Biaxial Mechanical Assessment of the Murine Vaginal Wall Using Extension–Inflation Testing

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Progress toward understanding the underlying mechanisms of pelvic organ prolapse (POP) is limited, in part, due to a lack of information on the biomechanical properties and microstructural composition of the vaginal wall. Compromised vaginal wall integrity is thought to contribute to pelvic floor disorders; however, normal structure–function relationships within the vaginal wall are not fully understood. In addition to the information produced from uniaxial testing, biaxial extension–inflation tests performed over a range of physiological values could provide additional insights into vaginal wall mechanical behavior (i.e., axial coupling and anisotropy), while preserving in vivo tissue geometry. Thus, we present experimental methods of assessing murine vaginal wall biaxial mechanical properties using extension–inflation

protocols. Geometrically intact vaginal samples taken from 16 female C57BL/6 mice underwent pressure–diameter and force–length preconditioning and testing within a pressure–myograph device. A bilinear curve fit was applied to the local stress–stretch data to quantify the transition stress and stretch as well as the toe- and linear-region moduli. The murine vaginal wall demonstrated a nonlinear response resembling that of other soft tissues, and evaluation of bilinear curve fits suggests that the vagina exhibits pseudoelectricity, axial coupling, and anisotropy. The protocols developed herein permit quantification of biaxial tissue properties. These methods can be utilized in future studies in order to assess evolving structure–function relationships with respect to aging, the onset of prolapse, and response to potential clinical interventions. [DOI: 10.1115/1.4037559]

Keywords: vaginal wall, women's health, mechanical testing, pelvic floor disorders

Introduction

Pelvic organ prolapse (POP) is a global health concern characterized by a loss of pelvic support that allows for the descent of the female pelvic organs (uterus, cervix, and vaginal wall) into the pelvic cavity. This may result in protrusion of the pelvic organs through the vaginal opening [1] and lead to issues such as incomplete bladder emptying, pressure, and defecatory dysfunction. As a result, many women experience sexual dysfunction, pain, discomfort, poor self-image, limitations in physical activity, and a reduced quality of life [2]. Over 40% of women are affected by POP. Of those, 11% require surgical intervention [3], and 30% of those surgical cases result in re-operation [4], leading to an annual direct cost of over 1 billion dollars [5]. In addition, patients typically develop POP between the ages of 50–70. Therefore, without adequate treatment, many women could potentially be dealing with POP for 30–50% of their lifespans [6].

The lack of effective treatment options arises, in part, from a limited understanding of the basic science of the vaginal wall and surrounding pelvic support structures. Furthermore, there is particular lack of understanding with respect to the biomechanical properties and microstructural composition of the vaginal wall. For example, levator ani injury is well established as a potential mechanism of POP [7,8]; however, a large proportion of prolapse cases exhibit no evidence of muscle injury [9]. Additionally, POP has also been associated with weakness or failure of the vaginal wall in conjunction with the cardinal and uterosacral ligaments, which are heavily involved in providing level I support that is often absent in cases of prolapse [10–12]. Despite these findings, the underlying mechanism that leads to POP has yet to be ascertained. Work to date has predominantly relied on uniaxial testing [13–21]. Uniaxial experiments can provide meaningful data but do not preserve the in vivo geometry, account for the anisotropy, or consider the axial coupling that most soft tissues exhibit [22]. Recent work has demonstrated the importance of biaxial assessment by evaluating anisotropy and axial coupling through planar biaxial tests on excised rat vaginal tissue [23,24]. Biaxial experiments performed on swine uterosacral/cardinal ligaments as well as a recent review by Baah-Dwomoh et al. further indicate the importance of biaxial properties of pelvic tissues [12,25,26]. Alternatively, studies in vascular biomechanics have biomechanically phenotyped central arteries using biaxial protocols involving cyclic pressure–diameter and axial force–length tests [27,28]. These protocols enable preservation of in vivo tissue geometry and native cell–matrix interactions while performing biaxial assessment in which stress or stretch can be applied simultaneously as well as independently along two perpendicular axes. Ultimately, biaxial extension–inflation tests recreate the multi-axial loads that the tissue experiences in the body and permit assessment under a variety of physiological conditions of interest. Additionally, recent work has demonstrated that animal models, including rodent models, offer valuable insights into fundamental

