

# Anisotropy of the Passive and Active Rat Vagina Under Biaxial Loading

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**Abstract**—Pelvic organ prolapse, the descent of the pelvic organs from their normal anatomical position, is a common condition among women that is associated with mechanical alterations of the vaginal wall. In order to characterize the complex mechanical behavior of the vagina, we performed planar biaxial tests of vaginal specimens in both the passive (relaxed) and active (contracted) states. Specimens were isolated from virgin, female Long-Evans rats ( $n = 16$ ) and simultaneously stretched along the longitudinal direction (LD) and circumferential direction (CD) of the vagina. Tissue contraction was induced by electric field stimulation (EFS) at incrementally increasing values of stretch and, subsequently, by KCl. On average, the vagina was stiffer in the CD than in the LD ( $p < 0.001$ ). The mean maximum EFS-induced active stress was significantly higher in the CD than in the LD ( $p < 0.01$ ). On the contrary, the mean KCl-induced active stress was lower in the CD than in the LD ( $p < 0.01$ ). When comparing the mean maximum EFS-induced active stress to the mean KCl-induced active stress, no differences were found in the CD ( $p = 0.366$ ) but, in the LD, the mean active stress was much higher in response to the KCl stimulation ( $p < 0.001$ ). Collectively, these results suggest that the anisotropic behavior of the vaginal tissue is determined not only by collagen and smooth muscle fiber organization but also by the innervation.

**Keywords**—Vagina, Biaxial tests, Anisotropy, Contractility, Mechanical properties.

## INTRODUCTION

Pelvic organ prolapse (POP) represents a major public health concern in adult women worldwide. Prolapse occurs when a pelvic organ, such as the bladder or

uterus, descends from its normal position. This can cause urinary incontinence, fecal incontinence, pelvic discomfort, sexual dyspareunia, and a decreased quality of life.<sup>12,26</sup> Treatments for POP include non-surgical management techniques such as pelvic floor muscle training, the use of pessaries, and lifestyle changes as well as surgical options.<sup>1</sup> Surgeries have limited success, though, with nearly 30% of POP surgical patients undergoing additional operations to treat recurrent prolapse.<sup>37</sup> While factors such as age, weight, and parity are strongly associated with the development of POP, the exact underlying causes of POP remain unknown.<sup>11,48</sup> However, several studies have indicated that the structural and mechanical properties of the vagina are altered in women with POP.

The vagina is comprised of four layers: the epithelium, the subepithelium, the muscularis, and the adventitia.<sup>33</sup> Structurally, the most important layers are the subepithelium, which is made up of collagen, and the muscularis, which contains longitudinally and circumferentially oriented smooth muscle cells.<sup>53</sup> The collagen content determines the passive mechanical properties of the vagina, and the smooth muscle content dictates the active, or contractile, mechanical properties. There is a wide range of contradictory findings in regard to structural changes of the vagina associated with POP. Vaginal specimens with POP have been reported to have both increased<sup>33</sup> and decreased<sup>31</sup> levels of type III collagen, the predominant collagen throughout the vaginal wall. Several studies have reported significantly decreased fractions of smooth muscle in vaginal tissue with POP.<sup>3,7,23,49</sup> In addition, collagen and smooth muscle have been reported being less organized in prolapsed tissues.<sup>8,23</sup> Of course, changes in collagen and smooth muscle

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content and orientation lead to altered passive and active mechanical properties of the vagina.

