with respect to time ($p<0.001$) and state ($p<0.001$) for volume. Volume increased significantly ($p<0.05$) at the passive state and at D18 compared to NP and D6 ($p<0.001$) (Fig. 7.1D).

### 7.3.2 Biaxial Contractility

All samples exhibited spontaneous phasic contractions throughout the active protocols. Three-way ANOVA (Time, Axial-Extension, Pressure) determined significant differences in circumferential and axial contractility with time ($p<0.001$) and circumferential contractility with pressure ($p<0.001$). Circumferential contraction decreased significantly ($p<0.001$) at the highest pressure (P= 12.0 mmHg) compared to the lowest pressure (P= 6.0 mmHg). Change in diameter with maximum decreased significantly ($p<0.01$) at D15 and D18 compared to NP, D6, D12, and PP (Fig. 7.2A). Change in force with contraction decreased ($p<0.001$) from NP to early pregnancy time points (D6, D12). Axial contractility increased ($p<0.001$) at D15-PP compared to D6 and D12. Additionally, axial force increased in PP compared to the NP state (Fig. 7.2B).
Wavelength and frequency differed significantly (\(p<0.001\)) with increasing gestation and postpartum. Wavelength decreased (\(p<0.05\)) from NP to D12 to D15 and postpartum induced a significant increase in wavelength compared to all time points. Frequency increased from the NP and D6 time points from D12 through D18. However, PP frequency significantly decreased compared to all pregnancy time points.

**Figure 7.2:** Biaxial maximum contractile behavior of the murine cervix through gestation and postpartum (\(n=5\) per group) at the estimated physiologic (EP) length and mean physiologic pressure (\(P = 9.0\) mmHg) with significance indicated by: \(p<0.05\); \(p<0.01\); \(p<0.001\). Where the groups are represented by nulliparous (NP; white with closed circles), Day 6 (D6; light gray with squares), Day 12 (D12; dark gray with triangles), Day 15 (D15; white with squares), Day 18 (D18; light gray with triangles), and postpartum (PP; dark gray with circles). (A) Maximum circumferential contraction induced a significantly greater total change in outer diameter at D15 and D18 compared to NP, D6, D12, and PP. (B) Maximum axial contraction significantly decreased at D6 and D12 compared to NP followed by a significant increase at D15, D18, and PP compared to early and mid-pregnancy time points (D6, D12). (C) The wavelength of contractions decreased from NP to D12 and D15. However, postpartum induced a significant increase in wavelength compared to all time points. (D) Frequency increased from the NP and D6 time points from D12 through D18. However, PP frequency significantly decreased compared to all pregnancy time points.
increased \( (p<0.001) \) from all time points compared to PP (Fig. 7.2C). Frequency of contractions increased \( (p<0.05) \) from NP and early-pregnancy (D6) to D12-D18. Additionally, PP frequency decreased significantly \( (p<0.001) \) compared to all pregnancy time points, however, the NP group did not differ significantly (Fig. 7.2D).

**Figure 7.3:** Changes in circumferential \( (\Delta T_{\theta \theta}) \) and axial \( (\Delta T_{zz}) \) stress representing the active cSMC contribution for each time point wherein nulliparous (NP) is represented by white with close circles, Day 6 (D6) by light gray with squares, Day 12 (D12) by dark gray and triangles, Day 15 (D15) by white with squares, Day 18 (D18) light gray with triangles, and postpartum (PP) by dark gray with circles. (A) Active circumferential stress \( (\Delta T_{\theta \theta}) \) decreased significantly at D15 compared to NP, early to mid-pregnancy (D6, D12), and PP. Additionally, \( \Delta T_{\theta \theta} \) at D6 increased significantly against D18. (B) Axial stress \( (\Delta T_{zz}) \) decreased from the NP state throughout pregnancy followed by a significant increase at PP where NP and PP axial stress exceeded D12, D15, and D18 axial stress. Additionally, D15 and D18 decreased significantly compared to D6. Where significance is denoted by: \( p<0.05^* \), \( p<0.01^{**} \), and \( p<0.001^{***} \).

Three-way ANOVA (Time, Axial-Extension, Pressure) determined significant differences with time \( (p<0.001) \) for the total change in circumferential \( (\Delta T_{\theta \theta}) \) and axial \( (\Delta T_{zz}) \) stress (Fig. 7.3). \( \Delta T_{\theta \theta} \) at D15 decreased significantly \( (p<0.001) \) compared to NP, D6, D12, and PP. Additionally, \( \Delta T_{\theta \theta} \) at D15 decreased \( (p<0.01) \) compared to D6. Contrastingly, NP and PP \( \Delta T_{zz} \) increased \( (p<0.01) \) compared to D12, D15, and D18. \( \Delta T_{zz} \) decreased \( (p<0.05) \) from D15-D18 compared to D6. Total change in stress differed with respect to circumferential and axial loading directions. In the NP and PP state axial stress
increased ($p<0.05$) compared to circumferential stress (Fig. 7.4A, 4F). However, axial stress decreased ($p<0.05$) compared to circumferential stress in D15 and D18 (Fig. 7.4D, 7.4E).

**Figure 7.4:** Absolute active change in stress with respect to loading direction at the estimated physiologic (EP) length and mean physiologic pressure for each time point ($n=5$ per group). Where circumferential loading direction is represented by white with closed circles and axial direction by gray with squares. Significance values are represented by: $p<0.05^*$; $p<0.01^{**}$; $p<0.001^{***}$. (A) Nulliparous axial stress increased significantly compared to the circumferential stress. (B, C) In D6 and D12 the axial and circumferential stretch did not reach significance. (D, E) However, late pregnancy time points (D15, D18) resulted in a significant increase in circumferential stress compared to axial. (F) Similar to the nulliparous state, PP axial stress exceeded circumferential stress.
### 7.3.3 Biaxial Passive Mechanics

Friedman rank sum test determined no significant differences \((p>0.05)\) with circumferential material stiffness (Fig. 7.5). However, axial material stiffness and compliance differed with respect to time points (Fig. 7.5). Axial stiffness increased significantly \((p<0.05)\) at D18 compared to NP. Compliance increased significantly \((p<0.05)\) at D18 compared to PP. Further, circumferential stiffness and stress increased compared to axial stiffness and stress for all time points (Fig. 7.5, 7.6).

**Figure 7.5:** Passive material stiffness and compliance at the estimated physiologic (EP) length for each group \((n=5\) per group). Where nulliparous (NP) is represented by white with close circles, Day 6 (D6) by light gray with squares, Day 12 (D12) by dark gray and triangles, Day 15 (D15) by white with squares, Day 18 (D18) light gray with triangles, and postpartum (PP) by dark gray with circles. (A) Passive circumferential material stiffness did not significantly differ with time point. Circumferential stiffness exceeded axial stiffness for all time points. (B) Passive axial material stiffness significantly increased at D18 compared to the NP state. (C) Compliance decreased at D18 compared to PP, however, no other groups achieved significance. Significance values are denoted by: \(p<0.05\); \(p<0.01\); \(p<0.001\).
Herein, this Chapter described for the first time the biaxial maximum contractility and passive mechanical properties of the murine cervix in a physiologically relevant loading environment through pregnancy and early postpartum.

Throughout pregnancy the cervix must remain stiff and closed to maintain the fetus yet soften and dilate prior to delivery for safe passage of the fetus [16, 24-26]. To perform
these disparate functions, the cervix undergoes dramatic remodeling of cSMC and ECM constituents [16, 24-26, 64]. In the 1940s, research proposed a minimal or secondary role of the cSMCs in cervical function due to the high collagen content and relatively low cSMC content [33]. Over the following years, research sought to characterize the contribution of collagen to cervical function with minimal focus on the mechanical role of cSMC [21, 28, 36, 38, 64, 73, 74, 102, 111]. Current research, however, highlights the independent contractile function of the cervix from the rest of the reproductive tract and emphasizes the importance of ECM-cSMC relationships [19, 20, 22, 40, 135]. Previous work primarily utilized uniaxial methods to determine contractility and mechanics of the cervix [19-23, 73, 76, 111]. Uniaxial testing methods are limited to independently assess direction dependent properties without accounting for tissue anisotropy [20, 22, 40]. Adapting and utilizing biaxial inflation-extension contractility and passive mechanical protocols for the parous murine cervix provided a tool to elucidate the evolving multiaxial cervical contractility and passive biomechanics through pregnancy [3, 53, 151, 160].

Biaxial maximum contractility protocols revealed a significant increase in circumferential contractility at D15 ($p<0.001$) and D18 ($p<0.01$) compared to the NP state, early- to mid-pregnancy (D6, D12), and PP (Fig. 7.2). Although PP circumferential contractility decreased significantly compared to D15 ($p<0.001$) and D18 ($p<0.01$), axial contractility did not significantly differ from late pregnancy and significantly ($p<0.05$) increased compared to NP, D6, and D12. Interestingly, wavelength increased and frequency decreased significantly at PP compared to gestational time points (Fig 7.2). Potentially, the postpartum involution process requires significantly greater axial contractions to assist the reproductive tissues to return to nonpregnant geometry as the
collagen and ECM constituents remodel [21, 28, 64, 75, 170]. Additionally, axial contractility significantly decreased from the nonpregnant state through D12 ($p<0.001$) followed by a significant increase in force at D15 and D18. Early to mid-pregnancy may experience a decrease in contractile potential prior to preparation of labor as a function of maintaining the fetus. In pregnant rats on gestation day 9-13, the cervix exhibited decreased responsiveness to oxytocin, a contractile agonist that promotes labor [22].

Absolute circumferential active stress contribution to cervical mechanical function increased significantly at D15 and D18 (Fig 7.3, 7.4). Increased circumferential cSMC contribution compared to axial contribution in late pregnancy may be indicative of cervical ripening and preparation for dilation and shortening prior to labor [20]. Alternatively, increased load of the fetal pups and circumferential tension on the cervix may require greater circumferential contractile compensation to maintain the pups within the uterus [6]. Further, active anisotropy in the murine cervix altered with increasing gestation (Fig. 7.6). In the NP and PP state active axial stress increased ($p<0.05$) compared to the circumferential stress, however, D15 and D18 demonstrated an inverse in circumferential and axial stresses (Fig 7.6). Interestingly, passive circumferential stress exceeded passive axial stress at all time points. Vink et al. proposed a sphincter-like function of the cervix which may be represented by the increase in circumferential stress at later pregnancy time points [19, 135]. Interestingly, a decrease in axial stress compared to circumferential stress in pregnancy may play a role in the progression of CI. Maladaptive remodeling may exacerbate the decrease in axial active contribution and result in cervical shortening and effacement [11, 135].
Circumferential material stiffness did not significantly differ with increasing gestational age or postpartum. Axial stiffness increased at D18 compared to the nulliparous group and compliance increased at D18 compared to PP (Fig. 7.5). Comparatively, prior uniaxial studies reported increased stiffness at D6 and a subsequent loss of stiffness through D18 in the murine cervix [21, 64]. Potentially, material stiffness and compliance calculations made around the mean physiologic pressure of the nulliparous murine cervix may not recapitulate the in vivo loading environment for all time points.

**Figure 7.7:** Relative changes (not to scale) in estradiol (blue) and progesterone (pink) compared to the relative changes in compliance (green) and contractile force with maximum contraction (gray) with respect to the nulliparous states (NP), postpartum (PP), and Day 6 (D6), Day 12 (D12), Day 15 (D15), and Day 18 (D18) gestation. Wherein D12 corresponds with the first phase of cervical remodeling, softening, and D18 corresponds to the ripening and dilation phases. Interestingly, compliance and estradiol peak at D18 gestation and contractile force plateaus at D15 gestation correlating with a spike in progesterone. In the PP time point compliance decreased with decreasing levels of steroid hormones, however, contractile force remained elevated compared to the NP state. Estradiol and progesterone levels adapted from Nallasamy et al. [64].

Additionally, data variability and distribution in the passive mechanics samples may be a
result of biological variability in an outbred strain. Power analysis reports a sample size of \( n=13 \) per group to determine significance between D6 and late gestation for circumferential and axial material stiffness. However, remaining time points require greater than \( n=20 \) samples per group to detect significance. Sample size for compliance averages \( n=15 \) per group to determine significance between NP and gestational time points, D6 and late gestation, and D12 against PP. Interestingly, compliance peaked at D18 corresponding with peaks in estradiol during pregnancy and diminished in PP with decreases in estrogen and progesterone (Fig. 7.7). Additionally, contractile force increased significantly at D15 corresponding with peaks in progesterone (Fig. 7.7). However, contractile force remained elevated in PP suggesting that steroid hormones alone do not drive the change in smooth muscle machinery during pregnancy and postpartum.

In summary, the Chapter determined the evolving biaxial maximum contractility and passive mechanics of the murine cervix through gestation and early postpartum. This study presents an independent multiaxial contribution of the active cSMC to cervical function throughout pregnancy. Understanding the role of cervical smooth muscle cell contractility and passive mechanics through pregnancy will inform mechanisms of normal physiology and potential etiologies of CI, as well as aid in the development of diagnostic imaging tools and treatments.