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structure–function relationships in the pelvic floor [12,17,22,26,29–34]. In particular, genetically modified mice missing crucial genes for elastic fiber assembly readily develop prolapse with increasing age or immediately after vaginal birth [16,31,32,35–46]. Further, rodent models of vaginal wound healing offer valuable insights into potential mechanisms of surgical failure in POP patients, including the role of altered steroid hormones on collagen production and mechanical response [30,47,48]. Hence, it is valuable to develop protocols which can assess the biaxial structure–function relationships of the vagina in these rodent models to elucidate dynamic structure–function relationships. Thus, in this paper we present biaxial mechanical testing methods for assessing the mechanical properties of the murine vaginal wall using extension–inflation tests. We hypothesize that, like most soft tissues [22], the vaginal wall exhibits pseudoelasticity, axial coupling, and anisotropy.

Methods

Specimen Preparation. Female C57BL/6 mice ($n = 16$) were euthanized at 4–6 months of age while in estrus in accordance with Tulane University Institutional Animal Care and Use Committee guidelines. All mice underwent one freeze/thaw cycle, after which the entire reproductive tract was excised. Singular cuts at the end of each of uterine horn and past the vaginal introitus were utilized to maximize essential tissue (Fig. 1). During dissection, stain lines were placed along the tissue every 3-mm using India Ink (Dick Blick Art Materials, Galesburg, IL). The *in vivo* axial stretch was estimated by measuring the *in situ* to *ex vivo* change in distance between these lines after 15 min of equilibration in Hank's Balanced Salt Solution (HBSS) containing 136.9 M NaCl, 5.4 M KCl, 491.9 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 440.9 mM KH_2PO_4 , 338.1 mM Na_2HPO_4 , 405.7 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.26 M CaCl_2 , 4.17 M NaHCO_3 , and 5.46 M dextrose [27]. The vagina was then isolated from the surrounding tissue by cutting the sample at the border between the cervix and vagina (Figs. 1 and 2). Postexcise, samples were frozen in HBSS until the day of testing. Note that Rubod et al. showed the mechanical properties of frozen and fresh vaginas were not statistically different [49].

Mechanical Testing. Samples were mounted onto 5-mm-diameter metal cannulas within a pressure-myograph system (Danish MyoTechnologies, Aarhus, Denmark) containing HBSS at 37 °C and secured using two 6-0 silk sutures on each end. Initial unloaded dimensions were measured using a hand-held digital micrometer immediately following cannulation, before the tissue was connected to the pressure transducer. The unloaded length

Fig. 2 Postexcise murine reproductive system demonstrating the point at which the bifurcated external os (two rings) of the cervix merges into the vaginal canal (one ring)

was defined as the point at which the vaginal tissue began to buckle (Fig. 3). Due to the highly compliant nature of the vagina, samples were frequently collapsed at 0 mm Hg. Therefore, pilot studies were conducted, and the unloaded pressure (i.e., pressure at which the vagina was not collapsed) was selected as 4 mm Hg for all samples. The outer diameter at this pressure was selected as the unloaded outer diameter (i.e., the reference diameter) and was measured using an Eclipse TS100 video-microscope (Nikon, Melville, NY). The force was zeroed while the vagina was in the unloaded configuration.

Specimens underwent five cycles of pressure–diameter (p – d) preconditioning ($P = 0$ to 25 mm Hg at the estimated *in vivo* axial stretch) and five cycles of axial force–length (f – l) preconditioning (from 4% below to 4% above the estimated *in vivo* axial stretch) at a rate of 2 mm Hg/s to ensure that hysteresis was minimized and mechanical results were consistent and repeatable. The *in vivo* axial stretch was then re-estimated by identifying the axial stretch at which the force remained nearly constant in response to changes in luminal pressure as described previously [27,28], and the vagina was preconditioned for another five p – d and f – l cycles. Following a 15-min equilibration period (tissue held at 2 mm Hg while in the *in vivo* axial stretch), the estimated unloaded length was re-evaluated and adjusted when necessary while the pressure transducer was set to 0 mm Hg. Specimens then underwent three pressure–diameter tests (pressure cycled from 0 to 25 mm Hg), while axial length was held constant at different axial extensions