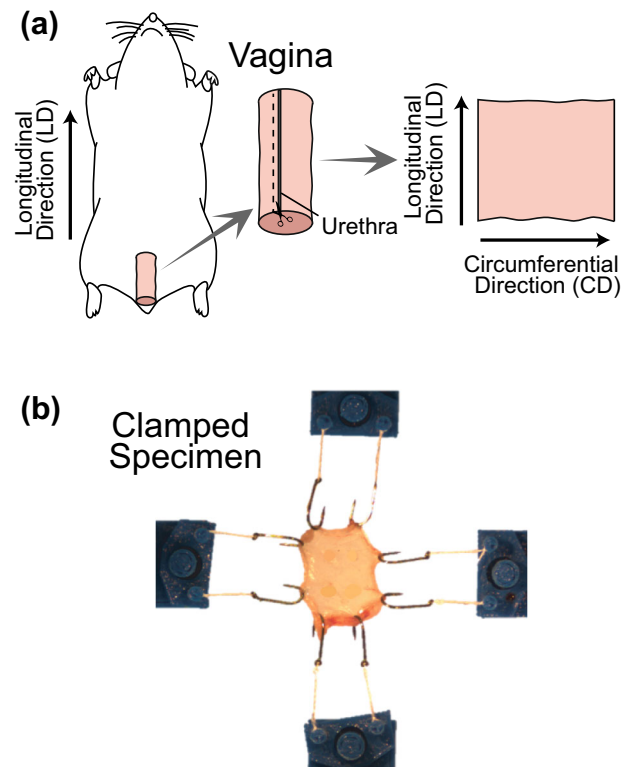
The influence of several factors on the passive mechanics of the vagina has been investigated including prolapse,<sup>20,25,39,44</sup> pregnancy and parity,<sup>17,28,41,46,52</sup> age,<sup>9</sup> menopause and hormones,<sup>15,18,22,29,30</sup> and weight.<sup>32</sup> Additionally, a few studies have been conducted to observe how the passive mechanical behavior of the vagina differs regionally across the entire vagina.<sup>45,51</sup> With some exceptions,<sup>24,38,50</sup> the overwhelming majority of these studies were conducted using uniaxial tensile tests, in which vaginal strips that were cut along either the longitudinal direction (LD) or the circumferential direction (CD) of the vagina were tested. In addition to the passive mechanical behavior of the vagina, the presence of smooth muscle in the tissue calls for the characterization of the active, or contractile, mechanical properties. Experimental studies on the active mechanical properties have been performed using exclusively uniaxial tests. Contractions have been induced using high concentrations of  $K^+$  for direct membrane depolarization,<sup>4,5,16,19,53</sup> electric field stimulation (EFS) for nerve-mediated responses,<sup>54</sup> various agonists and antagonists for receptor-mediated responses, or combinations thereof.<sup>6,21,27,35,36,47</sup> Finally, there have been several *in vivo* tests that measured the mechanical properties of the vagina using inflation or suction techniques.<sup>2,10,13,14,43</sup> Although these tests better emulated *in vivo* loading conditions, they provided data that cannot be used to distinguish and quantify the active and passive mechanical properties of the vaginal tissue.

In this study, we present the first planar biaxial characterization of both passive and active mechanical properties of the vagina using the rat as an animal model. Both passive and EFS-induced active stress data in the LD and CD of the vagina are collected at incrementally increasing stretch values. Following EFS, tissue contractions of the vaginal tissue are also induced by high concentrations of  $K^+$  in order to compare nerve-mediated mechanical responses to those caused by direct membrane depolarization. The findings of this rigorous mechanical characterization, which includes the study of the anisotropic behavior of the vagina in both the passive and active states, will serve to develop new effective treatments for POP. These may include vaginal electrical stimulation methods, surgical grafts with contractile properties, and drug treatments that could potentially prevent or reverse tissue alterations that are associated with POP.

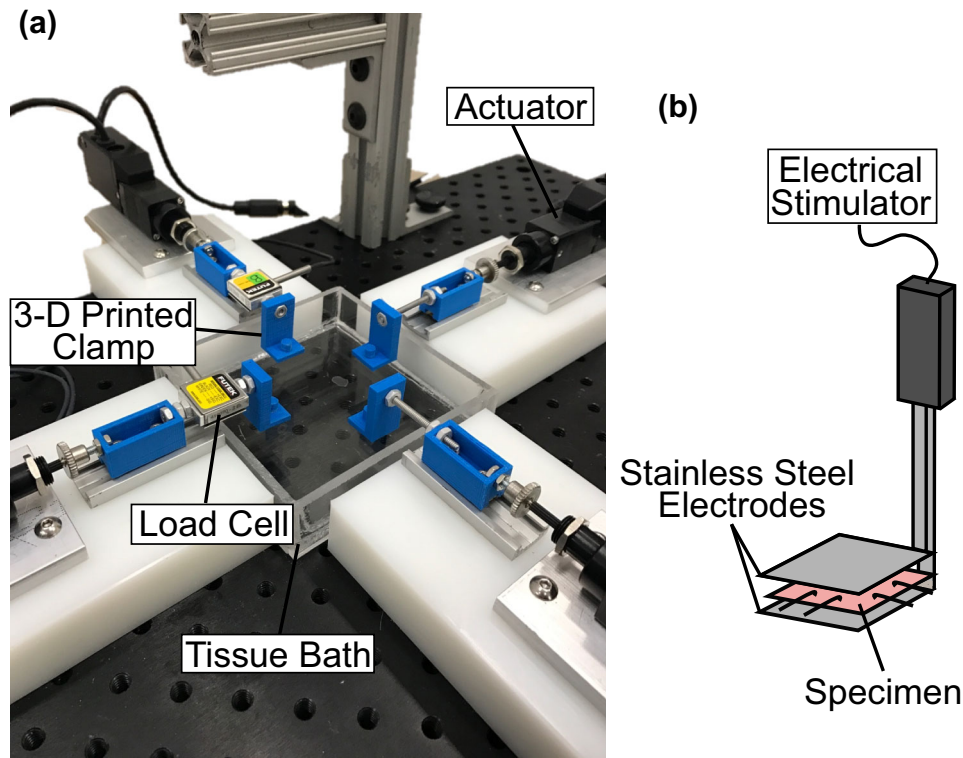
## MATERIALS AND METHODS

This study was conducted with the approval of the Institutional Animal Care and Use Committee (IA-CUC) at Virginia Tech. A total of sixteen adult female