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Chapter 8: Future Directions

8.1 The Role of Elastic Fibers on Cervical Contractility and Passive Mechanics through Pregnancy

The ability of the cervix to remodel in response to mechanical loading and hormones is critical for function during pregnancy and postpartum [5, 12, 26, 111]. Mechanical factors coupled with genetic factors such as connective tissue disorders can accelerate disease progression [42, 43, 53, 97, 156, 157]. Specifically, elastic fiber deficiency is a theorized factor for maladaptive cervical remodeling during pregnancy and postpartum [11, 13]. Therefore, elastic fiber deficiency coupled with load from the fetus during pregnancy may induce maladaptive remodeling of the cervix. Additionally, elastic fibers may interact with smooth muscle cells and a balanced relationship preserves contractile function [158]. However, there is a need to elucidate the exact role of elastic fibers in contractility and passive mechanical properties of the cervix through pregnancy. Therefore, I propose a project utilizing pregnant Fibulin-5 wildtype (WT) and haploinsufficient (HET) mice to determine the role of elastic fibers in cervical contractility, basal mechanics, and passive mechanics through pregnancy. I hypothesize that the elastic fiber deficiency in the HET genotype will become exacerbated with the mechanical loads of pregnancy resulting in decreased cervical contractility and increased passive stiffness with respect to the wildtype.

With Tulane University Institutional Care and Use Committee approval, WT and HET female mice will be bred and sacrificed at the estrus phase in the nulliparous mouse and Days 6, 12, 15, and 18 gestation. Using methods from Chapters 6 and 7, cervixes from the WT and HET animals will undergo a biaxial maximum contractility protocol at
the estimated physiologic length, a basal mechanical testing protocol, and a passive mechanical testing experiments [3, 50]. After mechanical testing, specimens will be prepared for second harmonic generation (SHG) imaging of collagen and elastic fibers [3, 64]. SHG imaging can be analyzed to calculate fiber angles within the layers of the cervix [3, 24]. An appropriate strain energy function will be informed by incorporating the collagen and elastin fiber orientations with the mechanical properties and of the cervix through pregnancy [52]. If successful, this study would fulfill a need to describe the role of elastic fibers within the cervix during pregnancy. Additionally, this study would provide a foundational step for growth and remodeling models of the murine cervix through pregnancy. Future experiments could incorporate ultrasound imaging of \textit{in vivo} cervical geometry at matching time points to inform a 3D finite element model of the cervix in pregnancy [6, 171].

8.2 The Effect of Maternal Obesity on Cervical Contractility and Passive Mechanics

Obesity is a global health condition defined as body mass index (BMI) $\geq 30$ kg/m$^2$ with increased risk for co-morbidities such as hypertension, Type 2 diabetes, heart disease, osteoarthritis, and cancer [172-174]. With growing trends in obesity, the prevalence of overweight and obese pregnancies is on the rise [175]. Pre-pregnancy obesity is linked to higher risks of maternal health complications such as preeclampsia, gestational diabetes, and gestational hypertension [175, 176]. Interestingly, the effect of obesity on preterm birth (PTB), specifically spontaneous PTB, remains debated. Many studies report obesity as a protective mechanism against spontaneous PTB, however, rates of medically indicated PTB is greater in obese mothers [177, 178]. Further, research suggests that high body mass
index (BMI $\geq 30$ kg/m$^2$) is linked to longer term pregnancies, slower rates of cervical ripening in spontaneous and induced labor, and a greater dose requirement of oxytocin during labor induction [179, 180]. Adipose is an endocrine tissue associated with increased levels of circulating steroid hormones such as estradiol in obesity [181]. The increased levels of estrogen could alter the balance of estrogen and progesterone in pregnancy resulting in an imbalance of normal biochemical progression of pregnancy in preparation of labor [182]. Additionally, reproductive tissues are sensitive to changes in steroid hormones with observed changes in microstructural contents and smooth muscle cell activity through the estrous cycle and pregnancy [18, 40, 61, 62]. The increased levels of estradiol may lead to dysfunction of the cSMC during the dilation and ripening phases. Adiponectin or leptin, hormones produced by adipose tissue, may also play a role in altering contractile potential in smooth muscle cells. Increasing doses of leptin induced lower myometrial contractility in vitro and adiponectin decreased murine vascular tone [183, 184]. Potentially, prolonged labor and cervical ripening with maternal BMI may be related to a loss of cSMC function. Therefore, I propose a project that investigates the role of obesity on the in vivo and in vitro contractile behavior of the cervix and hypothesize that obesity will decrease cSMC contractile potential.

Firstly, I propose the use of a mouse model for obesity wherein the C57BL6 mice on a normal chow and high fat diet (HFD) will be utilized. C57BL6 mice are considered the best strain for investigating metabolic diseases, develop insulin resistance, and gain body mass on a high fat diet [185]. Firstly, with Tulane University IACUC approval, nulliparous mice from the control and HFD group will be anesthetized and undergo the intracervical pressure measurement protocols outlined in Chapters 4, 5, and 6 [140, 160].
At the following estrus phase, the mice will be euthanized and a maximum contractility followed by a passive mechanical testing protocol performed. A separate cohort of control and HFD mice will be bred and utilized for *in vivo* catheter experiments at Day 6, Day 12, Day 15, and Day 18 gestation [140]. Research suggests that obesity induced a proinflammatory effect in early- to mid-pregnancy, however, inflammatory upregulation resolved in late pregnancy [186]. This suggests the importance to investigate contractile behavior in early- to mid-pregnancy (Day 6 and Day 12). Animals will be immediately sacrificed to prevent abortion of the fetal pups and the tissues harvested and randomly allocated for biochemical assays and immunohistochemistry. An additional cohort of control and HFD animals at Days 6, 12, 15, and 18 gestation will be utilized for biaxial maximum contractility and passive mechanical testing protocols of the cervix [3, 160]. Following mechanical testing, samples will be allocated into immunohistochemistry and biochemical assay groups. Utilizing immunohistochemistry, the smooth muscle cells would be targeted αSMA and fibroblasts with Vimentin. Additionally, qPCR will be utilized to determined upregulation of collagen type I, collagen type III, elastin, inflammatory factors such as interleukin-6 and prostaglandins [180, 186, 187]. Upon completion, I expect an increase in *in vivo* baseline pressure, a decrease in *in vivo* maximum contractile pressure, and a decrease in contractile frequency within the HFD/obese group. Further, a decrease in maximum circumferential and axial contractility within the obese group for all time points is expected. Frequency of contractions and maximum generated force *in vitro* at D18 is expected to decrease in the HFD group. With respect to biochemical assays, I expect an increase of inflammatory factors in early pregnancy (Day 6) for the HFD group followed by a homeostasis at later pregnancy time points. Further, I expect an
increase in αSMA positive cells and a decrease in Vimentin positive cells in the control group. If successful, this study would be the first, to the author’s knowledge, to describe in vivo contractility as well as biaxial maximum contractility and passive mechanics in the murine cervix. This study may elucidate the factors prescribing the “protective mechanism” for spontaneous PTB and insights into prolonged cervical ripening in obese patients [177, 179].
Appendix


Materials:
1) Absorbent Pad
2) Tools:
   a) Forceps
   b) Scissors
   c) Micro-Scissors
   d) Angled Tweezers
   e) Straight Tweezers
3) Aluminum Foil
4) Tape
5) Tex Wipes
6) Petri Dish
7) Syringe
8) Hank’s Buffered Saline Solution (HBSS)
9) Permanent Marker
10) Gloves
11) India ink

Set Up:
*Everything from this point forward should be done with gloves on*
1) Place diaper on worktop under microscope
2) Fill petri dish halfway with HBSS solution
3) Fill syringe with HBSS solution
4) Set aside Tex Wipe for tissue disposal
5) Place mouse on sheet of foil ventral side up, taping the front and back paws and tail to the foil
6) Place mouse and foil under the microscope on top of the absorbent pad
7) Turn microscope lights on and adjust microscope to appropriate magnification

Methods:
1) Using tweezers and scissors, lift skin and make incision at base of the abdomen.
   a) Be careful not to puncture intestines; you will have to tie off the punctured intestine with a suture and wash the cavity thoroughly with saline
2) Cut superiorly to just below rib cage, then cut laterally from the top of the original incision to the lateral most part of abdomen
3) Cut laterally from base of original incision to the lateral most part of abdomen
4) Carefully move the intestines out of cavity by lifting from below
   a) Be careful not to cut intestines
5) Under the microscope, remove superficial fat by pulling lightly on the fat with angled tweezer and cutting away excess with micro-scissors.
6) Place all removed tissue on designated Tex Wipe
7) Make sure that each uterine horn is visible.
8) Remove superficial fat and muscle so that pubic bone is visible
9) Place closed scissors between vagina and pubic bone (the pubic symphysis) and carefully separate the tissue and bone
   a) Make sure that scissors are visible on inferior side of pubic bone
10) Cut pubic symphysis
11) Cut away at the protruding bone at least to the outer edges of the vagina to allow better access.
12) Start removing the bladder and urethra from the ventral side of the vagina using tweezers and micro-scissors as a dissection method to separate the two.
   a) Hold bladder with forceps to create tension.
   b) Aim sharp part of forceps toward urethra in order to protect vagina.
13) Once the bladder is completely dissected away, cut base and remove from cavity.
14) Using angled tweezers and micro-scissors as necessary remove the urethra and clitoris
   a) The clitoris can be determined by the black grainy texture the tissue will take on as the dissections moves inferior from the urethra
   b) Be careful not to remove tissue from the vaginal introitus
15) Once entire reproductive system is visible get out india ink to mark 3mm dots.
16) Using the calipers measure a 3 mm piece of suture under the microscope
17) Using the 3mm piece of suture as a measuring tool, the india ink, and the angled tweezers, place dots on each uteri, spaced 3 mm apart, leaving a little room between the ovary and uterus.
   a) Place as many dots as necessary to reach cervix.
      1) Located where the uteri begin to bifurcate
18) Figure 4 below shows the path of the dots.
   a) Go from end of one uterus to center of cervix then use the center cervix dot to start dot path up other uterus.
   b) Finally use center cervix dot to start dot path down vagina to introitus.
19) Allow india ink to dry then begin separating repro tract from surrounding fat and tissue.
20) Using micro-scissors with the angled tweezers, separate the uterine horns and cervix from the surrounding connective tissue.
21) Carefully separate the cervix and vagina from the colon
22) Clean the vagina as close to the vaginal introitus as possible
23) Using micro-scissors cut around the vaginal introitus
   a) Some hair and skin remaining around the external portion of the vagina is acceptable
24) Cut the uterine horns above the bifurcation from the cervix/vagina
25) Place dissected tissue in petri dish with HBSS solution
26) Estimate In Vivo Stretch
   a) Write down the lengths of each organ based on previous measurements
   b) Wait 15 minutes post explant and re-measure space between dots using caliper.
   c) Record measurements for each dot distance. Enter values into excel spreadsheet.
Clean-up:
1) Fill large beaker with soap and warm water, place tools in bath and let sit for 5 minutes.
   a) Clean and dry tools thoroughly
2) Place Tex Wipe over dissected mouse
3) Fold aluminum foil over mouse and all removed tissue.
4) Place wrapped mouse in ziploc bag-label parts removed and date of dissection.

Figure 5: Pubic symphysis cut away with bladder and urethra still attached. Figure from Abramowitch et al. 2009 [1].
A.2 Cervix and Uterus Passive Test Protocol

**Find Unloaded Geometry**

1. Unloaded Length ($l^{\text{unloaded}}$)
   a. Look for length at which vessel appears very buckled/collapsed
      Turn on the pressure and set equal to 0 mmHg
   b. Record unloaded length from suture to suture
   c. Record the diameter at 0 mmHg

**CIRCUMFERENTIAL PRECONDITIONING 1 (Pressure-Inflation)**

1. Pressure should be set to 0 mmHg
2. Length should be at the estimated physiologic (EP) length
3. Set gradient to 2.0 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 200 mmHg and back down to 0 mmHg again
4. Repeat sequence for 5 cycles.
5. CREATE NEW FILE FOR PD PRECONDITIONING NAMED “DATE-CER-PDPC1”

**Axial PRECONDITIONING 1 (Force-Extension)**

1. Enter 1/3 max pressure (67 mmHg) in pressure window for P1 and P2
2. Press “Start” in lower right corner of Myoview software interface
3. Use axial length knob to adjust vessel to from 2% below the EP length to 2% above) the EP length at 10 microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
4. Once 5 cycles have been completed press “stop” button in lower right-hand corner
5. CREATE NEW FILE FOR FL PRECONDITIONING NAMED “DATE-CER-FLPC1”

**Finding Estimated Physiologic (EP) Stretch**

1. Adjust tissue to estimated EP length ($L = 6.0$ mm)
2. Press “Start” in lower right corner of Myoview software interface to record
3. In Myoview software interface assess pressure vs force values about the EP length from 0 to 200 mmHg
4. The experimentally determined EP stretch ($\lambda_{z,exp}^E$) will be the stretch value that displays a mostly flat line of force values over a range of pressures (i.e. constant force over a range of pressures)
5. If increasing the stretch only results in near constant slopes at increasing levels of force and the tissue exhibits extreme stress relaxation, the EP stretch has been greatly overestimated—take the tissue back down and start again

**Circumferential PRECONDITIONING 2 (Pressure-Inflation)**

1. Pressure should be set to 0 mmHg
2. Length should be at the estimated EP length
3. Press “Start” in lower right corner of Myoview software
4. Set gradient to 2 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 200 mmHg and back down to 0 mmHg again
5. Repeat sequence for 5 cycles.
6. Press “stop” button in lower right-hand corner and CREATE NEW FILE FOR P-D PRECONDITIONING NAMED “DATE-CER-PDPC2”