Fig. 1 Murine reproductive system pre- (left) and postexcise (right) with white lines denoting the border between the cervix and vagina and approximate distances from the border to the base of the vagina and split of the uterine horns

Fig. 3 Vaginal sample mounted onto cannulas in preparation for testing. The unloaded length (left) can be found by identifying the point at which the ridges on either side of where the urethra was removed from (white dotted lines added for emphasis) begin to buckle. Note that the ridges become more linear in the *in vivo* configuration (right).

Fig. 4 Schematic of the biaxial testing protocol for pressure (top) and force (bottom) over time. The protocol consists of: (I) five cycles of pressure–diameter preconditioning from 0 to 25 mm Hg, (II) five cycles of force–length preconditioning with pressure held at 2 mm Hg and the stretch ranging from –4% to 4% of the unloaded length, (III) equilibration period with the pressure held at 2 mm Hg at the estimated in vivo stretch, (IV) pressure–diameter tests performed at (a) –4% in vivo stretch, (b) in vivo stretch, and (c) 4% in vivo stretch over 0–25 mm Hg, and (V) force–length tests: (a) pressure held at 2 mm Hg, (b) 8 mm Hg, (c) 12 mm Hg, and (d) 25 mm Hg over the range of in vivo stretches.

around the estimated in vivo axial stretch (4% below, 4% above, and at the in vivo axial stretch), followed by four axial force–length tests (axial length cycled from 4% below to 4% above the in vivo axial stretch) while pressure was held constant ($P = 2, 8, 17$, and 25 mm Hg) as shown in Fig. 4 [27,28]. Following testing, a ring (~ 0.5 mm thick) was cut transversely from the center of the vagina, at the location at which the tissue diameter was tracked during mechanical testing. The ring was then placed in HBSS, and following a 30-min equilibration period, was imaged with a Moticam 580 microscope camera (Motic, Richmond, BC, Canada). The anterior section of the ring was cut radially, and the near zero-stress state was imaged 30 min later.

Fig. 5 Schematic of a bilinear curve fit wherein the built-in MATLAB function *lsqcurvefit* is used to identify the moduli of the toe and linear regions as well as the transition stress and stretch at the transition point

Data Analysis. Previous studies presented results from either the loading or unloading cycle of mechanical tests [27,28]. Thus, testing data were taken from the last of both loading and unloading cycles for the f – l and p – d tests in order to evaluate the pseudoelastic behavior of the vaginal wall. Following Amin et al., raw data were divided into intervals of pressure for p – d tests and axial stretch for f – l tests and averaged over those intervals using a custom MATLAB code [27]. Mean circumferential and axial Cauchy stresses and stretches were calculated from the processed biaxial data using the following equations [50]:

$$\langle t_{\theta\theta} \rangle = \frac{P_i r_i}{r_o - r_i} \quad (1)$$

$$\langle t_{zz} \rangle = \frac{P_i \pi r_i^2 + L}{\pi (r_o^2 - r_i^2)} \quad (2)$$

$$\lambda_\theta = \frac{r}{R} \quad (3)$$

$$\lambda_z = \frac{l}{L} \quad (4)$$

$$\lambda_r = \frac{l}{\lambda_\theta \lambda_z} \quad (5)$$

where $t_{\theta\theta}$ and t_{zz} are the mean circumferential and axial Cauchy stresses, respectively. P_i is the pressure measured by the pressure transducer, r_i is the deformed inner radius, and r_o is the deformed outer radius calculated from online measurements of the outer diameter. L is the unloaded length; r and R are the deformed and undeformed “middle” (average) radii, respectively; λ_z , λ_θ , and λ_r are the axial, circumferential, and radial stretches, respectively; and l is the deformed length. Radial stretch and the deformed inner diameter were calculated based on conservation of volume. For example,

$$V = \pi(R_o^2 - R_i^2)L \quad (6)$$

Fig. 6 Testing data (mean $\pm$ SEM) from pressure–diameter (a) and (b) and force–length (c) protocols conducted on $n = 16$ specimens. Estimation of the in vivo axial stretch ratio based on the near constancy of the transducer-measured force–pressure response during the cyclic pressure–diameter testing (b), and typical force–length responses for which the intersection in the force–axial stretch data, denoted by the black line, reveals the in vivo axial stretch (c) as described previously in Refs. [52] and [55].