Long-Evans rats, aged between 70 and 89 days, were used for this study. After euthanizing each rat *via* decapitation, the entire vagina was immediately isolated. During the dissection, the vagina was continuously kept moist with Krebs–Ringer bicarbonate buffer. The vaginal canal was opened into a flat specimen by placing a cut along the urethra, which lies along the longitudinal axis of the vagina. Once cut, the vagina showed little to no residual stress and, for this reason, it was easily flattened out. It was then trimmed to be approximately square with sides parallel to the LD and the CD of the vagina (Fig. 1). Two small hooks (size 18 dry fly hook, Mustad) that were connected *via* a silk thread were placed on each side of the specimen. The specimen was then secured to a custom-built biaxial testing machine described in detail elsewhere (Fig. 2a).<sup>55</sup> The linear actuators (T-NA08A25, Zaber Technologies, Inc.) of the biaxial system had a maximum travel length of 25 mm and micro-step size resolution of  $0.048\ \mu\text{m}$  while the two load cells (FSH02663, Futek Advanced Sensor Technology, Inc) had a maximum load capacity of 50 g and accuracy of  $\pm 0.1\%$ .



**FIGURE 1.** Schematic of specimen preparation. (a) The rat vagina is cut along the urethra in the LD to isolate a square specimen with sides that are parallel to LD and CD of the vagina. (b) The vaginal tissue specimen is hooked to custom-made clamps.



**FIGURE 2.** (a) Custom-built biaxial tensile machine consisting of four linear actuators, two load cells, four 3-D printed clamps, and a bath. (b) Schematic of the electrodes used for EFS.

**TABLE 1.** Mean  $\pm$  SD of the rat vagina specimen dimensions ( $n = 16$ ).

	Initial side-length (mm)	Hook distance (mm)	Cross-sectional area (mm <sup>2</sup> )
LD	10.54 $\pm$ 1.18	7.04 $\pm$ 0.72	2.67 $\pm$ 0.88
CD	11.10 $\pm$ 1.11	6.62 $\pm$ 0.87	2.55 $\pm$ 1.05

The thickness was measured to be 0.38  $\pm$  0.13 mm..

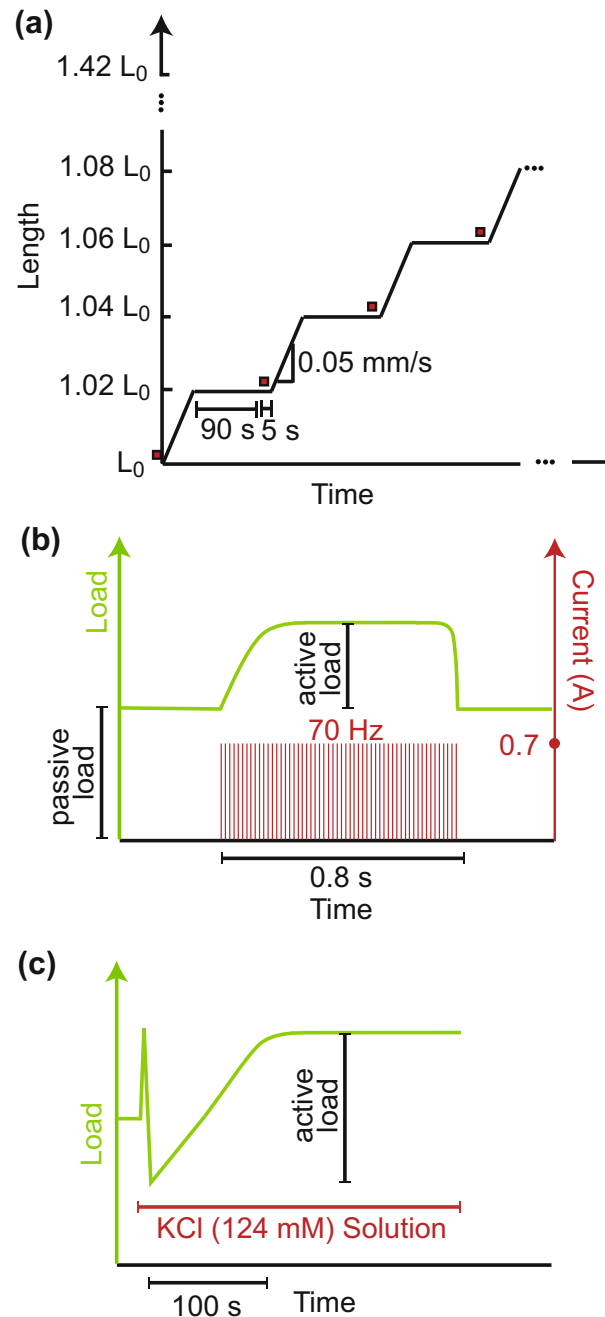
Once mounted on the biaxial testing machine, each specimen was submerged in a bath of Krebs–Ringer bicarbonate buffer with calcium (2.00 mM). Preliminary experiments to evaluate the pH variation occurring in the bath were performed. The pH changes were considered negligible, and oxygenation through bubbling of carbogen was not employed to avoid interference with the force measurements. In order to establish the initial configuration more consistently across different specimens, each specimen was stretched at 0.05 mm/s displacement rate until a pre-load of approximately 4 mN was recorded in both axial loading directions. It was then allowed to relax for 30 min before being re-stretched at 0.05 mm/s displacement rate until a 4 mN preload was reached in both directions. In this configuration, a picture of each specimen was quickly taken using a CMOS camera (DCC1545 M, Thorlabs) equipped with lens (59-871, Edmund Optics) to measure the specimen dimensions