Axial PRECONDITIONING 2 (Force-Elongation)
6. Enter 1/3 max pressure (67 mmHg) in pressure window for P1 and P2
7. Press “Start” in lower right corner of Myoview software interface
8. Use axial length knob to adjust vessel to from 2% below the EP length to 2% above) the EP length at 10 microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
6. Once 5 cycles have been completed press “stop” button in lower right-hand corner
7. CREATE NEW FILE FOR FL PRECONDITIONING NAMED “DATE-CER-FLPC2”

Equilibrate
1. Set tissue to EP length and pressure (10 mmHg) for 10 minutes

PRESSURE-INFLATION TESTING
1. 2% below EP length
   a. Make sure vessel is at the 2% below the experimentally determined EP length
   b. Make sure pressure is at 0 mmHg
   c. Press “Start” in lower right corner of Myoview software interface
   d. Increase pressure from 0 mmHg to 200 mmHg and back to 0 mmHg using sequencer
      i. 5 mmHg increments for 2s
      ii. Hold the 5-0 mmHg step for 10 secs to ensure the pressure actually reaches 0 mmHg
   e. Repeat sequence for 3 cycles
   f. Once 5 cycles have been completed press “stop” button in lower right-hand corner
      i. CREATE NEW FILE NAMED “DATE-CER-PD1”
2. EP length
   a. Make sure vessel is at the experimentally determined basal EP length
   b. Make sure pressure is at 0 mmHg
   c. Press “Start” in lower right corner of Myoview software interface
   d. Increase pressure from 0 mmHg to 200 mmHg and back to 0 mmHg using sequencer
      i. 5 mmHg increments for 2s
ii. Hold the 5-0 mmHg step for 10 sec to ensure the pressure actually reaches 0 mmHg

e. Repeat sequence for 3 cycles

f. Once 5 cycles have been completed press “stop” button in lower right-hand corner

   i. CREATE NEW FILE NAMED “DATE-CER-PD2”

3. 2% above EP length
   a. Make sure vessel is at the 2% above the EP length
   b. Make sure pressure is at 0 mmHg
   c. Press “Start” in lower right corner of Myoview software interface
   d. Increase pressure from 0 mmHg to 200 mmHg and back to 0 mmHg using sequencer
      i. 5 mmHg increments for 2s
      ii. Hold the 5-0 mmHg step for 10 sec to ensure the pressure actually reaches 0 mmHg
   e. Repeat sequence for 3 cycles
   f. Once 5 cycles have been completed press “stop” button in lower right-hand corner
      i. CREATE NEW FILE NAMED “DATE-CER-PD3”

FORCE-ELONGATION TESTING

1. At nominal pressure (10 mmHg)
   a. Make sure pressure is at 10 mmHg
   b. Make sure vessel is -2% EP length
   c. Press “Start” in lower right corner of Myoview software interface
      a. Use axial length knob to stretch vessel up to 2% above the experimentally determined EP length ($l_{exp}^{EP}$) length and back down to 2% below the experimentally determined EP length at 10 microns/s
      i. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
      a. Repeat stretch for 3 cycles
   f. Once 3 cycles have been completed press “stop” button in lower right-hand corner
      vii. CREATE NEW FILE FOR FL NAMED “DATE-CER-FL1”

2. At 1/3 max pressure (67 mmHg)
   a. Make sure pressure is at 1/3 of Max Pressure
   b. Make sure vessel is at -2% EP length
   c. Press “Start” in lower right corner of Myoview software interface
   d. Use axial length knob to stretch vessel up to 2% above the experimentally determined EP length ($l_{exp}^{EP}$) length and back down to 2% below the experimentally determined EP length at 10 microns/s
i. Each mark on knob denotes .05mm so time between marks should be 5 seconds.
   a. Repeat stretch for 3 cycles
f. Once 3 cycles have been completed press “stop” button in lower right-hand corner
   vii. CREATE NEW FILE FOR FL NAMED “DATE-CER-FL2”

3. At 2/3 max pressure (133 mmHg)
   a. Make sure pressure is at 2/3 of Max Pressure
   b. Make sure vessel is at -2% EP length
   c. Press “Start” in lower right corner of Myoview software interface
   d. Use axial length knob to stretch vessel up to 2% above the experimentally determined EP length ($l_{exp}^{EP}$) length and back down to 2% below the experimentally determined EP length at 10 microns/s
      i. Each mark on knob denotes .05mm so time between marks should be 5 seconds.
         a. Repeat stretch for 3 cycles
   f. Once 3 cycles have been completed press “stop” button in lower right-hand corner
      vii. CREATE NEW FILE FOR FL NAMED “DATE-CER-FL3”

4. At Max Pressure (200 mmHg)
   a. Make sure pressure is at Max Pressure
   b. Make sure vessel is -2% EP length
   c. Press “Start” in lower right corner of software interface
   d. Use axial length knob to stretch vessel up to 2% above the experimentally determined EP length ($l_{exp}^{EP}$) length and back down to 2% below the experimentally determined EP length at 10microns/s
      i. Each mark on knob denotes .05mm so time between marks should be 5 seconds.
         a. Repeat stretch for 3 cycles
   f. Once 3 cycles have been completed press “stop” button in lower right-hand corner
      vii. CREATE NEW FILE FOR FL NAMED “DATE-CER-FL4”
A.3 Cervix *In Vivo* Pressure Measurement Protocol

**Tool Preparation:**

1) Retrieve large blue cart from storage area in hallway across from 431
2) Retrieve plate warmer, pressure calibration device (near mechanical testing device), and surgical instruments (yellow box in drawer labeled Cervix Catheter: flashlight, lubrication, two sets of tweezers, eye drops, alcohol pads, metal platform, surgical pad, heating pad, 3mL syringe, and gloves) and load onto front of cart
   a. Heating pad and heating pad power source may be located on bench near the AD instruments boxes
3) Retrieve AD instruments Powerlab and bridge amp and load onto back of the cart
   Retrieve laptop and charger (lock code 1214) and load next to AD instrument equipment
4) Put small black stool on the bottom rack of cart and retrieve the pole with gripping arm (Bayer lab bench or Bayer lab imaging room)
5) Load the gripping arm and notebook with testing sheets
6) **LASTLY** load the catheter carefully on top of notebooks away from water bath

**Set-up:**

1) Transport the cart the surgical suite 435
2) Place cart in front of sink near the anesthesia station
3) Plug in water bath to outlet closest to the sink and turn on to warm water to 37°C
   a. Fill large beaker with 500mL-750mL DI water
4) Retrieve red anesthesia chamber from drying rack and connect waste line and gas line to the chamber
5) Make sure all lines are in the “closed/horizontal position”
6) Check isoflurane levels
   a. If ¼ to empty refill to at least ½
7) Check oxygen levels
   a. Back-up air in histology room. Immediately request a 100% O2 tank purchase with Rebecca and pick-up of old tank.
8) Place AD instruments Powerlab and Bridge amp on top shelf and plug into red outlet by isoflurane machine. USB computer connection should fall to the right.
9) Place computer to far right of the surgical area and plug in USB cord connection to AD Instruments boxes
10) Place metal platform at preferred height (about eye level with the vagina when sitting) and cover with heating pad followed by surgical pad
11) Utilize nose-cone with small cuts to layer to fit mouse head but not too large to allow for leaks (green preferred). Make sure waste line is connected to Miller Lab waste container and place over surgical pad. Try to put magnetic square under surgical pad leaving enough heating pad available to warm the mouse.
12) Place gripping arm on the right and grip flashlight in hand
13) Place surgical tool kit next to red anesthesia chamber or in nearby drawer with easy access: lay out tweezers, eye gel, alcohol pads, lubricant, and ear punch (only for new animal that will be used continuously)
14) Once water bath holding constant temperature at or slightly below 37 C: connect tubing from pressure calibration device to Y connector, fill syringe with 37C water from bath and screw into connector.
   a. After this step remove any long sleeves and discharge static (grab back of metal chair or door knob in room).
15) Open catheter box and gently thread catheter out of spiral casing and keep the electronic component within packaging to act as an anchor. DO NOT GRIP CATHETER BY THE PROBE. Keep at least two inches distance between fingers and probe. Gently lay catheter across the packaging and keep dry.
16) Make sure third end of Y connector is open (screw left to open) and gently release some water from syringe to fill Y chamber. Tip: hold y connector flat to prevent flow into tubing or out of opening.
17) Gently, gripping the catheter two inches below the probe insert into the open end of the Y connector. Make sure catheter is submerged and not pressing on the walls. Carefully screw the connector to the right to close off the opening around the catheter. Tight enough to prevent water from escaping but gentle enough to not put great amount of pressure on the catheter.
18) Place entire interface within the water bath and place the 3-way valve on the pressure calibration device under the left front corner of the bath to prevent the lid from pressing on the catheter wiring.
19) Set a timer for 30 minutes.
20) Using another cart retrieve mice from DCM on the seventh floor using the service elevator.
21) Return to room allow mice to equilibrate and turn on heating pad (~10 minutes)
22) Calibrate catheter at 6 mmHg and 60 mmHg

Anesthesia
1) Check and record stage of estrous
2) Record mouse mass
3) Anesthetize mouse in chamber at 2% isoflurane
4) Check for depth of anesthesia by toe pinch
5) Gently transfer mouse onto the pad and place belly down with nose in cone
6) Apply eye gel to both eyes
7) If animal has not been previously tested: use ear punch to identify mouse and record identifying mark
8) Flip mouse onto back and position flashlight directed towards the vagina
Catheter Measurements

1) Apply lubrication to angled forceps and gently open the vaginal cavity until the cervix can be visualized
2) Gently thread the catheter to the center of the cervix
   a. Avoid using too much pressure
   b. The catheter is flexible and likely to reflect off cervix into vaginal tissue
3) At the cervix the first black mark on the catheter should reach the vaginal opening. Second marking denotes the end of the uterine horn
4) Within a few minutes the cervix should begin to contract regularly up to 50mmHg
5) Upon confirmation of cervical placement make a note within the program
6) Lower isoflurane percentage to 1.5%
7) Allow mouse to equilibrate for 10 minutes and record data
8) Following equilibration, record for an additional ten minutes
9) Remove catheter from mouse and place within Y connector in water bath
10) Redirect isoflurane to chamber and transport mouse to cage for recovery
11) Watch mouse for signs of distress after waking up
12) After allowing catheter to equilibrate for 10 mins in bath, apply known pressure and measure on software to determine any measurement offset
13) Clean catheter in small beaker of Tergazyme for 15 mins
14) To perform on another mouse: repeat process starting with 30 min in water bath and calibration

Clean-Up

1) Clean catheter in small beaker of Tergazyme for 15 mins
2) Dispose of surgical pad and any biological hazards
3) Turn off equipment, pour out water, and return devices to cart
4) Weigh the waste containers: Waste must be replaced after reaching 200g above first measurement
5) Clean out anesthesia box
6) Store cart in corner of room
7) Return mice to the in-house quarantine
8) Return cart to main lab space (415) and place equipment back in proper location
9) Charge heating pad
10) Lock computer up to bench
A.4 Isolation of Cervix from Reproductive Tract for Active Protocols

1) Place dissected reproductive tract in petri dish with 4°C Hank’s Balanced Saline Solution (HBSS)
2) Using sharp scissors to make cuts immediately inferior to the bifurcation of the uterine horns
3) Next make a cut superior to the vaginal introitus and a cut inferior to the external os of the cervix (Figure 4.2.A, 4.2.B)
4) Place cervical complex in a separate dish with 4°C HBSS under the microscope
5) Using micro-scissors and blunt straight tweezers to dissect away fat and connective tissue from the outer layer
6) To ensure cannulation, the septum between the uterine canals was cut with micro-scissors (Figure 4.2.C)
7) Utilizing 6-0 silk suture and suture forceps, pull the cervical complex over the cannula and tie down with sutures
   a) Pull the vaginal end over the 3.0 mm diameter cannula
   b) Pull the uterine end over the 0.85 mm diameter cannula

![Diagram of reproductive tract with labels for Ovaries, Uterine Horns, Cervix, Vagina, Top View Uterine Segment, Bottom View Vaginal Opening, and External Os]
A.5 Cervix Maximum Contractility and Passive Test Protocol

**Testing Protocol Outline**

- Max Contract at -1%, Estimated Physiologic (EP) Length, +1%
- Passive
  - Pressure-Inflation/Pressure-Diameter Test
  - Force-Elongation/Force-Length Test

_Ultrasound imaging performed at the unloaded configuration for the end of the active test and end of the passive test_

**Find Unloaded Geometry**

1. Unloaded Length ($l_{unloaded}$)
   a. Look for length at which vessel appears very buckled/collapsed
   b. Turn on the pressure and set equal to 0 mmHg
   c. Record unloaded length from suture to suture
   d. Record the diameter at 0 mmHg
2. Unloaded Pressure
   a. Manually increase pressure from 0 to 5 mmHg in increments of 1 mmHg while observing the vessel
   b. _Make sure pressure gradient is at 1.5 mmHg/s_
      1. Look for the pressure at which the vessel no longer appears collapsed and at which there is the biggest jump in OD.
   c. Record the identified pressure and OD as first uncollapsed point
      1. Set pressure to 3 mmHg, record the OD, and zero the force (this is the new zero point—i.e. 3 mmHg is the UP)

**PD PRECONDITIONING 1** (Pressure-Inflation)

1. Pressure should be set to 0 mmHg
2. Length should be at the estimated EP length
3. Set gradient to 1.5 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 18+UP mmHg and back down to 0mmHg again
   a. Interval of 0-18 +UP mmHg with 30 sec hold time
4. Repeat sequence for 5 cycles.
   a. CREATE NEW FILE FOR P-D PRECONDITIONING NAMED “DATE-CER-Day Gestation_Max-pdpc1”