Fig. 7 Circumferential Cauchy stress–stretch curves (mean $\pm$ SEM of $n = 16$ specimens) calculated from p – d testing data for loading (filled) and unloading (unfilled) cycles at the estimated in vivo axial stretch, denoting the effect of cycle on vaginal wall mechanical behavior

$$r_i = \sqrt{r_o^2 - \frac{\bar{V}}{\pi l}} \quad (7)$$

where R_o is the undeformed outer radius; R_i is the undeformed inner radius calculated from the unloaded wall thickness determined during opening angle experiments; r_i is the deformed outer radius; r_o is the deformed inner radius; and $\bar{V}$ is the mean volume. A bilinear curve fit (Fig. 5) was applied to the stress–stretch data to quantify the moduli in the toe- and linear-regions as well as the stress and stretch at the transition point between the toe- and linear-regions [51]. The in vivo axial stretch was confirmed by identifying the point at which the

force–axial stretch responses intersect (Fig. 6(c)) as described previously in Ref. [52]. The opening angle was measured from the midpoint of the inner wall to the outer tips of the opened sections using the angle tool in ImageJ (NIH, Bethesda, MD) [50].

Statistical Analysis. In order to evaluate the effect of pseudoelasticity (i.e., loading versus unloading cycle), and axial coupling (i.e., p – d tests at 4% below, 4% above, and at the in vivo axial stretch and f – l tests at 2, 8, 17, and 25 mm Hg) on vaginal wall mechanical behavior, two-way analysis of variance (ANOVA) tests with repeated measures were conducted for each output of the bilinear fits. Comparisons with $p < 0.05$ were considered significant. Anisotropy (i.e., circumferential versus axial direction) was evaluated using paired, two-tailed student t -tests (12 comparisons). A Bonferroni correction was applied, and comparisons with $p < 0.0042$ ($p < 0.05/12$) were considered significant. Data are presented as mean $\pm$ standard error of the mean (SEM).

Results

Mechanical assessment of the murine vaginal wall revealed a nonlinear behavior resembling that of other soft tissues (Fig. 6) [28,53,54]. The vagina additionally demonstrated a similar behavior to arteries, wherein the measured axial force remained nearly constant in response to changes in luminal pressure at the estimated in vivo axial stretch ($\lambda_z^{iv} = 1.15 \pm 0.005$) (Fig. 6(b)) (cf. Fig. 2 in Ref. [28]) [55]. The values of λ_z^{iv} were confirmed as the value of axial stretch where the f – λ_z responses overlapped for multiple luminal pressures (Fig. 6(c)) [52]. The average values for unloaded outer diameter, length, thickness, and opening angle were $4952.25 \pm 35.40 \mu\text{m}$, $4.96 \pm 0.04 \text{ mm}$, $769.86 \pm 49.86 \mu\text{m}$, and $74.41 \pm 17.67 \text{ deg}$, respectively.

Evaluation of Vaginal Pseudoelastic Properties. Bilinear fits were applied to the circumferential stress versus circumferential stretch ($t_{\theta\theta} - \lambda_{\theta}$) curves (Fig. 7) calculated from the pressure–diameter tests. Average values are reported in Table 1. Two-way ANOVA tests with repeated measures revealed that

Table 1 Average bilinear fit results for the $t_{\theta\theta} - \lambda_{\theta}$ curve. Data from the $n = 16$ specimens are presented as mean $\pm$ SEM.