using ImageJ (NIH, Bethesda, MD). Specifically, the average of two distances between the hooks that were placed directly opposite of each other along the LD or CD was considered to be the initial side-length of the specimen in that direction (Table 1). In this initial configuration, the specimen was then electrically stimulated in the LD and CD using a high-power pulse stimulator (701C, Aurora Scientific, Inc.) through custom made stainless steel electrode plates (Fig. 2b). The electrode plates were designed such that one plate could be positioned above the specimen and the other plate below the specimen. The plates were large enough to cover the entire specimen without being directly in contact with it. This design ensured that the direction of the electric current would be perpendicular to both the LD and CD and applied through the specimen thickness. The EFS consisted of one train of 2 ms pulses delivered at 70 Hz for 0.8 s, with 700 mA of current intensity. These EFS parameters were selected

solely because they produced maximum forces in preliminary tests but they may not be the most physiologically relevant.

Each specimen was then stretched simultaneously in each direction by increasing its length by 2% of its initial length in each direction for 21 consecutive increments at 0.05 mm/s displacement rate up to a final stretch of 42% of its initial length. The specimen was held at each stretch for 90 s and, at the end of each holding period, the forces in the LD and CD were measured. These forces were termed the “passive forces” of the specimen, i.e. the forces that resulted from the application of the stretches prior to the EFS. Note that these are not fully passive forces because the smooth muscle was not chemically passivated. Obtaining force data on fully passivated specimens using this incremental protocol would require extensive tissue washing and additional time which would introduce more variability in the force measurements. After each holding period, the specimen was electrically stimulated using the same methods and parameters presented above and the contraction forces in the LD and CD were measured. At each stretch, the “active force” in the LD or CD was defined as the difference between the maximum force reached *via* EFS and the passive force at that stretch. A schematic of this testing protocol is presented in Figs. 3a and 3b.

After electrically stimulating the specimen multiple times, the specimen was unloaded and then stretched at a 0.05 mm/s displacement rate until the load reached about 4 mN and it was then allowed to relax for 5 min. While recording force data, the testing solution was removed and replaced with Krebs–Ringer bicarbonate buffer with calcium (2.00 mM) and a high concentration of potassium chloride (124 mM) (Fig. 3c).<sup>21</sup> The specimen remained in the bath until the force in the LD and CD reached a maximum value and began to decrease, which typically took 1–2 min. The “active force” due to the KCl in the LD or CD was calculated as the difference between the maximum force reached with the KCl stimulation and the passive force recorded immediately after the KCl solution was added (Fig. 3c). Specimen thickness was measured immediately after testing in five different locations through the use of a CCD laser displacement sensor (LK-G82, Keyence, Inc.) with an accuracy of 7.5  $\mu\text{m}$  (Table 1). It was assumed that changes in thickness during biaxial testing were negligible. Cross sectional area was measured as the average of distances between hooks on the same side of the specimen multiplied by specimen thickness. Passive and active force data in the LD or CD were divided by the cross-sectional area that was perpendicular to the LD or CD, respectively, to obtain (nominal) passive and active stress data.



**FIGURE 3.** (a) Schematic of the incremental stretching protocol used to obtain passive and EFS-induced active force data simultaneously along the LD and CD. Red square symbols denote the application of EFS. (b) Schematic of the load and applied EFS at each stretch. (c) Schematic of the load and applied KCl stimulation following the incremental stretching protocol and EFS.

Maximum active stresses induced by EFS or KCl, stretches at which the maximum EFS-induced active stresses were achieved, and maximum EFS-induced active stresses normalized by KCl induced active stresses were compared between the LD and CD using a Wilcoxon signed-rank test. Similarly, in each direc-



tion, the maximum EFS-induced and KCl-induced active stresses were compared. Differences in passive stresses and EFS-induced active stresses were evaluated with 2-way ANOVA using direction and stretch as factors. Statistical analysis was performed with GraphPad Prism 6.0, and differences were considered significant when  $p < 0.05$ .