**Finding EP Stretch**

1. Adjust tissue to estimate _in vivo_ length (usually helpful to set to unloaded pressure)
2. Press “Start in lower right corner of Myoview software interface to record
3. In software interface assess pressure vs force values about the estimated *in vivo* length from UP to 18+UP mmHg
4. The experimentally determined EP stretch ($\lambda_{\text{EP}}^{\text{Exp}}$) will be the stretch value that displays a mostly flat line of force values over a range of pressures (i.e. constant force over a range of pressures)
   a. If increasing the stretch only results in near-constant slopes at increasing levels of force the EP stretch has been overestimated—take the tissue back down and start again.
   b. CREATE NEW FILE FOR FINDING EP LENGTH NAMED “DATE-CER-DX_Max-IVL”

**Circumferential PRECONDITIONING** (Pressure-Diameter)
1. Pressure should be set to 0 mmHg
2. Length should be at the EP length
3. Press “Start” in lower right corner of software interface
4. Set gradient to 1.5 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 18+UP mmHg and back down to 0mmHg again
   a. Interval of 0-18 +UP mmHg with 30 sec hold time
5. Repeat sequence for 5 cycles.
6. Once 5 cycles have been completed press “stop” button in lower right-hand corner
   a. CREATE NEW FILE FOR P-D PRECONDITIONING NAMED “DATE-CER-DX_Max-pdpc2”

**Axial PRECONDITIONING** (Force-Length)
1. Enter 1/3 max pressure + UP (6mmHg+ UP) in pressure window for P1 and P2
2. Press “Start” in lower right corner of myoview
3. Use axial length knob to adjust vessel to from 1% below the in vivo length to 1% above) the in vivo length at 10 microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
4. Once 5 cycles have been completed press “stop” button in lower right-hand corner
   a. CREATE NEW FILE FOR FL PRECONDITIONING NAMED “DATE-CER-DX_Max-flpc”

**Re-Find Unloaded Geometry**
1. Unloaded Length ($l_{\text{unloaded}}$)
   a. Set tissue to in vivo length
   b. Set to unloaded pressure
   c. Decreases axial length at 0.01mm/sec until there is minimal to no change in the slope of the force-This is the unloaded length
   d. **Record** unloaded length and the diameter
   e. **Zero** the force

**Smooth Muscle PRECONDITIONING**
1. Set pressure to the mean measured in vivo pressure + UP (9mmHg +UP)
2. Adjust tissue (from unloaded length) to where force is close to zero
3. Let the tissue equilibrate until a steady force and outer diameter is achieved (~5 minutes)
4. Add 20 mM of KCl to bath
5. Let tissue contract for at least 5 minutes
   a. CREATE NEW FILE FOR SMC PRECONDITIONING NAMED “DATE-CER-DX_Max-SMCpc”
6. Wash out solution and add fresh active KRB
   a. If force/outer diameter do not return to baseline value perform a secondary wash

**Equilibrate**
1. Set tissue to *in vivo* length and pressure (9+UP mmHg) for 10 minutes
2. CREATE NEW FILE FOR EQUILIBRATION NAMED “DATE-CER-DX_Max-EQUIL”

**Maximum Contraction**
*Randomize lengths and pressures below is used as an example*

**Estimated Physiologic Length + Below Pressure**
1. Verify length is at the EP length
2. Set pressure to the below mean pressure (6 + UP)
   1. Press “Start in lower right corner of interface to record
   2. Record Baseline Force and Outer Diameter
   3. Contract with 20mM of KCl for 5 mins
   4. Record maximally contracted Force and Outer Diameter
3. Wash out solution and add fresh active KRB
   1. If force/outer diameter do not return to baseline value perform a secondary wash
4. Press “stop” button in lower right-hand corner and CREATE NEW FILE NAMED “DATE-CER-DX_Max-EPL-BelowP”

**Estimated Physiologic Length + Mean Physiologic Pressure**
1. Verify length is at the EP length
2. Set pressure to the mean pressure (9 + UP)
3. Press “Start in lower right corner of interface to record
   a. Record Baseline Force and Outer Diameter
4. Contract with 20mM of KCl for 5 mins
   a. Record contracted Force and Outer Diameter
5. Wash out solution and add fresh active KRB
   a. If force/outer diameter do not return to baseline value perform a secondary wash
   b. Press “stop” button in lower right-hand corner and CREATE NEW FILE NAMED “DATE-CER-DX_Max-EPL-IVP”
**Estimated Physiologic Length + Above Pressure**

1. Verify length is at the EP length
2. Set pressure to one standard deviation above the mean pressure (12 + UP)
3. Press “Start in lower right corner of interface to record
   a. Record Baseline Force and Outer Diameter
4. Contract with 20mM of KCl for 5 mins
   a. Record contracted Force and Outer Diameter
5. Wash out solution and add fresh active KRB
   a. If force/outer diameter do not return to baseline value perform a secondary wash
   b. Press “stop” button in lower right-hand corner and CREATE NEW FILE NAMED “DATE-CER-DX_Max-EPL-AboveP”

**1% Below Estimated Physiologic Length + Below Pressure**

1. Verify length is at the -1% EP length
2. Set pressure to the below mean pressure (6 + UP)
3. Press “Start in lower right corner of interface to record
   a. Record Baseline Force and Outer Diameter
4. Contract with 20mM of KCl for 5 mins
   a. Record contracted Force and Outer Diameter
5. Wash out solution and add fresh active KRB
6. If force/outer diameter do not return to baseline value perform a secondary wash
   a. Press “stop” button in lower right-hand corner
   b. CREATE NEW FILE NAMED “DATE-CER-DX_Max-BelowL-BelowP”

**1% Below Estimated Physiologic Length + In Vivo Pressure**

a. Verify length is at the -1% EP length
b. Set pressure to the below mean pressure (9 + UP)
c. Press “Start
   i. Record Baseline Force and Outer Diameter
d. Contract with 20mM of KCl for 5 mins
e. Record contracted Force and Outer Diameter
f. Wash out solution and add fresh active KRB
g. If force/outer diameter do not return to baseline value perform a secondary wash
   a. Press “stop” button in lower right-hand corner
   ii. CREATE NEW FILE NAMED “DATE-CER-DX_Max-BelowL-IVP”

**1% Below Estimated Physiologic Length + Above Pressure**

1. Verify length is at the -1% EP length
2. Set pressure to the above mean pressure (12 + UP)
3. Press “Start
   a. Record Baseline Force and Outer Diameter
4. Contract with 20mM of KCl for 5 mins
   a. Record contracted Force and Outer Diameter
5. Wash out solution and add fresh active KRB
a. If force/outer diameter do not return to baseline value perform a secondary wash
6. Press “stop” button in lower right-hand corner
   a. CREATE NEW FILE NAMED “DATE-CER-DX_Max-BelowL-AboveP”

1% Above Estimated Physiologic Length + Below Pressure
1. Verify length is at the +1% EP length
2. Set pressure to the below mean pressure (6 + UP)
3. Press “Start in lower right corner interface
   a. Record Baseline Force and Outer Diameter
4. Contract with 20mM of KCl for 5 mins
   a. Record contracted Force and Outer Diameter
5. Wash out solution and add fresh active KRB for 5 mins
   a. If force/outer diameter do not return to baseline value perform a secondary wash
6. Press “stop” button in lower right-hand corner
   a. CREATE NEW FILE NAMED “DATE-CER-DX_Max-AboveL-BelowP”

1% Above Estimated Physiologic Length + In Vivo Pressure
1. Verify length is at the +1% EP length
2. Set pressure to the mean pressure (9 + UP)
3. Press “Start in lower right corner of myoview to record
   a. Record Baseline Force and Outer Diameter
4. Contract with 20mM of KCl for 5 mins
   a. Record contracted Force and Outer Diameter
5. Wash out solution and add fresh active KRB
   a. If force/outer diameter do not return to baseline value perform a secondary wash
6. Press “stop” button in lower right-hand corner
   a. CREATE NEW FILE NAMED “DATE-CER-DX_Max-AboveL-IVP”

1% Above Estimated Physiologic Length + Above Pressure
1. Verify length is at the +1% EP length
2. Set pressure to the above physiologic mean pressure (12 + UP)
3. Press “Start to record
   a. Record Baseline Force and Outer Diameter
4. Contract with 20mM of KCl for 5 mins
   a. Record contracted Force and Outer Diameter
5. Wash out solution and add fresh active KRB
   a. If force/outer diameter do not return to baseline value perform a secondary wash
6. Press “stop” button in lower right-hand corner
   a. CREATE NEW FILE NAMED “DATE-CER-DX_Max-AboveL-AboveP”

FORCE-LENGTH TESTING (Tare Pressure)
i. At nominal pressure (3 mmHg + UP)
ii. Make sure vessel is -1% EP length
iii. Press “Start” in lower right corner of Myoview software interface
iv. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   1. Each mark on knob denotes .05mm so time between marks should be 5 seconds.
   2. Repeat stretch for 3 cycles
v. Once 3 cycles have been completed press “stop” button in lower right-hand corner
   1. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Max-FL1”

FORCE-LENGTH TESTING (1/3 Max Pressure)

i. At 1/3 max pressure (6 + UP)
ii. Make sure vessel is -1% EP length
iii. Press “Start” in lower right corner of Myoview software interface
iv. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   1. Each mark on knob denotes .05mm so time between marks should be 5 seconds.
   2. Repeat stretch for 3 cycles
v. Once 3 cycles have been completed press “stop” button in lower right-hand corner
   1. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Max-FL2”

FORCE-LENGTH TESTING (2/3 Max Pressure)

1. At 2/3 max pressure (12 + UP)
2. Make sure vessel is -1% EP length
3. Press “Start” in lower right corner of Myoview software interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
   a. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Max-FL3”

FORCE-LENGTH TESTING (Max Pressure)

1. At Max pressure (12 + UP)
2. Make sure vessel is -1% EP length
3. Press “Start” in lower right corner of Myoview software interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05mm so time between marks should be 5 seconds.
b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
   a. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Max-FL3”

**Ultrasound**
- Return vessel to unloaded length and pressure
- Using 40Mhz transducer take images of the cervix mounted within the DMT

**Passive Protocol**
- After basal testing induce the passive state with 2mM (0.015g in 20 mL KRB) of EGTA in calcium free Krebs Ringer Buffer for 30 minutes at the EP length

**Record Length and Diameter -> New Unloaded**

**PD PRECONDITIONING 1 (Pressure-Inflation)**
1. Pressure should be set to 0 mmHg
2. Length should be at the estimated EP length
3. Set gradient to 1.5 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 18+UP mmHg and back down to 0mmHg again
4. Interval of 0-18 +UP mmHg with 30 sec hold time
   1. Repeat sequence for 5 cycles.
5. CREATE NEW FILE FOR P-D PRECONDITIONING NAMED “DATE-CER-Day Gestation_Passive-pdpc1”

**Finding EP Stretch**
1. Adjust tissue to estimate in vivo length (usually helpful to set to unloaded pressure)
2. Press “Start in lower right corner of Myoview software interface to record
3. In software interface assess pressure vs force values about the estimated EP length from UP to 18+UP mmHg
4. The experimentally determined EP stretch ($\lambda_{z,exp}^{EP}$) will be the stretch value that displays a mostly flat line of force values over a range of pressures (i.e. constant force over a range of pressures)
   a. If increasing the stretch only results in near-constant slopes at increasing levels of force the EP stretch has been overestimated—take the tissue back down and start again.
5. CREATE NEW FILE FOR FINDING EP LENGTH NAMED “DATE-CER-DX_Passive-IVL”

**Circumferential PRECONDITIONING (Pressure-Diameter)**
1. Pressure should be set to 0 mmHg
2. Length should be at the EP length
3. Press “Start” in lower right corner of software interface
4. Set gradient to 1.5 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 18+UP mmHg and back down to 0mmHg again
5. Interval of 0-18 +UP mmHg with 30 sec hold time
   a. Repeat sequence for 5 cycles.
6. Once 5 cycles have been completed press “stop” button in lower right-hand corner
7. CREATE NEW FILE FOR P-D PRECONDITIONING NAMED “DATE-CER-DX_Passive-pdpc2”

Axial PRECONDITIONING (Force-Length)
1. Enter 1/3 max pressure + UP (6mmHg+ UP) in pressure window for P1 and P2
2. Press “Start” in lower right corner of interface
3. Use axial length knob to adjust vessel to from 1% below the EP length to 1% above
   the EP length at 10 microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds
4. Once 5 cycles have been completed press “stop” button in lower right-hand corner
5. CREATE NEW FILE FOR FL PRECONDITIONING NAMED “DATE-CER-DX_Passive-flpc”

Re-Find Unloaded Geometry and Equilibrate
i. Unloaded Length ($l_{unloaded}$)
   1. Set tissue to EP length
   2. Set to unloaded pressure
   3. Decreases axial length at 0.01mm/sec until there is minimal to no change in
      the slope of the force-This is the unloaded length
   4. Record unloaded length and the diameter
   5. Zero the force

2. Set tissue to EP length and mean physiologic pressure (9+UP mmHg) for 10 minutes
3. CREATE NEW FILE FOR EQUILIBRATION NAMED “DATE-CER-DX-Passive-UL-EQUIL”