Cycle	Test type	BP_x	BP_y (kPa)	E_T (kPa)	E_L (kPa)
Loading	4% below	1.10 ± 0.02	2.30 ± 0.16	22.19 ± 6.96	267.31 ± 23.08
	in vivo	1.09 ± 0.02	2.35 ± 0.19	26.14 ± 7.94	249.60 ± 19.98
	4% above	1.08 ± 0.02	2.42 ± 0.14	23.35 ± 5.29	233.70 ± 22.74
Unloading	4% below	1.12 ± 0.02	2.93 ± 0.19	66.64 ± 9.69	453.60 ± 46.74
	in vivo	1.11 ± 0.02	3.08 ± 0.20	65.24 ± 10.71	405.84 ± 44.41
	4% above	1.11 ± 0.02	3.04 ± 0.16	60.17 ± 8.59	453.79 ± 77.97

Table 2 Average bilinear fit results for the $t_{zz}-\lambda_\theta$ curve. Data from the $n = 16$ specimens are presented as mean $\pm$ SEM.

Cycle	Test type	BP_x	BP_y (kPa)	E_T (kPa)	E_L (kPa)
Loading	4% below	1.11 $\pm$ 0.02	1.39 $\pm$ 0.18	7.52 $\pm$ 2.23	77.10 $\pm$ 9.35
	in vivo	1.10 $\pm$ 0.02	3.35 $\pm$ 0.47	16.34 $\pm$ 4.75	113.57 $\pm$ 9.60
	4% above	1.07 $\pm$ 0.02	6.16 $\pm$ 1.13	17.13 $\pm$ 4.83	130.44 $\pm$ 12.90
Unloading	4% below	1.13 $\pm$ 0.02	1.62 $\pm$ 0.17	21.64 $\pm$ 3.25	152.30 $\pm$ 24.51
	in vivo	1.11 $\pm$ 0.02	3.33 $\pm$ 0.40	31.56 $\pm$ 4.40	171.76 $\pm$ 16.30
	4% above	1.11 $\pm$ 0.02	5.96 $\pm$ 1.11	40.43 $\pm$ 6.25	250.53 $\pm$ 40.14

Table 3 Average bilinear fit results for the $t_{zz}-\lambda_z$ curve. Data from the $n = 16$ specimens are presented as mean $\pm$ SEM.

Cycle	Test type	BP_x	BP_y (kPa)	E_T (kPa)	E_L (kPa)
Loading	2 mm Hg	1.16 $\pm$ 0.02	2.23 $\pm$ 0.34	36.43 $\pm$ 6.89	122.31 $\pm$ 23.05
	8 mm Hg	1.16 $\pm$ 0.02	3.82 $\pm$ 0.41	55.28 $\pm$ 7.68	139.80 $\pm$ 24.05
	17 mm Hg	1.15 $\pm$ 0.02	5.13 $\pm$ 0.44	73.32 $\pm$ 8.86	148.39 $\pm$ 21.75
	25 mm Hg	1.15 $\pm$ 0.02	6.38 $\pm$ 0.51	87.00 $\pm$ 9.16	164.98 $\pm$ 21.21
Unloading	2 mm Hg	1.17 $\pm$ 0.02	2.17 $\pm$ 0.27	33.14 $\pm$ 5.29	135.86 $\pm$ 27.62
	8 mm Hg	1.16 $\pm$ 0.02	3.49 $\pm$ 0.32	47.86 $\pm$ 5.61	149.22 $\pm$ 27.24
	17 mm Hg	1.16 $\pm$ 0.02	5.05 $\pm$ 0.38	64.62 $\pm$ 6.76	164.69 $\pm$ 25.26
	25 mm Hg	1.16 $\pm$ 0.02	6.52 $\pm$ 0.48	80.58 $\pm$ 8.02	192.26 $\pm$ 27.15

cycle (loading versus unloading) had a significant effect on all four outputs of the $t_{\theta\theta}-\lambda_\theta$ curve bilinear fits: stretch at the transition point between the toe and linear region (BP_x), stress at the transition point (BP_y), toe-region modulus (E_T), and linear-region modulus (E_L). Additionally, bilinear fits were applied to the $t_{zz}-\lambda_\theta$ values (Fig. 8) determined from the pressure–diameter tests (Table 2). Two-way ANOVA tests with repeated measures revealed that cycle had a significant effect on transition stretch, toe-region modulus, and linear-region modulus of the $t_{zz}-\lambda_\theta$ curves; however, cycle did not significantly affect the transition stress. Finally, bilinear fits were applied to the $t_{zz}-\lambda_z$ values (Fig. 9) calculated from the force–length tests (Table 3). Two-way ANOVA tests with repeated measures revealed that, similar to the $t_{zz}-\lambda_\theta$ data, cycle had a significant effect on the transition stretch, toe modulus, and linear modulus, of the $t_{zz}-\lambda_z$ curve, but was not found to significantly affect the transition stress.