## RESULTS

The mean passive stress-stretch data collected from  $n = 16$  specimens in both loading directions are shown in Fig. 4. The rat vaginal tissue exhibits the typical non-linear stress-stretch behavior of soft biological tissues. The 2-way ANOVA revealed that *direction* ( $p < 0.001$ ) and *stretch* ( $p < 0.0001$ ) significantly affected the passive stress. As expected, the mean passive stress increased as the stretch increases. More interestingly, the tissue resulted to be anisotropic, being, on average, stiffer in the CD than in the LD.

Force vs. time data collected during the first, middle, and last EFSs corresponding to stretch values of  $\lambda = 1$ ,  $\lambda = 1.2$ , and  $\lambda = 1.42$  are shown for a representative specimen in Fig. 5. After the EFS was applied ( $t$  is approximately 1 s in Figs. 5a, 5b, and 5c) the vagina contracted producing gradual increases in forces in the LD and CD until the stimulation ended ( $t$  is approximately 1.8 s in Figs. 5a, 5b, and 5c) and, at that time, the forces returned to their passive values. At  $\lambda = 1$ , the specimen was pre-loaded so that the initial passive forces acting along each direction were nearly identical, around 4 mN (Fig. 5a). As the stretch increased, the difference between the passive forces along the CD and LD increased with higher passive

forces in the CD than in the LD (Figs. 5b and 5c)). The active forces were typically higher in the CD than in the LD at each stretch value. The differences of both active and passive forces in the two directions demonstrate the anisotropy of the vaginal tissue.

The mean active stresses in response to EFS at each stretch value are shown in Fig. 6. The 2-way ANOVA revealed that the stretch ( $p < 0.0001$ ) and direction ( $p < 0.0001$ ) factors significantly affected the active stress. EFS-induced active stresses were higher in the CD than in the LD, increasing with stretch until the maximum values were reached. These stresses then decreased even though the stretch continued to increase. The maximum active stress induced by EFS was about 60% higher in the CD than in the LD ( $p < 0.01$ ). The maximum active stress along the LD was measured at a stretch of about 1.32 on average, while the maximum active stress along the CD was

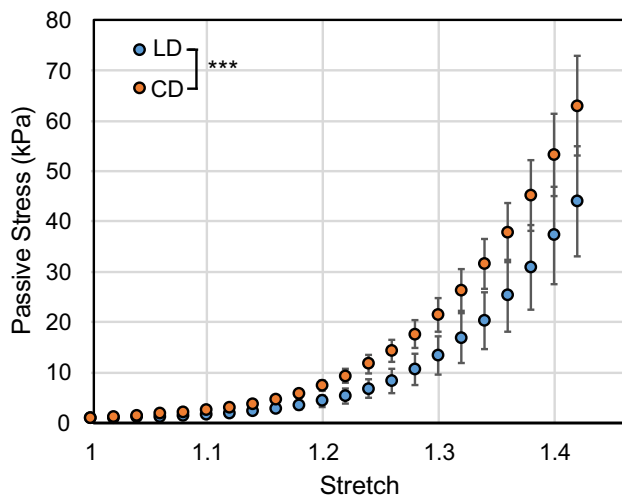


FIGURE 4. Mean ( $\pm$  S.E.M.) passive stress vs. stretch data along the LD and CD of the vaginal specimens ( $n = 16$ ). \*\*\* $p < 0.001$ .

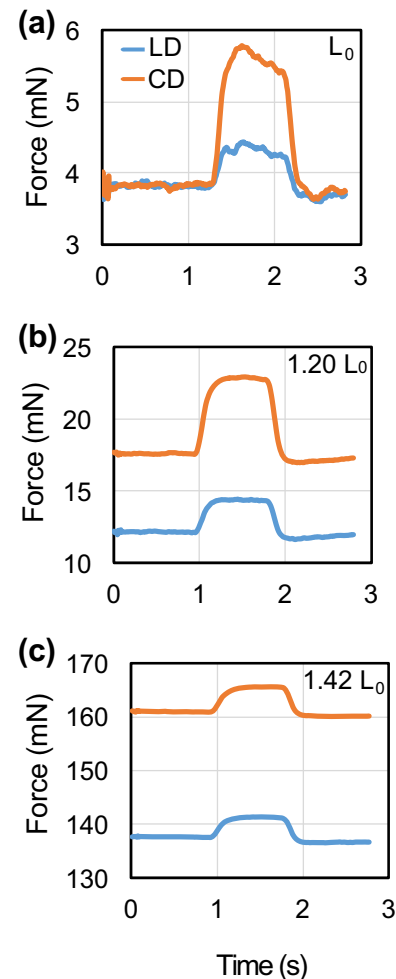


FIGURE 5. EFS induced forces of a representative specimen at stretch values of (a) 1, (b) 1.2, and (c) 1.42. The time scales are adjusted so that  $t = 0$  represents approximately 1 s prior to the application of the EFS. Thus, the forces at  $t = 0$  are the passive forces.