PRESSURE-DIAMETER TESTING (1% Below EP Length)
a. Make sure vessel is at the 1% below the experimentally determined in vivo length
b. Make sure pressure is at 0 mmHg
c. Press “Start” in lower right corner of myoview
d. Increase pressure from 0 mmHg to 18+UP mmHg and back to 0 mmHg using sequencer
   a. 1 mmHg increments for 1s
   b. Hold the 1-0 mmHg step for 10 secs to ensure the pressure actually reaches 0 mmHg
   c. Repeat sequence for 3 cycles
e. Once 3 cycles have been completed press “stop” button in lower right-hand corner
f. CREATE NEW FILE NAMED “DATE-CER-DX-Passive-PD1”

PRESSURE-DIAMETER TESTING (EP Length)
a. Make sure vessel is at the experimentally determined EP length
b. Make sure pressure is at 0 mmHg
c. Press “Start” in lower right corner of myoview
d. Increase pressure from 0 mmHg to 18+UP mmHg and back to 0 mmHg using sequencer
   a. 1 mmHg increments for 1s
   b. Hold the 1-0 mmHg step for 10 sec to ensure the pressure actually reaches 0 mmHg
   c. Repeat sequence for 3 cycles
e. Once 3 cycles have been completed press “stop” button in lower right-hand corner
f. CREATE NEW FILE NAMED “DATE-CER-DX-Passive-PD2”

PRESSURE-DIAMETER TESTING (1% Above EP Length)

a. Make sure vessel is at the 1% above the EP length
b. Make sure pressure is at 0 mmHg
c. Press “Start” in lower right corner of myoview
d. Increase pressure from 0 mmHg to 18+UP mmHg and back to 0 mmHg using sequencer
   a. 1 mmHg increments for 1s
   b. Hold the 1-0 mmHg step for 10 sec to ensure the pressure actually reaches 0 mmHg
   c. Repeat sequence for 3 cycles
e. Once 3 cycles have been completed press “stop” button in lower right-hand corner
f. CREATE NEW FILE NAMED “DATE-CER-DX-Passive-PD1”

FORCE-LENGTH TESTING (Tare Pressure)

1. At nominal pressure (3 mmHg + UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Passive-FL1”

FORCE-LENGTH TESTING (1/3 Max Pressure)

1. At 1/3 max pressure (6 + UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner

6. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Passive-FL2”

**FORCE-LENGTH TESTING (2/3 Max Pressure)**
1. At 2/3 max pressure (12 + UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Passive-FL3”

**FORCE-LENGTH TESTING (Max Pressure)**
1. At max pressure (18+ UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE FOR FL NAMED “DATE-CER-DX-Passive-FL4”

**Ultrasound**
- Return vessel to unloaded length and pressure
- Using the ultrasound 40Mhz transducer take images of the cervix mounted within the inflation-extension device
A.6 Cervix Basal Tone and Passive Test Protocol

**Testing Protocol Outline**

- Max Contract at *in vivo*
- Basal (PD+FL)
- Passive (PD+FL)

*Ultrasound imaging performed at the unloaded configuration for the passive control and elastase treated sample*

**Find Unloaded Geometry**

1. **Unloaded Length** ($l_{unloaded}$)
   a. Look for length at which vessel appears very buckled/collapsed Turn on the pressure and set equal to 0 mmHg
   b. Record unloaded length from suture to suture
   c. Record the diameter at 0 mmHg

2. **Unloaded Pressure**
   a. Manually increase pressure from 0 to 10 mmHg in increments of 1 mmHg while observing the vessel
   b. *Make sure pressure gradient is at 1.5 mmHg/s*
      a. Look for the pressure at which the vessel no longer appears collapsed and at which there is the biggest jump in OD.
   c. Record the identified pressure and OD as first uncollapsed point
   d. Set pressure to 4 mmHg, record the OD, and **zero the force and Record tare force** (this is the new zero point—i.e. 4 mmHg is the UP)

**Pre-PRECONDITIONING** (Pressure-Diameter)

1. Pressure should be set to 0 mmHg
2. Length should be at the estimated in vivo length
3. Set gradient to 1.5 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 28+UP mmHg and back down to 0mmHg again
   a. Interval of 0-28 +UP mmHg with 30 sec hold time
4. Repeat sequence for 5 cycles.

**Finding EP Stretch**

1. Adjust tissue to estimate in vivo length (usually helpful to set to unloaded pressure)
2. Press “Start in lower right corner of myoview to record
3. In software assess pressure vs force values about the estimated *in vivo* length from UP to 28 mmHg
4. The experimentally determined EP stretch ($\lambda_{z,exp}^{EP}$) will be the stretch value that displays a mostly flat line of force values over a range of pressures (i.e. constant force over a range of pressures)
Circumferential PRECONDITIONING (Pressure-Diameter)
1. Pressure should be set to 0 mmHg
2. Length should be at the EP length
3. Press “Start” in lower right corner of myoview
4. Set gradient to 1.5 mmHg, run preconditioning sequence that takes pressure from 0 mmHg to 28+UP mmHg and back down to 0 mmHg again
   a. Interval of 0-28+UP mmHg with 10 sec hold time
2. Repeat sequence for 5 cycles.
3. Once 5 cycles have been completed press “stop” button in lower right-hand corner
   a. CREATE NEW FILE FOR P-D PRECONDITIONING NAMED “DATE-CER-basal-PDPC”

Axial PRECONDITIONING (Force-Length/Force-Elongation)
1. Enter 1/3 max pressure + UP (9 mmHg + UP) in pressure window for P1 and P2
2. Press “Start” in lower right corner of myoview
3. Use axial length knob to adjust vessel to from 1% below the in vivo length to 1% above) the in vivo length at 10 microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
4. Once 5 cycles have been completed press “stop” button in lower right-hand corner
5. CREATE NEW FILE FOR FL PRECONDITIONING NAMED “DATE-CER-basal-flpc”

Re-Find Unloaded Geometry
1. Unloaded Length (\(l^{unloaded}\))
   a. Set tissue to in vivo length
   b. Set to unloaded pressure
   c. Decreases axial length at 0.01 mm/sec until there is minimal to no change in the slope of the force-This is the unloaded length
   d. Record unloaded length and the diameter

SMOOTH MUSCLE PRECONDITIONING
1. Set pressure to the mean measured in vivo pressure + UP (10 mmHg + UP)
2. Adjust tissue (from unloaded length) to where force is close to zero
3. Add 20 mM of KCl to bath
4. Let tissue contract for at least 5 minutes
5. Wash out solution and add fresh active KRB
   a. If force/outer diameter do not return to baseline value perform a secondary wash

Re-Equilibrate and Verify EP length/stretch
1. Set tissue to EP length and pressure (10 mmHg) for 10 minutes
2. Increase pressure from 0 to 28 mmHg+Up to verify the in vivo length – adjust as needed

**Maximum Contraction**
1. Verify length is at the *in vivo*
2. Set pressure to mean measured *in vivo* + UP
   a. Press “Start in lower right corner of myoview to record
   b. Record Baseline Force and Outer Diameter
   c. Contract with 20mM of KCl for 5 mins
   d. Record contracted Force and Outer Diameter
3. Wash out solution and add fresh active KRB
   1. If force/outer diameter do not return to baseline value perform a secondary wash
   2. When tissue returns to basal state record Baseline Force and Outer Diameter
4. Press “stop” button in lower right-hand corner and CREATE NEW FILE NAMED “DATE-CER-EPL-IVP”
5. Repeat for pressures one standard deviation below and above the mean physiologic pressure

**PRESSURE-DIAMETER TESTING (1% Below EP Length)**
1. Make sure vessel is at the 1% below the experimentally determined EP length
2. Make sure pressure is at 0 mmHg
3. Press “Start” in lower right corner of myoview
4. Increase pressure from 0 mmHg to 28+UP mmHg and back to 0 mmHg using sequencer
   a. 1 mmHg increments for 1s
   b. Hold the 1-0 mmHg step for 10 secs to ensure the pressure actually reaches 0 mmHg
   c. Repeat sequence for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE NAMED “DATE-CER-Basal-PD1”

**PRESSURE-DIAMETER TESTING (EP Length)**
1. Make sure vessel is at the 1% below the experimentally determined EP length
2. Make sure pressure is at 0 mmHg
3. Press “Start” in lower right corner of myoview
4. Increase pressure from 0 mmHg to 28+UP mmHg and back to 0 mmHg using sequencer
   a. 1 mmHg increments for 1s
   b. Hold the 1-0 mmHg step for 10 secs to ensure the pressure actually reaches 0 mmHg
   c. Repeat sequence for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE NAMED “DATE-CER-Basal-PD”

**PRESSURE-DIAMETER TESTING (1% Above EP Length)**
1. Make sure vessel is at the 1% above the experimentally determined EP length
2. Make sure pressure is at 0 mmHg
3. Press “Start” in lower right corner of myoview software interface
4. Increase pressure from 0 mmHg to 28+UP mmHg and back to 0 mmHg using sequencer
   a. 1 mmHg increments for 1s
   b. Hold the 1-0 mmHg step for 10 secs to ensure the pressure actually reaches 0 mmHg
   c. Repeat sequence for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE NAMED “DATE-CER-Basal-PD3”

FORCE-LENGTH TESTING (Tare Pressure)
1. At tare pressure (3+ UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE FOR FL NAMED “DATE-CER-Basal-FL1”

FORCE-LENGTH TESTING (1/3 Max Pressure)
1. At 1/3 Max pressure (9 + UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE FOR FL NAMED “DATE-CER-Basal-FL2”

FORCE-LENGTH TESTING (2/3 Max Pressure)
1. At 1/3 Max pressure (18 + UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE FOR FL NAMED “DATE-CER-Basal-FL3”

FORCE-LENGTH TESTING (Max Pressure)
1. At max pressure (28+ UP)
2. Make sure vessel is -1% below EP length
3. Press “Start” in lower right corner of myoview interface
4. Use axial length knob to stretch vessel up to 1% above the experimentally determined EP length and back down to 1% below the EP length at 10microns/s
   a. Each mark on knob denotes .05 mm so time between marks should be 5 seconds.
   b. Repeat stretch for 3 cycles
5. Once 3 cycles have been completed press “stop” button in lower right-hand corner
6. CREATE NEW FILE FOR FL NAMED “DATE-CER-Basal-FL4”

Passive Treatment Protocol
- After basal testing induce the passive state with 2mM (0.015g in 20 mL KRB) of EGTA in calcium free Krebs Ringer Buffer for 30 minutes at the in vivo length
- Repeat testing protocol – First evaluate new in vivo and unloaded configuration – After perform PD- and FL test
A.7 Data Analysis MATLAB Codes

A.7.1 Averaging Raw Data Point

Sample Compiler Script

```matlab
%Sample Compiler – All For One

test_dates = ['082820'];

test = 'Passive' %Passive or Active

for f = 1:length(test_dates) %iterate through samples
    Test = strcat(test_dates(f))
    Location='CER-PP-
    Pd_id = [1:3];
    for a= 1: length(Pd_id)
    %
    [filename, data] = Input_Data( Test, Location, Pd_id, a, test)
    [AVG_Array, Array] = AVG_Data_P_0_9_CER (filename, data)
    [AVG_Array, Array] = AVG_Data_P_10_15_CER( data, AVG_Array )
    [AVG_Array, Array] = AVG_Data_P_16_20( data, AVG_Array )
    %
    end
    end

Pressure Diameter Data Averaging Code:

```function` [ AVG_Array, Array ] = AVG_Data_P_0_9 (filename, data) %Averages Data Within A Pressure Range % date_cer = input('Date?') % Location = input('UCER or VCER?') % Type = input('control or elastase?') % Test = input( 'pd4? pd6? pd8? or pde4 etc?') % filename = strcat(date_cer,'-',Location,Test);

```
data = xlsread(filename);