Evaluation of Vaginal Axial Coupling. Two-way ANOVA tests with repeated measures were also used to evaluate the role of axial coupling in vaginal wall mechanics. Comparison by test type (i.e., $p-d$ tests at 4% below, 4% above, and at the in vivo axial stretch) of the $t_{\theta\theta}-\lambda_\theta$ data revealed no significant effect on any of the four bilinear fit parameters. Comparisons of the $t_{zz}-\lambda_\theta$ bilinear fits, however, revealed a significant effect of test type on transition stretch, transition stress, toe modulus, and linear modulus. Test type (i.e., $f-l$ tests at 2 mm Hg, 8, 17, and 25 mm Hg) was also shown to significantly affect the transition stress, transition stretch, toe modulus, and linear modulus of the $t_{zz}-\lambda_z$ curves.

Interaction Between Pseudoelasticity and Axial Coupling. The interaction term between cycle and test type was statistically

Table 4 Reported p values from paired t -tests (12 comparisons with a Bonferroni correction) comparing toe and linear moduli between the circumferential (–4%, in vivo, 4%) and axial (2 mm Hg, 8 mm Hg, 17 mm Hg, and 25 mm Hg) directions. Significance is denoted by *.

	Toe			Linear		
	–4%	in vivo	4%	–4%	in vivo	4%
2 mm Hg	0.1	0.2	0.1	0.001*	0.001*	0.009
8 mm Hg	0.004	0.01	0.001*	0.004*	0.006	0.03
17 mm Hg	0.0004*	0.0008*	0.0001*	0.005	0.007	0.03
25 mm Hg	0.00005*	0.00009*	0.0000009*	0.01	0.02	0.08

significant for the transition stress of the $t_{zz}-\lambda_\theta$ curve ($p=0.027$). Interaction terms for the remaining three bilinear fit outputs of the $t_{zz}-\lambda_\theta$ curve were not significant, nor were they significant for any of the four outputs of both $t_{\theta\theta}-\lambda_\theta$ and $t_{zz}-\lambda_z$ curves.

Evaluation of Vaginal Wall Anisotropy. To evaluate the anisotropy of the murine vaginal wall, all combinations of circumferential and axial toe and linear modulus (12 comparisons) from the loading cycle of $p-d$ and $f-l$ tests were compared using paired two-tailed t -tests (Table 4). Paired t -tests revealed a significantly higher linear modulus in the circumferential direction compared to the axial when comparing the $p-d$ test conducted at 4% below the in vivo axial stretch and the $f-l$ tests conducted at both 2 mm Hg and 8 mm Hg. The comparison between the $p-d$ test conducted at the in vivo axial stretch and the $f-l$ test conducted at 2 mm Hg also demonstrated a significantly higher linear modulus in the circumferential direction. The toe-region modulus, however, was

Fig. 8 Axial Cauchy stress versus circumferential stretch curves (mean $\pm$ SEM of $n = 16$ specimens) calculated from $p-d$ testing data for loading (filled) and unloading (unfilled) cycles at 4% above (gray) and below (black) the in vivo axial stretch, denoting the effect of cycle and axial coupling on vaginal wall mechanical behavior

significantly smaller in the circumferential direction for all comparisons between p - d tests conducted at both 4% below and at the in vivo axial stretch and f - l tests conducted at both 17 mm Hg and 25 mm Hg. The toe-region modulus was additionally significantly smaller in the circumferential direction for comparisons between the p - d test conducted at 4% above the in vivo axial stretch and f - l tests conducted at 8 mm Hg, 17 mm Hg, and 25 mm Hg.