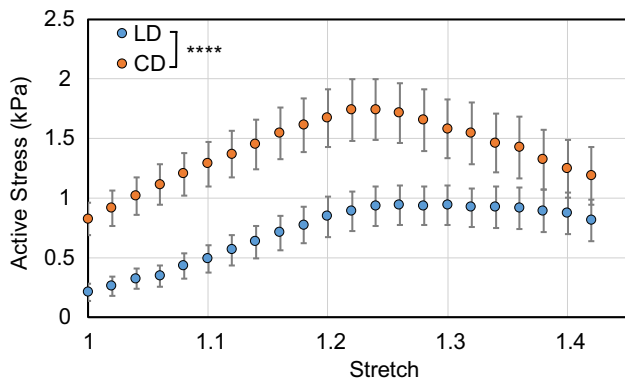


FIGURE 6. Mean ( $\pm$  S.E.M.) EFS induced active stresses vs. stretches in the LD and CD ( $n = 16$ ). \*\*\*\* $p < 0.0001$ .

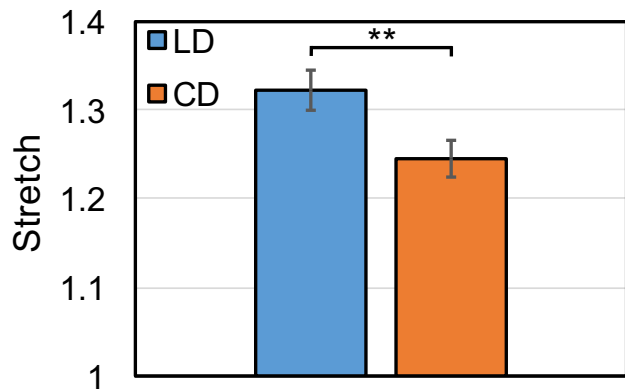


FIGURE 7. Mean ( $\pm$  SEM) stretch at which maximum EFS induced active force was recorded ( $n = 16$ ). \*\* $p < 0.01$ .

obtained at a stretch of around 1.25 on average (Fig. 7). This difference was found to be statistically significant ( $p < 0.01$ ).

In Fig. 8, the forces along the LD and the CD for a representative specimen that was stimulated using KCl are reported. During the first few seconds of KCl stimulation, the forces in both the LD and CD quickly increased and decreased due to the removal of the first solution and the addition of the KCl solution. Once the KCl solution was added, the forces in each direction gradually increased until plateauing after around 100 s.

On average, the KCl stimulation caused 60% higher active stresses in the LD than in the CD ( $p < 0.01$ ). The mean active stresses caused by the KCl stimulation were 3.46 kPa in the LD and 2.15 kPa in the CD (Fig. 9). When comparing the mean maximum active stress induced by EFS to the mean active stress induced by the KCl, no differences were found in the CD ( $p = 0.366$ ). On the other hand, in the LD, the mean active stress was much higher in response to the KCl stimulation than in response to EFS ( $p < 0.001$ ). All statistically significant differences found with respect

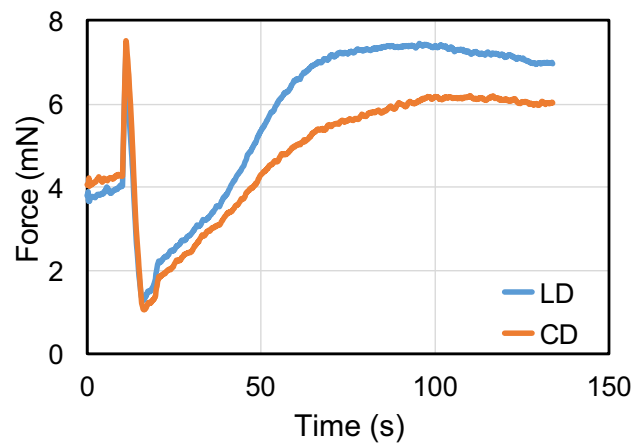


FIGURE 8. Force for a representative specimen during the application of the KCl stimulation. In this figure, the time scale is adjusted so that  $t = 0$  represents the time in which the solution used for EFS stimulation was removed before adding the KCl-based solution.

to stimulation methods and directions are noted in Fig. 9. Finally, the mean maximum EFS-induced active stress normalized by the KCl-induced active stress was significantly higher in the CD (96%) than the LD (40%) (Fig. 10).

## DISCUSSION

In this study, we presented the first characterization of the biaxial passive and active mechanical properties of the rat vagina. The mechanical properties of this reproductive organ are crucial to the proper function of the entire female pelvic floor. Indeed, alterations of these properties are implicated in the development of POP. The vagina is a tubular organ that, *in vivo*, is primarily loaded along the LD and CD with smooth muscle fibers that are mainly oriented along these directions. For these reasons, we performed planar biaxial tests, rather than the commonly used uniaxial tests. During mechanical testing, we stimulated the vaginal tissue through nerve fibers using EFS and through direct membrane depolarization using KCl. Our findings demonstrated that the vaginal tissue is highly anisotropic: the tissue in the CD is significantly stiffer than in the LD in the passive state (Fig. 4) and EFS-induced active state (Fig. 6).