```
%AVERAGING OD
    d = size(data); %Variable d = to size of data set
    %
    e = length(data);
    Array = zeros(d); %Creates an array of zeros the size of data
    AVG_Array = zeros(14,5); %Creates an array of zeros to input averaged data

    e = length(data); %e = length of data matrix
    min_pressure = min(data(:,8)); %Finds min pressure
```
for i = 2:e; %Reiterates through length of data struct from 2
    (Raw data starts at row 2)
    for n=1:e; %Reiterates through length of data struct from 1 so that averaged array starts at row 1
        if data(i,8)>min_pressure; %If data is greater than lower bounds - 0.5
            if data(i,8)<min_pressure+0.25; %And if data is less than upper bounds
                Array(n,1)=data(i,2); %Row 1 = OD
                Array(n,2)=data(i,8); %Row 2 = Pressure
                Array(n,3)=data(i,10); %Row 3 = Force
                Array(n,4)=data(i,17); %Row 4 = Length
                Array(n,5)=data(i,18); %Row 5 = Axial Stretch
                if Array(n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
                    AVG_P = mean(P_Point);
                    AVG_F = mean(F_Point);
                    AVG_L = mean(L_Point);
                    AVG_S = mean(S_Point);
                    if Array(n,1)>0;
                        OD_Point(n,1)=Array(n,1);
                        P_Point(n,1)=Array(n,2);
                        F_Point(n,1)=Array(n,3);
                        L_Point(n,1)=Array(n,4);
                        S_Point(n,1)=Array(n,5);
                        AVG_OD = mean(OD_Point);
                        AVG_P = mean(P_Point);
                        AVG_F = mean(F_Point);
                        AVG_L = mean(L_Point);
                        AVG_S = mean(S_Point);
                else
                    error('Try Again')
                end
            end
        end
    end
    end
end
%5Pressure
for i = 2:e;
    for n=1:e;
        if data(i,8)>3.5;
            if data(i,8)<4.25;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
                if Array(n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
AVG_P = mean(P_Point);
AVG_F = mean(F_Point);
AVG_L = mean(L_Point);
AVG_S = mean(S_Point);

AVG_Array(2,1)=AVG_OD;
AVG_Array(2,2)=AVG_P;
AVG_Array(2,3)=AVG_F;
AVG_Array(2,4)=AVG_L;
AVG_Array(2,5)=AVG_S;
else
    error('Try Again!')
end

AVG_Array(3,1)=AVG_OD;
AVG_Array(3,2)=AVG_P;
AVG_Array(3,3)=AVG_F;
AVG_Array(3,4)=AVG_L;
AVG_Array(3,5)=AVG_S;
else
    error('Try Again!')
end

AvG%10 Pressure
for i = 2:e;
    for n=1:e;
        if data(i,8)>4;
            if data(i,8)<6;
                Array(n,1)=data(i,8);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,8);
                Array(n,1)=data(i,8);
                if Array (n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
                    AVG_P = mean(P_Point);
                    AVG_F = mean(F_Point);
                    AVG_L = mean(L_Point);
                    AVG_S = mean(S_Point);
                    AVG_Array(3,1)=AVG_OD;
                    AVG_Array(3,2)=AVG_P;
                    AVG_Array(3,3)=AVG_F;
                    AVG_Array(3,4)=AVG_L;
                    AVG_Array(3,5)=AVG_S;
                else
                    error('Try Again!')
                end
            end
        end
    end
end
end
%15 Pressure

for i = 2:e;
    for n=1:e;
        if data(i,8)>6;
            if data(i,8)<7.5;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
                if Array(n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
                    AVG_P = mean(P_Point);
                    AVG_F = mean(F_Point);
                    AVG_L = mean(L_Point);
                    AVG_S = mean(S_Point);
                else
                    error('Try Again!')
                end
            end
        end
    end
end

for i = 2:e;
    for n=1:e;
        if data(i,8)>8;
            if data(i,8)<9.25;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
                if Array(n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
                    AVG_P = mean(P_Point);
                    AVG_F = mean(F_Point);
            end
        end
    end
end
AVG_L = mean(L_Point);
AVG_S = mean(S_Point);

AVG_Array(5,1)=AVG_OD;
AVG_Array(5,2)=AVG_P;
AVG_Array(5,3)=AVG_F;
AVG_Array(5,4)=AVG_L;
AVG_Array(5,5)=AVG_S;
else
    error('Try Again')
end
end
end

save('P_0_8');

end

function [AVG_Array, Array] = AVG_Data_P_10_15(data, AVG_Array)
%
% d = size(data);  %Variable d = to size of data set
% e = length(data);
%      Array = zeros(d); %Creates an array of zeros the size of data
%      AVG_Array = zeros(X,5);  %Creates an array of zeros to input averaged data
%
% e = length(data);  %e = length of data matrix
% min_pressure = min(data(:,8));  %Finds min pressure
load('P_0_8');
%20 Pressure
%25 Pressure
for i = 2:e;
    for n=1:e;
        if data(i,8)>9;
            if data(i,8)<10.25;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
                if Array(n,1)>0;
                    OD_POINT(n,1)=Array(n,1);
                    P_POINT(n,1)=Array(n,2);
                    F_POINT(n,1)=Array(n,3);
                    L_POINT(n,1)=Array(n,4);
                    S_POINT(n,1)=Array(n,5);
                end
            end
        end
    end
end
AVG_OD = mean(OD_POINT);
AVG_P = mean(P_POINT);
\[ AVG_F = \text{mean}(F_{\text{Point}}); \]
\[ AVG_L = \text{mean}(L_{\text{Point}}); \]
\[ AVG_S = \text{mean}(S_{\text{Point}}); \]

\[ AVG_{\text{Array}}(6,1) = AVG_{\text{OD}}; \]
\[ AVG_{\text{Array}}(6,2) = AVG_{\text{P}}; \]
\[ AVG_{\text{Array}}(6,3) = AVG_{\text{F}}; \]
\[ AVG_{\text{Array}}(6,4) = AVG_{\text{L}}; \]
\[ AVG_{\text{Array}}(6,5) = AVG_{\text{S}}; \]

else
\[ \text{error('Try Again')} \]
end

end
end
end
end

%30 Pressure

for \( i = 2:e; \)
    for \( n=1:e; \)
        if \( \text{data}(i,8)>11; \)
            if \( \text{data}(i,8)<12.5; \)
                \( \text{Array}(n,1) = \text{data}(i,2); \)
                \( \text{Array}(n,2) = \text{data}(i,8); \)
                \( \text{Array}(n,3) = \text{data}(i,10); \)
                \( \text{Array}(n,4) = \text{data}(i,17); \)
                \( \text{Array}(n,5) = \text{data}(i,18); \)
                if \( \text{Array}(n,1)>0; \)
                    \( \text{OD}_{\text{Point}}(n,1) = \text{Array}(n,1); \)
                    \( \text{P}_{\text{Point}}(n,1) = \text{Array}(n,2); \)
                    \( \text{F}_{\text{Point}}(n,1) = \text{Array}(n,3); \)
                    \( \text{L}_{\text{Point}}(n,1) = \text{Array}(n,4); \)
                    \( \text{S}_{\text{Point}}(n,1) = \text{Array}(n,5); \)
                \end
                \[ \text{AVG}_{\text{OD}} = \text{mean}(\text{OD}_{\text{Point}}); \]
                \[ \text{AVG}_{\text{P}} = \text{mean}(\text{P}_{\text{Point}}); \]
                \[ \text{AVG}_{\text{F}} = \text{mean}(\text{F}_{\text{Point}}); \]
                \[ \text{AVG}_{\text{L}} = \text{mean}(\text{L}_{\text{Point}}); \]
                \[ \text{AVG}_{\text{S}} = \text{mean}(\text{S}_{\text{Point}}); \]
                \[ \text{AVG}_{\text{Array}}(7,1) = \text{AVG}_{\text{OD}}; \]
                \[ \text{AVG}_{\text{Array}}(7,2) = \text{AVG}_{\text{P}}; \]
                \[ \text{AVG}_{\text{Array}}(7,3) = \text{AVG}_{\text{F}}; \]
                \[ \text{AVG}_{\text{Array}}(7,4) = \text{AVG}_{\text{L}}; \]
                \[ \text{AVG}_{\text{Array}}(7,5) = \text{AVG}_{\text{S}}; \]
            \end
            else
                \[ \text{error('Try Again')} \]
            end
        end
    end
end

for \( i = 2:e; \)
    for \( n=1:e; \)
        if \( \text{data}(i,8)>13.5\)
if data(i,8)<14.25;
Array(n,1)=data(i,2);
Array(n,2)=data(i,8);
Array(n,3)=data(i,10);
Array(n,4)=data(i,17);
Array(n,5)=data(i,18);
if Array (n,1)>0;
   OD_Point(n,1)=Array(n,1);
P_Point(n,1)=Array(n,2);
   F_Point(n,1)=Array(n,3);
   L_Point(n,1)=Array(n,4);
   S_Point(n,1)=Array(n,5);
   AVG_OD = mean(OD_Point);
   AVG_P = mean(P_Point);
   AVG_F = mean(F_Point);
   AVG_L = mean(L_Point);
   AVG_S = mean(S_Point);
   AVG_Array(8,1)=AVG_OD;
   AVG_Array(8,2)=AVG_P;
   AVG_Array(8,3)=AVG_F;
   AVG_Array(8,4)=AVG_L;
   AVG_Array(8,5)=AVG_S;
else
   error('Try Again!')
end
end
end

%50 Pressure

for i = 2:e;
   for n=1:e;
      if data(i,8)>14.0
         if data(i,8)<15.5;
            Array(n,1)=data(i,2);
            Array(n,2)=data(i,8);
            Array(n,3)=data(i,10);
            Array(n,4)=data(i,17);
            Array(n,5)=data(i,18);
            if Array (n,1)>0;
               OD_Point(n,1)=Array(n,1);
               P_Point(n,1)=Array(n,2);
               F_Point(n,1)=Array(n,3);
               L_Point(n,1)=Array(n,4);
               S_Point(n,1)=Array(n,5);
               AVG_OD = mean(OD_Point);
               AVG_P = mean(P_Point);
               AVG_F = mean(F_Point);
               AVG_L = mean(L_Point);
               AVG_S = mean(S_Point);
               AVG_Array(9,1)=AVG_OD;
               AVG_Array(9,2)=AVG_P;
               AVG_Array(9,3)=AVG_F;
               AVG_Array(9,4)=AVG_L;
               AVG_Array(9,5)=AVG_S;
         else
            error('Try Again!')
         end
      end
   end
end
AVG_Array(9,2)=AVG_P;
AVG_Array(9,3)=AVG_F;
AVG_Array(9,4)=AVG_L;
AVG_Array(9,5)=AVG_S;
else
    error('Try Again')
end
end
da = size(data);
e = length(data);
Array = zeros(d);
AVG_Array = zeros(41,5);
min_pressure = min(data(:,8));
load('P_10_15');
for i = 2:e;
    for n=1:e;
        if data(i,8)>15.25;
            if data(i,8)<16.5;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
            end
            if Array (n,1)>0;
                OD_Point(n,1)=Array(n,1);
                P_Point(n,1)=Array(n,2);
                F_Point(n,1)=Array(n,3);
                L_Point(n,1)=Array(n,4);
                S_Point(n,1)=Array(n,5);
            end
            AVG_OD = mean(OD_Point);
            AVG_P = mean(P_Point);
            AVG_F = mean(F_Point);
            AVG_L = mean(L_Point);
            AVG_S = mean(S_Point);
        end
    end
end
end
function [AVG_Array, Array] = AVG_Data_P_16_20( data, AVG_Array )
% d = size(data);  %Variable d = to size of data set
% e = length(data);
% Array = zeros(d);  %Creates an array of zeros the size of data
% AVG_Array = zeros(41,5);  %Creates an array of zeros to input averaged data

% e = length(data);  %e = length of data matrix
% min_pressure = min(data(:,8));  %Finds min pressure
load('P_10_15');
%20 Pressure
%25 Pressure
for i = 2:e;
    for n=1:e;
        if data(i,8)>15.25;
            if data(i,8)<16.5;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
            end
            if Array (n,1)>0;
                OD_Point(n,1)=Array(n,1);
                P_Point(n,1)=Array(n,2);
                F_Point(n,1)=Array(n,3);
                L_Point(n,1)=Array(n,4);
                S_Point(n,1)=Array(n,5);
            end
            AVG_OD = mean(OD_Point);
            AVG_P = mean(P_Point);
            AVG_F = mean(F_Point);
            AVG_L = mean(L_Point);
            AVG_S = mean(S_Point);
AVG_Array(10,1)=AVG_OD;
AVG_Array(10,2)=AVG_P;
AVG_Array(10,3)=AVG_F;
AVG_Array(10,4)=AVG_L;
AVG_Array(10,5)=AVG_S;
else
    error('Try Again')
end
end

%30 Pressure
for i = 2:e;
    for n=1:e;
        if data(i,8)>16.25;
            if data(i,8)<17.5;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
                if Array(n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
                    AVG_P = mean(P_Point);
                    AVG_F = mean(F_Point);
                    AVG_L = mean(L_Point);
                    AVG_S = mean(S_Point);
                    AVG_Array(11,1)=AVG_OD;
                    AVG_Array(11,2)=AVG_P;
                    AVG_Array(11,3)=AVG_F;
                    AVG_Array(11,4)=AVG_L;
                    AVG_Array(11,5)=AVG_S;
                else
                    error('Try Again')
                end
            end
        end
    end
end

for i = 2:e;
    for n=1:e;
        if data(i,8)>17.5
            if data(i,8)<18.25;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
            end
        end
    end
end
Array(n,4)=data(i,17);
Array(n,5)=data(i,18);
if Array (n,1)>0;
    OD_Point(n,1)=Array(n,1);
P_Point(n,1)=Array(n,2);
F_Point(n,1)=Array(n,3);
L_Point(n,1)=Array(n,4);
S_Point(n,1)=Array(n,5);
    AVG_OD = mean(OD_Point);
    AVG_P = mean(P_Point);
    AVG_F = mean(F_Point);
    AVG_L = mean(L_Point);
    AVG_S = mean(S_Point);
    AVG_Array(12,1)=AVG_OD;
    AVG_Array(12,2)=AVG_P;
    AVG_Array(12,3)=AVG_F;
    AVG_Array(12,4)=AVG_L;
    AVG_Array(12,5)=AVG_S;
else
    error('Try Again')
end
end
end
end

%50 Pressure
for i = 2:e;
    for n=1:e;
        if data(i,8)>18
            if data(i,8)<19.5;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
                if Array (n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
                    AVG_P = mean(P_Point);
                    AVG_F = mean(F_Point);
                    AVG_L = mean(L_Point);
                    AVG_S = mean(S_Point);
                    AVG_Array(13,1)=AVG_OD;
                    AVG_Array(13,2)=AVG_P;
                    AVG_Array(13,3)=AVG_F;
                    AVG_Array(13,4)=AVG_L;
                    AVG_Array(13,5)=AVG_S;
                end
            end
        end
    end
end

for i = 2:e;
    for n=1:e;
        if data(i,8)>18
            if data(i,8)<19.5;
                Array(n,1)=data(i,2);
                Array(n,2)=data(i,8);
                Array(n,3)=data(i,10);
                Array(n,4)=data(i,17);
                Array(n,5)=data(i,18);
                if Array (n,1)>0;
                    OD_Point(n,1)=Array(n,1);
                    P_Point(n,1)=Array(n,2);
                    F_Point(n,1)=Array(n,3);
                    L_Point(n,1)=Array(n,4);
                    S_Point(n,1)=Array(n,5);
                    AVG_OD = mean(OD_Point);
                    AVG_P = mean(P_Point);
                    AVG_F = mean(F_Point);
                    AVG_L = mean(L_Point);
                    AVG_S = mean(S_Point);
                    AVG_Array(13,1)=AVG_OD;
                    AVG_Array(13,2)=AVG_P;
                    AVG_Array(13,3)=AVG_F;
                    AVG_Array(13,4)=AVG_L;
                    AVG_Array(13,5)=AVG_S;
                end
    end
end
else
    error('Try Again')
end
end
end

for i = 2:e;
    for n=1:e;
        if data(i,8)>18
            if data(i,8)>19.25
                for
                    for
                        if data(i,8)>22
                            Array(n,1)=data(i,2);
                            Array(n,2)=data(i,8);
                            Array(n,3)=data(i,10);
                            Array(n,4)=data(i,17);
                            Array(n,5)=data(i,18);
                            if Array (n,1)>0;
                                OD_Point(n,1)=Array(n,1);
                                P_Point(n,1)=Array(n,2);
                                F_Point(n,1)=Array(n,3);
                                L_Point(n,1)=Array(n,4);
                                S_Point(n,1)=Array(n,5);
                                AVG_OD = mean(OD_Point);
                                AVG_P = mean(P_Point);
                                AVG_F = mean(F_Point);
                                AVG_L = mean(L_Point);
                                AVG_S = mean(S_Point);
                                AVG_Array(14,1)=AVG_OD;
                                AVG_Array(14,2)=AVG_P;
                                AVG_Array(14,3)=AVG_F;
                                AVG_Array(14,4)=AVG_L;
                                AVG_Array(14,5)=AVG_S;
                            else
                                error('Try Again')
                            end
                        end
                    end
                end
            end
        end
    end
end
save('P_16_20');
data = xlswrite(filename,AVG_Array,1);
end

Averages Force-Elongation Data to 20 Points
%Average Force Length
Derek Bivona
%DMT Data
11/2015

%Data is sorted by axial stretch for each different pressure. The average
% diameter, pressure, force, length, and axial stretch are calculated per
% different axial stretch.

These files should contain either the loading or unloading phase of
the last cycle of the test.

filename = '082820-CER-PP-Passive-';
ver = '';
test = 'c'; % for elastase, '' for control

select loading or unloading cycle
type='loading';
% type='unloading';
P_unload_obs = 3;
FL_id = [1 2 3 4];

This section of code finds the maximum and minimum axial stretches
during the force-length tests to organize data points for averaging.

if test=='c'
    sel = strcat(filename,ver,'FL1');
else if test=='e'
    sel = strcat(filename,ver,'fle10');
end

b = 20; % for loading or unloading

Clear surge
clear AVG
clear column

data = xlsread(sel);
% data(rows, columns)
% columns 1,3,5,7,9,11,13,15 are time
% data(:,2) = Outer Diameter (um)
% data(:,4) = Inlet Pressure (mmHg)
% data(:,6) = Outlet Pressure (mmHg)
% data(:,8) = Mean Pressure (mmHg)
% data(:,10) = Force (mN)
% data(:,12) = Temperature (degree C)
% data(:,14) = pH
% data(:,16) = Flow
% data(:,17) = Length (mm)
% data (:,18) = Axial Stretch
% data (1,19) = Unloaded length (mm)
d = size(data);

Find closest value to unloaded pressure (USING MEAN PRESSURE)
% for inlet pressure change 8 to 4
closest = (data(:,8) - P_unload_obs);
pos_closest = closest(closest>0);
min_closest = min(pos_closest);
max_closest=max(pos_closest);
[row,col] = find(closest == max_closest);
P_unload_exp = data(row(length(row)),8);

%Shift inlet and mean pressures so that P_unload is the new zero
for n = 1:d(1,1);
data(n,4) = data(n,4) - P_unload_obs;
data(n,8) = data(n,8) - P_unload_obs;
end

%Define NEW maximum pressure for organization in the next step
P_max = max(data(:,8)); %derek's
P_max = 25;
P_min=min(data(:,8));

limits = xlsread(sel);
size_limit = size(limits);

if strcmp(type, 'unloading')
    upper = limits(1,18); %unloading
    lower = limits(size_limit(1,1),18); %unloading
elseif strcmp(type, 'loading')
    lower = limits(1,18); %loading
    upper = limits(size_limit(1,1),18); %loading
end

%This section of code loops through the four different force-length
%tests and outputs an excel spreadsheet for each test with the desired
%averaged values.
for i = 1:length(FL_id);
    if test=='c'
        sel = strcat(filename, 'FL',ver, int2str(FL_id(i)));
    else if test=='e'
        sel = strcat(filename, 'FL',ver, test, int2str(FL_id(i)));
    end
end

%clear variables each loop
clear seek
clear AVG
clear column
data = xlsread(sel);
d = size(data);

% Shift inlet and mean pressures so that P_unload is the new zero
for n = 1:d(1,1);
    data(n,4) = data(n,4) - P_unload_obs;
    data(n,8) = data(n,8) - P_unload_obs;
end

% This section of code calculates the limits of the intervals
int = (upper - lower)/b;
for t = 1:b+1
    B(t) = upper - (t-1)*int;
end

% This section of code reads in the axial stretch and organizes in
the % desired amount of steps (b = ...)
for j = 1:d(1,1);
    for q = 1:b
        if data(j,18) <= B(q) && data(j,18) >= B(q+1)
            seek(j,q) = data(j,18);
        end
    end
end

% The command sum(seek(:,a)~=0) outputs the number of non-zero
elements % in the 'a' column of the 'seek' matrix. This will allow the
program % to average the corresponding desired values based on the axial
stretch % intervals in the Excel file. The following loop performs this
% action.

% The average diameter is located in column 1, the average pressure
% is
% located in column 2, the average force is located in column 3, the
% average length is located in column 4, and the average axial
stretch
% is located in column 5.
column(1) = sum(seek(:,1)~=0)+1;
AVG(1,1) = mean(data(1:column(1)-1,2));
AVG(1,2) = mean(data(1:column(1)-1,8));
AVG(1,3) = mean(data(1:column(1)-1,10));
AVG(1,4) = mean(data(1:column(1)-1,17));
AVG(1,5) = mean(data(1:column(1)-1,18));
for n = 2:b-1;
    column(n) = column(n-1) + sum(seek(:,n)~=0);
    AVG(n,1) = mean(data(column(n-1):column(n)-1,2));
    AVG(n,2) = mean(data(column(n-1):column(n)-1,8));
    AVG(n,3) = mean(data(column(n-1):column(n)-1,10));
    AVG(n,4) = mean(data(column(n-1):column(n)-1,17));
AVG(n,5) = mean(data(column(n-1):column(n)-1,18));
end

col_PD = [2 8 10 17 18];

% preserve last data point
for t = 1:5;
    AVG(b,t) = data(d(1,1),col_PD(t));
end

% This section of code outputs an Excel file with the average data for
% each test.
if test=='c'
    filename2 = strcat(filename,ver,'p',int2str(FL_id(i)),'passive');
elseif test=='e'
    filename2 = strcat(filename,ver,test,int2str(FL_id(i)));
end

data = x1swrite(filename2,AVG,1);
end
A.7.2 Calculating Stress and Stretch

close all
clear all

%%Calculates PK Stress Strain and Outputs figures for PD Test

Organ='CER'
Date= '082820'
% CvsE=input('Control c or Elastase e?')
CvsE= 'c'

CERfilename =strcat(Date,'-CER-PP-Passive-passive')

Hero = load(strcat(CERfilename))

%% Unloaded Geometry
    Hero.passive.H = Hero.passive.H/1000;
    Hero.passive.OD = Hero.passive.OD/1000;
    Hero.passive.ID = Hero.passive.OD-(2*Hero.passive.H);
    Hero.passive.OR = Hero.passive.OD/2;
    Hero.passive.IR = Hero.passive.ID/2;
    Hero.passive.R = (Hero.passive.OR+Hero.passive.IR)/2;
    cross_sectional_area = pi*(Hero.passive.OR^2 - Hero.passive.IR^2);

%% Test 1
%Axial Stretch
    Hero.passive.biaxial_Pd.test1data.lambda_z =
    Hero.passive.biaxial_Pd.test1(:,5)./Hero.passive.L;

%Geometry
    Hero.passive.biaxial_Pd.test1data.od_mm =
    Hero.passive.biaxial_Pd.test1(:,2)./1000;
    Hero.passive.biaxial_Pd.test1data.or_mm =
    Hero.passive.biaxial_Pd.test1data.od_mm/2;
    Hero.passive.biaxial_Pd.test1data.ir_mm =
    sqrt(Hero.passive.biaxial_Pd.test1data.or_mm.^2 -
    (cross_sectional_area./(pi.*(Hero.passive.biaxial_Pd.test1data.lambda_z)))
    );
    Hero.passive.biaxial_Pd.test1data.r_mm =
    (Hero.passive.biaxial_Pd.test1data.or_mm +
    Hero.passive.biaxial_Pd.test1data.ir_mm)/2;

%Circ and Radial Stretch
    Hero.passive.biaxial_Pd.test1data.lambda_t =
    Hero.passive.biaxial_Pd.test1data.r_mm./Hero.passive.R;
    Hero.passive.biaxial_Pd.test1data.lambda_r =
    1./(Hero.passive.biaxial_Pd.test1data.lambda_t.*Hero.passive.biaxial_Pd
    .test1data.lambda_z);

%Pressure
    Hero.passive.biaxial_Pd.test1data.pressure =
    Hero.passive.biaxial_Pd.test1(:,1).*133.32;
```matlab
Hero.passive.biaxial_Pd.test1data.pressurekPa = Hero.passive.biaxial_Pd.Test1data.pressure./1000;