Discussion

In this study, we presented experimental methods to determine the biaxial mechanical properties of the murine vaginal wall using extension-inflation protocols. Supported by previous uniaxial studies [16,19,29,56], the results presented herein indicate that the vaginal wall exhibits nonlinear and anisotropic behavior [19]. Also observed were pseudoelasticity and axial coupling. Additionally, the vaginal wall exhibited a smaller value of in vivo axial stretch ($\lambda_z^{\text{iv}} = 1.15 \pm 0.005$) compared to that reported for both infrarenal aortas ($\lambda_z^{\text{iv}} = 1.47 \pm 0.20$) [57] and carotid arteries ($\lambda_z^{\text{iv}} = 1.69 \pm 0.005$) [58] in mice. Similarly, the average opening angle of the vaginal wall (74.41 ± 17.67 deg) was lower than that of the rabbit carotid artery (92.17 ± 11.43 deg) [59]. In vivo axial stretches and opening angles are thought to arise, in part, from the deposition of stable elastin during development and the continual turnover of collagen and smooth muscle at consistent preferred stretches within the ground matrix [60]. While vascular elastin is only produced during development, it has been suggested that the female reproductive system is uniquely able to produce functional elastin outside of this time period [61,62]. Production of elastin throughout maturity rather than only in development may, in part, account for the smaller values of axial stretch and opening angle observed in the vagina compared to those of vasculature [60]. The stability and turnover of elastin within the vagina, however, is currently unknown. Hence, there is a need to determine the prestretch and deposition time course of elastin within the vagina, as well as to ascertain how elastin deposition characteristics may affect collagen undulation and organization [60,63]. Additional microstructural differences between vasculature and vaginal tissue may also contribute to these observed variations. Vascular elastin is predominantly organized in lamellar structures [64,65] and makes up 15–30% of a vessel's dry weight (depending on location) [66], while vaginal elastin has been observed to be organized within isolated elastic fibers [31,63] and makes up 2.17% of vaginal tissue [67]. Although the tissues contain the same load bearing constituents, variations in organization and orientation may contribute to differences observed in mechanical behavior.

Although the in vivo axial stretch of the vaginal wall was determined in this study, there is still limited information on the mechanical loads that the vagina experiences in the body and throughout a woman's lifespan. For the protocols established herein, we leveraged values of resting in vivo pressure reported by van der Walt et al. in human patients [68]; however, future experimental data are needed to quantify patient-specific values over time, the changes induced by prolapse, and the relation to animal models. Information on the in vivo configuration will be useful in future work to calculate linearized material stiffness and in vivo geometry using a nonlinear constitutive relation [69].

Further, in this study, vaginal properties of pseudoelasticity and axial coupling were evaluated. Although not an intrinsic material property, pseudoelasticity is a "convenient description of the stress-strain relationship in a specific cyclic loading" [70]. Essentially, it is the assumption that soft tissues behave as one elastic material in loading and another in unloading, such that quantification of stress-stretch relations in both cycles via a constitutive model utilizes the same functional form of strain energy, simply with different sets of material parameters [70]. Unsurprisingly, the vaginal wall displayed pseudoelastic behavior. The changes in bilinear fit outputs observed between the loading and unloading cycles, however, were less pronounced in the $t_{zz} - \lambda_z$ curves obtained from the f - l tests compared curves obtained from the p - d

tests. This seemingly directional dependence of pseudoelastic properties may be, in part, due to the underlying microstructure. These results may indicate that elastic fibers are oriented predominantly in the longitudinal direction. The organization and orientation of collagen and elastic fibers within the vaginal wall, however, have yet to be determined [31,63]. Thus, future work is needed to rigorously quantify the microstructure of the vagina, including potential elastin-collagen interactions. The vaginal wall also exhibited axial coupling, however, test type did not have a significant effect on the bilinear fit outputs from the $t_{\theta\theta} - \lambda_{\theta}$ curves. Axial coupling appears to have less influence on circumferential stress compared to axial stress. This may be because the four different f - l tests were conducted over larger range of values than the three different p - d tests were (p - d tests at 4% below, 4% above, and at the in vivo axial stretch versus f - l tests at 2, 8, 17, and 25 mm Hg). Additionally, the mechanical protocols presented herein test for passive properties and, thus, do not account for basal smooth muscle tone. Smooth muscle tone has been suggested to play an important role in vaginal wall mechanics [71,72] and could potentially influence axial coupling [24]. Therefore, future work is needed to evaluate the active properties of the vagina extending the biaxial extension-inflation protocols developed herein [28,73,74].