Previous studies have sought to quantify the anisotropy of vaginal tissue in the passive state by comparing results of uniaxial tests collected from tissue strips that were cut along the LD and CD,<sup>34,40,45</sup> and, very recently, through inflation-extension testing.<sup>42</sup> The results from these studies have been conflicting, though. Pena *et al.* found that the vagina was stiffer in the LD than in the CD.<sup>40</sup> Rubod *et al.* reported that

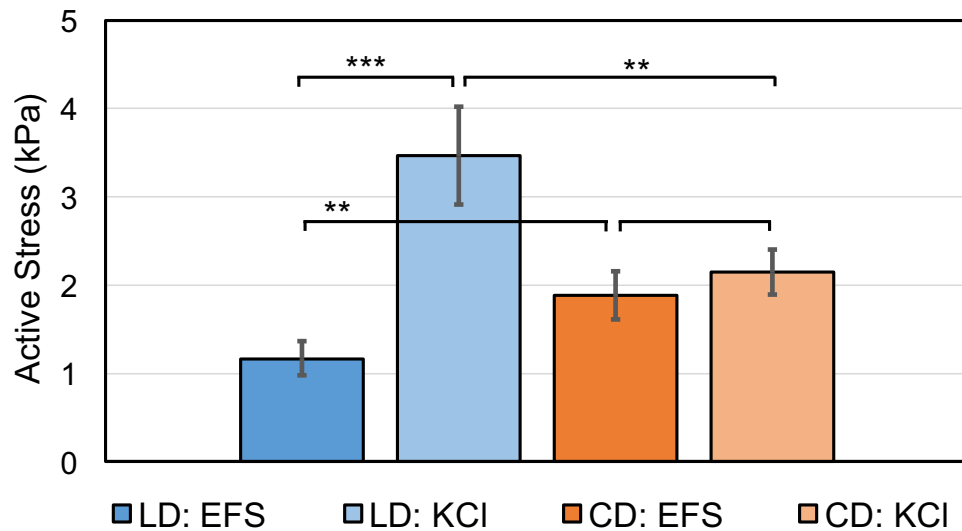


FIGURE 9. Mean ( $\pm$  SEM) maximum active stresses in response to EFS and active stresses in response to KCl stimulation in the LD and CD ( $n = 16$ ).  $**p < 0.01$  and  $***p < 0.001$ .

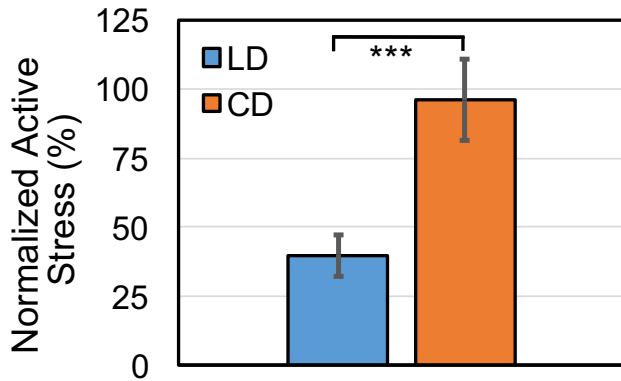


FIGURE 10. Mean ( $\pm$  SEM) maximum active stress obtained by EFS normalized by active stress obtained by KCl-induced stimulation in the LD and CD ( $n = 16$ ).  $***p < 0.001$ .

the anisotropy of the vaginal tissue was strain-dependent: there were no significant differences in stiffness at strains below 50%, but the tissue in the LD became stiffer at higher strains.<sup>34,45</sup> Similarly, Robison *et al.* also found the vagina to be stiffer in the LD at high strains, but found it stiffer in the CD at low strains.<sup>42</sup> In this study, we found that, on average, the rat vagina was significantly stiffer in the CD at strains between 0 and 42% (Fig. 4). However, it should be noted that not every specimen followed this trend, as there were some specimens that exhibited higher stresses in the LD than in the CD for the same stretches. Our findings align with those of Robison *et al.* but additional testing at higher strains would need to be performed to potentially observe the reported strain-dependent anisotropy of vaginal tissue. The stress values reached during our testing were lower than previously reported stress values at similar strains.<sup>41</sup> This is likely due to our

experimental protocol which included 90 s holding periods at each stretch level before collecting passive and active force data. The holding period at each stretch level was included to allow the specimen to relax. This was necessary so that changes in force due to relaxation would not interfere with the measurement of the active forces.