% 1st PK Stress Circ

Hero.passive.biaxial_Pd.test1data.PKstress_t = (Hero.passive.biaxial_Pd.test1data.pressure.*Hero.passive.biaxial_Pd.test1data.ir_mm)./...
    ((Hero.passive.biaxial_Pd.test1data.or_mm-Hero.passive.biaxial_Pd.test1data.ir_mm).*Hero.passive.biaxial_Pd.test1data.lambda_t)./1000;

% 1st PK Stress Axial
% TERM 1: Axial Transducer Force
Hero.passive.biaxial_Pd.test1data.StressZ_part1 = (Hero.passive.biaxial_Pd.test1(:,3)./1000)./(pi*(((Hero.passive.biaxial_Pd.test1data.or_mm.^2)-(Hero.passive.biaxial_Pd.test1data.ir_mm.^2))./1000000));
% TERM 2: Adjust Pressure to Force/Stress
Hero.passive.biaxial_Pd.test1data.StressZ_part2 = (Hero.passive.biaxial_Pd.test1data.pressure.*((Hero.passive.biaxial_Pd.test1data.ir_mm.^2))./1000000)./...
    (((Hero.passive.biaxial_Pd.test1data.or_mm-Hero.passive.biaxial_Pd.test1data.ir_mm)./1000).*((Hero.passive.biaxial_Pd.test1data.or_mm+Hero.passive.biaxial_Pd.test1data.ir_mm)./1000));

Hero.passive.biaxial_Pd.test1data.PKstress_z = (1./Hero.passive.biaxial_Pd.test1data.lambda_z).*((Hero.passive.biaxial_Pd.test1data.StressZ_part1+Hero.passive.biaxial_Pd.test1data.StressZ_part2)./1000);

% Test 2
% Axial Stretch
Hero.passive.biaxial_Pd.test2data.lambda_z = Hero.passive.biaxial_Pd.test2(:,5)./Hero.passive.L;

% Geometry
Hero.passive.biaxial_Pd.test2data.od_mm = Hero.passive.biaxial_Pd.test2(:,2)./1000;
Hero.passive.biaxial_Pd.test2data.or_mm = Hero.passive.biaxial_Pd.test2data.od_mm/2;
Hero.passive.biaxial_Pd.test2data.ir_mm = sqrt(Hero.passive.biaxial_Pd.test2data.or_mm.^2-
    (cross_sectional_area./(pi.*(Hero.passive.biaxial_Pd.test2data.lambda_z))));
Hero.passive.biaxial_Pd.test2data.r_mm = (Hero.passive.biaxial_Pd.test2data.or_mm + Hero.passive.biaxial_Pd.test2data.ir_mm)./2;

% Circ and RadialStretch
Hero.passive.biaxial_Pd.test2data.lambda_t = Hero.passive.biaxial_Pd.Test2data.r_mm./Hero.passive.R;
Hero.passive.biaxial_Pd.test2data.lambda_r = 1./(Hero.passive.biaxial_Pd.test2data.lambda_t.*Hero.passive.biaxial_Pd.test2data.lambda_z));```
%Pressure

Hero.passive.biaxial_Pd.test2data.pressure = Hero.passive.biaxial_Pd.test2(:,1).*133.32;
Hero.passive.biaxial_Pd.test2data.pressurekPa = Hero.passive.biaxial_Pd.test2data.pressure./1000;

%1st PK Stress Circ

Hero.passive.biaxial_Pd.test2data.PKstress_t = (Hero.passive.biaxial_Pd.test2data.pressure.*Hero.passive.biaxial_Pd.test2data.ir_mm)./(Hero.passive.biaxial_Pd.test2data.or_mm-Hero.passive.biaxial_Pd.test2data.ir_mm).*Hero.passive.biaxial_Pd.test2data.lambda_t)./1000;