Finally, in this study, vaginal wall anisotropy was evaluated. Comparisons of circumferential and axial toe- and linear-region moduli suggest that the longitudinal direction is more extensible in the low-strain regime but less extensible for higher ranges of stretch compared to the circumferential direction (Table 4). Although a previous uniaxial study on the vaginal wall did not report toe and linear moduli, it revealed that specimens tested in the longitudinal (axial) direction were stiffer and experienced higher maximal stresses than specimens tested along the transverse (circumferential) axis [19]. Although the findings of Peña et al. correspond with the more compliant circumferential toe modulus reported herein, they qualitatively demonstrate a stiffer linear modulus in the axial direction (cf. Fig. 2 in Ref. [19]). One possible explanation for the opposite trend observed for the linear modulus in the present study is the difference in testing methods as well as in sample type. Peña et al. performed uniaxial tensile tests on longitudinal and transverse rectangular strips of prolapsed human tissue, whereas the protocols described in the current study consist of intact, healthy, murine tissue tested biaxially with extension-inflation protocols [19]. Additionally, Peña and colleagues performed load-dependent tests of 2, 4, 6, and 8 N that resulted in axial stretches of up to 1.6, while our protocols were stretch-dependent from 4% below to 4% above the in vivo axial stretch and resulted in an average maximum axial stretch of 1.2 (with respect to the unloaded configuration). For additional comparison, the majority of samples in our study had maximum loads (measured by the force transducer) of around 100 mN. Thus, it is possible that the longitudinal collagen fibers may not have been fully engaged, accounting for the more compliant axial response in the linear region. Experimental observations from the current study also suggest that higher values of stretch may produce similar results to that of Peña et al. The aim of this study, however, was to develop mechanical testing protocols to quantify vaginal wall behavior under physiological conditions of interest, thus 4% axial stretch above the in vivo may be most physiologically relevant. Further, the toe region, which is argued to be more physiologically relevant, corresponded to the findings of Peña et al. Future work is needed to determine the physiologic range of stretch the vagina experiences throughout a woman's lifetime and how that may change with the onset of prolapse.

Limitations of our study include difficulties in finding the unloaded configuration (i.e., length and outer diameter). As shown in Figs. 7–9, stretch errors are larger than stress errors. Factoring in biological variability and uncertainty in the unloaded configuration could account for potential error. While pilot studies yielded a consistent and repeatable method to estimate the unloaded configuration, finding the unloaded configuration still poses a

Fig. 9 Axial Cauchy stress–stretch curves (mean $\pm$ SEM of $n = 16$ specimens) calculated from f – l testing data for loading (filled) and unloading (unfilled) cycles at 2 (black) and 25 mm Hg (gray), denoting the effect of cycle and axial coupling on vaginal wall mechanical behavior

challenge and introduces variability. As shown in Figs. 6(b) and 6(c), however, force–length tests can be used to confirm the in vivo axial stretch experimentally identified in pressure–diameter tests, thus ensuring that the sample is tested over a range of physiological values.

In summary, the experimental protocols we describe provide physiologically relevant methods for mechanically assessing the murine vaginal wall. Like most soft tissues, the vaginal wall was shown to exhibit pseudoelasticity, axial coupling, and anisotropy. Future work is needed to determine the microstructural organization, orientation, and constituent interactions, as well as to isolate the mechanical contribution of the load bearing constituents. The protocols developed in this study provide physiological means of assessing evolving structure–function relationships within the vaginal wall and could aid in the future investigation of biaxial mechanical properties needed to delineate the underlying mechanisms of pelvic organ prolapse and potential biomaterial interventions.

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Nomenclature

t_{zz} = axial Cauchy stress
 $t_{\theta\theta}$ = circumferential Cauchy stress
 λ_z = axial stretch
 λ_θ = circumferential stretch
 λ_z^{iv} = in vivo axial stretch

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