The mechanical properties of the vagina in the active state have been exclusively studied *via* uniaxial tests. In these tests, either longitudinal<sup>4-6,19,27,35</sup> or circumferential<sup>16,21,47,54</sup> strips of vaginal tissue were tested, and the active forces generated by various stimulation methods were recorded. Oh *et al.*<sup>36</sup> tested both longitudinal and circumferential strips of vaginal tissue *via* uniaxial tests and found no significant differences in the active responses in the LD and CD.<sup>36</sup> This is in contrast with the results of our biaxial tests as we found that, in response to EFS, the vagina contracted significantly more in the CD than in the LD as demonstrated by the higher active stresses in the CD (Figs. 5, 6 and 9). Moreover, in response to KCl, the vagina contracted more in the LD than in the CD, generating higher active stresses in the LD (Figs. 8 and 9). Before normalizing by cross sectional area, we found that our testing yielded higher, but comparable, EFS-induced active forces than tests by van Helden *et al.*<sup>54</sup>; this was expected since we used the entire vagina rather than strips of vaginal tissue. We found that the magnitude of the active forces generated by KCl in our tests were similar to those found in uniaxial testing when normalized by specimens' volume.<sup>19,53</sup>

In our study, we measured the active mechanical response of the entire rat vagina, rather than separating it into proximal and distal regions as done by

others. It has been shown that the vagina undergoes stronger contractions in the proximal than distal regions in response to EFS<sup>36,47,54</sup> and KCl-induced stimulation.<sup>5,6,36</sup> However, regional differences in peak active force due to KCl stimulation disappear after normalizing by cross sectional area of the smooth muscle<sup>5</sup> or by weight.<sup>36,47</sup> Thus, while our biaxial tests of the entire vagina better emulated physiological loading conditions and captured the coupling effects of contractions in the LD and CD, they did not measure the heterogeneous active mechanical properties of the vagina. Future biaxial tests should be conducted by localizing the EFS on the rat vagina or, alternatively, square specimens should be isolated from the distal and proximal regions of the vagina of large animal models. The heterogeneous deformation of the vaginal tissue could also be measured using non-contact strain measurement methods such as the digital image correlation method. In this study, strain was measured from the displacement of the hooks. It is well known that this is not the most accurate strain measurement method. In our experimental setup (Fig. 2b), the electrodes created a physical barrier and images of the specimens during testing could not be acquired for non-contact strain measurements. Moreover, attachment of markers or spraying paint to create speckle patterns requires the vaginal tissue to be slightly dry and this can affect tissue's viability. These limitations could be mitigated in the future through the use of transparent electrodes and fast dry paint and specimen preparation methods that will allow us to preserve the tissue's contractile properties.

The EFS contractions are frequency dependent, with increasing frequencies typically leading to higher active stresses.<sup>21,27,36,54</sup> To achieve the maximum EFS-induced contractions, we chose a frequency of 70 Hz after performing preliminary tests. The maximum EFS-induced active stresses occurred at optimal stretches, which we determined for each specimen and in both directions through our incremental stretching protocol (Fig. 7). KCl-induced active stresses were measured by keeping the specimen under low equi-biaxial loads and using a high concentration of KCl (124 mM). Active stresses induced by KCl were significantly higher in the LD than in the CD, while active stresses induced by EFS were higher in the CD (Fig. 9). EFS-induced active forces were normalized by KCl-induced active forces in order to quantify the approximate percentage of total muscle in each direction that was receptive to nerve-mediated stimulations. Normalized active stresses were significantly lower in the LD than in the CD, which could suggest that the vaginal tissue in the LD is less innervated than in the CD (Fig. 10). It remains unknown whether these directional differences in the active stresses are the re-

sult of different content and organization of smooth muscle cells and nerve fibers.

The role of smooth muscle contractions has largely been overlooked in previous studies on the mechanical properties of the vagina, and those who have accounted for it have done so *via* uniaxial tests. The results of our biaxial tests reveal the inherent anisotropy of the vaginal tissue and the coupling between the LD and CD in both the passive and active states. This study represents a springboard for future studies on the effect of pregnancy, parity, and prolapse on the active and passive mechanical behavior of vaginal tissue. The properties of the vagina should be considered in the development of new prevention and treatment methods for POP including vaginal electrical stimulation, physical therapies, drug treatments, surgical protocols, and graft materials.

## CONCLUSION

In this study, the passive and active mechanical properties of rat vaginal tissue were obtained using planar biaxial tensile testing. Tissue contraction for the measurement of active properties was induced using EFS at incrementally increasing stretch values followed by KCl. Results showed that the vagina was anisotropic in both the passive and active states. In the passive and EFS-induced active states, the vagina was stiffer along the CD than along the LD. In the KCl-induced active state, this trend was reversed. These results may suggest that the vaginal tissue in the LD is not as innervated as in the CD. Future studies should quantify the organization of smooth muscle cells and the innervation in the vaginal wall.

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## CONFLICTS OF INTEREST

The authors have no conflict of interest.

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