%1st PK Stress Axial

%TERM 1: Axial Transducer Force

Hero.passive.biaxial_Pd.test2data.StressZ_part1 = (Hero.passive.biaxial_Pd.test2(:,3)./1000)./(pi*((Hero.passive.biaxial_Pd.test2data.or_mm.^2)-(Hero.passive.biaxial_Pd.test2data.ir_mm.^2))./1000000));

%TERM 2: Adjust Pressure to Force/Stress

Hero.passive.biaxial_Pd.test2data.StressZ_part2 = ((Herp.passive.biaxial_Pd.test2data.pressure.*((Hero.passive.biaxial_Pd.test2data.ir_mm.^2))./1000000)./(Hero.passive.biaxial_Pd.test2data.or_mm-Hero.passive.biaxial_Pd.test2data.ir_mm)./1000).*((Hero.passive.biaxial_Pd.test2data.or_mm+Hero.passive.biaxial_Pd.test2data.ir_mm)./1000)

Hero.passive.biaxial_Pd.test2data.PKstress_z = (1./Hero.passive.biaxial_Pd.test2data.lambda_z).*((Hero.passive.biaxial_Pd.test2data.StressZ_part1+Hero.passive.biaxial_Pd.test2data.StressZ_part2)./1000);

% Test 3

%Axial Stretch

Hero.passive.biaxial_Pd.test3data.lambda_z = Hero.passive.biaxial_Pd.test3(:,5)./Hero.passive.L;

%Geometry

Hero.passive.biaxial_Pd.test3data.od_mm = Hero.passive.biaxial_Pd.test3(:,2)./1000;
Hero.passive.biaxial_Pd.test3data.or_mm = Hero.passive.biaxial_Pd.test3data.od_mm/2;
Hero.passive.biaxial_Pd.test3data.ir_mm = sqrt(Hero.passive.biaxial_Pd.test3data.or_mm.^2-(cross_sectional_area./(pi.*HERO.biaxial_Pd.test3data.lambda_z))));
Hero.passive.biaxial_Pd.test3data.r_mm = (Hero.passive.biaxial_Pd.test3data.or_mm + Hero.passive.biaxial_Pd.test3data.ir_mm)/2;

%Circ and RadialStretch

Hero.passive.biaxial_Pd.test3data.lambda_t = Hero.passive.biaxial_Pd.test3data.r_mm./Hero.passive.R;
Hero.passive.biaxial_Pd.test3data.lambda_r =
1./(Hero.passive.biaxial_Pd.test3data.lambda_t.*Hero.passive.biaxial_Pd.
test3data.lambda_z);

%Pressure
Hero.passive.biaxial_Pd.test3data.pressure =
Hero.passive.biaxial_Pd.test3(:,1).*133.32;
Hero.passive.biaxial_Pd.test3data.pressurekPa =
Hero.passive.biaxial_Pd.test3data.pressure./1000;

1st PK Stress Circ

Hero.passive.biaxial_Pd.test3data.PKstress_t =
(Hero.passive.biaxial_Pd.test3data.pressure.*Hero.passive.biaxial_Pd.te
st3data.ir_mm)./
((Hero.passive.biaxial_Pd.test3data.or_mm-Hero.passive.biaxial_Pd.test3data.ir_mm).*Hero.passive.biaxial_Pd.test3
data.lambda_t)./1000;

1st PK Stress Axial

%TERM 1: Axial Transducer Force
Hero.passive.biaxial_Pd.test3data.StressZ_part1 =
(Hero.passive.biaxial_Pd.test3(:,3)./1000)./(pi*(((Hero.passive.biaxial
_Pd.test3data.or_mm.^2))-
(Hero.passive.biaxial_Pd.test3data.ir_mm.^2))/.1000000);
%TERM 2: Adjust Pressure to Force/Stress
Hero.passive.biaxial_Pd.test3data.StressZ_part2 =
(Hero.passive.biaxial_Pd.test3data.pressure.*((Hero.passive.biaxial_Pd.
test3data.ir_mm.^2))/.1000000).
((Hero.passive.biaxial_Pd.test3data.or_mm-Hero.passive.biaxial_Pd.test3data.ir_mm)/1000).*(Hero.passive.biaxial
_Pd.test3data.or_mm+Hero.passive.biaxial_Pd.test3data.ir_mm)/1000));

Hero.passive.biaxial_Pd.test3data.PKstress_z =
(1./Hero.passive.biaxial_Pd.test3data.lambda_z).*(Hero.passive.biaxial
_Pd.test3data.StressZ_part1+Hero.passive.biaxial_Pd.test3data.StressZ_p
art2)/1000);

% SAVE FILE

%Save data in .mat file
if CvsE=='c'
file=strcat(CERfilename, '_1stPK_ss.mat')
save(file)
else if CvsE=='e'
file=strcat(Date,'-',Organ,'e','_1stPKss.mat')
save(file)
end

% PLOTS
subplot(2,2,1)
hold on
plot(Hero.passive.biaxial_Pd.test1data.lambda_t,Hero.passive.biaxial_Pd.
test1data.PKstress_t ,'o','MarkerSize', 5, 'Color', [0 0 0])
plot(Hero.passive.biaxial_Pd.test2data.lambda_t,Hero.passive.biaxial_Pd.test2data.PKstress_t, 'o', 'MarkerSize', 15, 'Color', [0 0 0])

plot(Hero.passive.biaxial_Pd.test3data.lambda_t,Hero.passive.biaxial_Pd.test3data.PKstress_t, 's', 'MarkerSize', 5, 'Color', [0.25 0.25 0.25])

ylabel(' \sigma_\vartheta (\text{kPa})')
xlabel(' \lambda_\vartheta')
subplot(2,2,2)
hold on

plot(Hero.passive.biaxial_Pd.test1data.lambda_z,Hero.passive.biaxial_Pd.test1data.PKstress_z, 'o', 'MarkerSize', 5, 'Color', [.25 .25 .25])
plot(Hero.passive.biaxial_Pd.test2data.lambda_z,Hero.passive.biaxial_Pd.test2data.PKstress_z, 'o', 'MarkerSize', 5, 'Color', [.25 .25 .25])
plot(Hero.passive.biaxial_Pd.test3data.lambda_z,Hero.passive.biaxial_Pd.test3data.PKstress_z, 'o', 'MarkerSize', 5, 'Color', [.25 .25 .25])

ylabel(' \sigma_z (\text{kPa})')
xlabel(' \lambda_z')
subplot(2,2,3)
hold on

plot(Hero.passive.biaxial_Pd.test1data.lambda_t,Hero.passive.biaxial_Pd.test1(:,3), 'o', 'MarkerSize', 5, 'Color', [.25 .25 .25])
plot(Hero.passive.biaxial_Pd.test2data.lambda_t,Hero.passive.biaxial_Pd.test2(:,3), 'o', 'MarkerSize', 5, 'Color', [.25 .25 .25])
plot(Hero.passive.biaxial_Pd.test3data.lambda_t,Hero.passive.biaxial_Pd.test3(:,3), 'o', 'MarkerSize', 5, 'Color', [.25 .25 .25])

ylabel('Force (\text{mN})')
xlabel('Pressure (\text{kPa})')
%Save Figure
if CvsE=='c'
filename = strcat(CERfilename,'-1stPK_ss')
savefig(filename)
print('-dtiff','-r300',filename);
else if CvsE=='e'
    filename = strcat(Date,'-', Organ,'e','1stPK_ss')
savefig(filename)
    print('-dtiff','-r300',filename);
end
end

hold off
A.7.3 Material Stiffness and Compliance

%Stiffness and Compliance for CERVIX 05 22 20 Cassie Conway
clc all;

clear all;

file = '110719-CER-PP-Max-passive_1stPK_ss';
load(file);

%Distensibility = (V_loaded/V_unloaded)/P
%Compliance = V_loaded/ P
%Compliance = DxV^2

%PD TEST 1 -1% Below
OD_PD1 = Hero.passive.biaxial_Pd.test1data.od_mm(3,:);
P_PD1 = Hero.passive.biaxial_Pd.test1data.pressurekPa(3,:);

od_PD1 = Hero.passive.biaxial_Pd.test1data.od_mm(7,:);
p_PD1 = Hero.passive.biaxial_Pd.test1data.pressurekPa(7,:);

C_PD1 = (od_PD1 - OD_PD1)./(p_PD1 - P_PD1);

%PD TEST 2 EPL
OD_PD2 = Hero.passive.biaxial_Pd.test2data.od_mm(3,:);
P_PD2 = Hero.passive.biaxial_Pd.test2data.pressurekPa(3,:);

od_PD2 = Hero.passive.biaxial_Pd.test2data.od_mm(7,:);
p_PD2 = Hero.passive.biaxial_Pd.test2data.pressurekPa(7,:);

C_PD2 = (od_PD2 - OD_PD2)./(p_PD2 - P_PD2);

%PD TEST 2 +1% Above
OD_PD3 = Hero.passive.biaxial_Pd.test3data.od_mm(3,:);
P_PD3 = Hero.passive.biaxial_Pd.test3data.pressurekPa(3,:);

od_PD3 = Hero.passive.biaxial_Pd.test3data.od_mm(7,:);
p_PD3 = Hero.passive.biaxial_Pd.test3data.pressurekPa(7,:);

C_PD3 = (od_PD3 - OD_PD3)./(p_PD3 - P_PD3);

save(strcat('Compliance',file),'C_PD1','C_PD2','C_PD3')

%%Pearson's Modulus
%Stress Above - Stress Below/ Stretch Above- Stretch Below

%PD Test 1 -1%
PKstress_t_Below_PD1 =
Hero.passive.biaxial_Pd.test1data.PKstress_t(3,:);
Stretch_t_Below_PD1 = Hero.passive.biaxial_Pd.test1data.lambda_t(3,:);

PKstress_t_Above_PD1 =
Hero.passive.biaxial_Pd.test1data.PKstress_t(7,:);
Stretch_t_Above_PD1 = Hero.passive.biaxial_Pd.test1data.lambda_t(7,:);

PM_t_PD1 = (PKstress_t_Above_PD1 -
PKstress_t_Below_PD1)./(Stretch_t_Above_PD1-Stretch_t_Below_PD1);
%PD Test 2 EPL
PKstress_t_Below_PD2 =
Hero.passive.biaxial_Pd.test2data.PKstress_t(3,:);
Stretch_t_Below_PD2 = Hero.passive.biaxial_Pd.test2data.lambda_t(3,:);

PKstress_t_Above_PD2 =
Hero.passive.biaxial_Pd.test2data.PKstress_t(7,:);
Stretch_t_Above_PD2 = Hero.passive.biaxial_Pd.test2data.lambda_t(7,:);

PM_t_PD2 = (PKstress_t_Above_PD2 -
PKstress_t_Below_PD2)./(Stretch_t_Above_PD2 - Stretch_t_Below_PD2);

%PD TEST 3 1% Above
PKstress_t_Below_PD3 =
Hero.passive.biaxial_Pd.test3data.PKstress_t(3,:);
Stretch_t_Below_PD3 = Hero.passive.biaxial_Pd.test3data.lambda_t(3,:);

PKstress_t_Above_PD3 =
Hero.passive.biaxial_Pd.test3data.PKstress_t(7,:);
Stretch_t_Above_PD3 = Hero.passive.biaxial_Pd.test3data.lambda_t(7,:);

PM_t_PD3 = (PKstress_t_Above_PD3 -
PKstress_t_Below_PD3)./(Stretch_t_Above_PD3 - Stretch_t_Below_PD3);

%Save File and Variables
save(strcat('Circ_Material_Stiffness',file),'PM_t_PD1','PM_t_PD2','PM_t_PD3')

%%
%AXIAL PEARSONS MODULI
%PD Test 1 -1%
PKstress_z_Below_PD1 =
Hero.passive.biaxial_Pd.test1data.PKstress_z(3,:);

PKstress_z_Above_PD1 =
Hero.passive.biaxial_Pd.test1data.PKstress_z(7,:);

PM_Z_PD1 = (PKstress_z_Above_PD1 -
PKstress_z_Below_PD1)./(Stretch_t_Above_PD1 - Stretch_t_Below_PD1);

%PD Test 2 EPL
PKstress_z_Below_PD2 =
Hero.passive.biaxial_Pd.test2data.PKstress_z(3,:);

PKstress_z_Above_PD2 =
Hero.passive.biaxial_Pd.test2data.PKstress_z(7,:);

PM_Z_PD2 = (PKstress_z_Above_PD2 -
PKstress_z_Below_PD2)./(Stretch_t_Above_PD2 - Stretch_t_Below_PD2);

%PD TEST 3 1% Above
PKstress_z_Below_PD3 = Hero.passive.biaxial_Pd.test3data.PKstress_z(3,:);

PKstress_z_Above_PD3 = Hero.passive.biaxial_Pd.test3data.PKstress_z(7,:);

PM_Z_PD3 = (PKstress_z_Above_PD3 - PKstress_z_Below_PD3)./(Stretch_t_Above_PD3 - Stretch_t_Below_PD3);

%Sav e File and Variables
save(strcat('Axial_Material_Stiffness',file),'PM_Z_PD1','PM_Z_PD2','PM_Z_PD3')
References:


[120] B. Garipcan, S. Maenz, T. Pham, U. Settmacher, K.D. Jandt, J. Zanow, J. Bossert, Image Analysis of Endothelial Microstructure and Endothelial Cell Dimensions of


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