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Mechanical Impact of Parturition-Related Strains on Rat Pelvic Striated Sphincters.

Pamela Duran, BS¹, Samuel Ward, PhD^{2,3}, Karen L. Christman, PhD^{1,*}, and Marianna Alperin, MD, MS^{4,*}

¹Department of Bioengineering, Sanford Consortium for Regenerative Medicine, University of California San Diego, La Jolla, CA

²Department of Orthopaedic Surgery, University of California San Diego, La Jolla, CA

³Department of Radiology, University of California San Diego, La Jolla, CA

⁴Department of Obstetrics, Gynecology, and Reproductive Sciences, Division of Female Pelvic Medicine and Reconstructive Surgery, University of California San Diego, La Jolla, CA

Abstract

Aims: To define operational resting sarcomere length (L_s) of the rat external urethral (EUS) and anal (EAS) sphincters and to determine mechanism of parturition-related injury of EUS and EAS using a simulated birth injury (SBI) vaginal distention model.

Methods: EUS and EAS of 3-months old Sprague-Dawley control and injured rats were fixed *in situ*, harvested; and microdissected for L_s measurements and assessment of ultrastructure. EUS and EAS function was determined at baseline, and immediately and 4 weeks following SBI, using leak point pressure (LPP) and anorectal manometry (ARM), respectively. Operational L_s were compared to species-specific optimal L_s using one-sample Student's t-test. Data (mean $\pm$ SD) were compared between groups and time points using repeated measures one-way ANOVA, followed by Tukey's post-hoc pairwise comparisons, with significance set to 0.05.

Results: The operational L_s of both sphincters (EUS: 2.09 ± 0.07 μ m, EAS: 2.02 ± 0.03 μ m) was significantly shorter than optimal rat L_s of 2.4 μ m. Strains imposed on EUS and EAS during SBI resulted in significant sarcomere elongation and disruption, compared to the controls (EUS: 3.09 ± 0.11 μ m, EAS: 3.37 ± 0.09 μ m). Paralleling structural changes, LPP and ARM measures were significantly lower acutely (LPP: 21.5 ± 1.0 cmH₂O, ARM: 5.1 ± 2.31 cmH₂O) and 4 weeks (LPP: 27.7 ± 1.3 cm H₂O, ARM: 2.5 ± 1.0 cmH₂O) following SBI relative to the baseline (LPP: 43.4 ± 8.5 cmH₂O, ARM: 8.2 ± 2.0 cmH₂O), $P<0.05$.

Corresponding Author: Marianna Alperin, MD, MS, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0863, Phone: +1 858-534-5015, Fax: +1 858-534-4704, malperin@ucsd.edu.

*Co-senior authors.

Ethics approval statement

Materials and Methods

Study Groups

The University of California, San Diego Institutional Animal Care and Use Committee approved all study procedures.

Conclusions: Analogous to humans, the resting L_s of rat EUS and EAS favors their sphincteric function. The insult experienced by these muscles during parturition leads to sarcomere hyperelongation, myofibrillar disruption, and dysfunction of the sphincters long-term.

Keywords

external urethral sphincter; external anal sphincter; rat; birth injury; stress urinary incontinence; fecal incontinence

Introduction

Stress urinary incontinence (SUI) and fecal incontinence (FI) are the most common pelvic floor disorders (PFDs), affecting 16% and 9% of the U.S. women, respectively.¹ Vaginal delivery has been established as the key risk factor for PFDs.^{1,2} Even though the precise causative link between childbirth and PFDs is yet to be elucidated, our current understanding is that the mechanical demands placed on maternal soft tissues damage pelvic connective tissues, nerves, and smooth and skeletal muscles, in turn, causing failure of the continence mechanisms.^{1,2}

Maintenance of urinary and anal continence involves multiple components of the female pelvic floor. The older theories emphasized urethral hypermobility due to the failure of the extrinsic to the urethra supportive structures, as the key factor in the SUI pathogenesis.^{3,4} Conversely, subsequent studies identified external urethral sphincter (EUS) dysfunction as the strongest predictor of SUI.⁵ In terms of fecal continence, the external anal sphincter (EAS) is a major contributor to the proper function of the anal sphincter complex, with obstetrical EAS lacerations recognized as a major cause of FI in women.⁶ However, EAS dysfunction can occur in parous women without such injuries.⁷ To date, the pathways leading to FI in the absence of EAS lacerations is not well understood. Taken together, the above clinical findings underscore the importance of investigating the pathogenesis of pelvic sphincteric muscles' dysfunction.

Current treatments for SUI and FI are delayed and compensatory, as they do not address the underlying pathophysiology leading to sphincter dysfunction. Investigations focused on the mechanisms of maternal birth injury, which are essential for the development of novel preventative and therapeutic approaches, necessitate the use of experimental models. The rat is the most extensively utilized model in PFDs research. Despite this, the structural design of the rat pelvic sphincters has not been determined to date. The importance of the length of muscle functional units, sarcomeres, lies in the fact that it determines the length-tension relationship fundamental to the skeletal muscles' function (Figure 1). The operational sarcomere length (L_s) indicates the *in vivo* length, at which the muscle operates to suit its functional needs and often differs from the optimal L_s , at which the overlap between actin and myosin allows for the maximal force production. To our knowledge, the operational L_s of the rat EUS and EAS has not been determined to date.

Secondly, studies in the limb muscles demonstrate that the primary cause of mechanical skeletal muscle injury is sarcomere hyperelongation and the associated myofibrillar disruption. These acute events result in decreased overlap between actin and myosin (Figure

1), inflammation, fibrosis, ultimately leading to muscle dysfunction.⁸ We have previously identified that excessive strains during simulated birth injury (SBI) lead to sarcomere hyperelongation of the rat pelvic floor muscles.⁹ Published studies examining the impact of SBI on the rat pelvic sphincters focus on important mechanisms of muscle dysfunction, specifically hypoxia and pudendal neuropathy.^{10–12} However, the mechanical impact of parturition-related strains on the EUS and EAS has not been determined to date.

Thus, the objectives of the current study were two-fold: to determine the operational L_s and the mechanical impact of SBI on the rat pelvic sphincteric muscles. We hypothesized that analogous to the human EAS¹³, the rat EUS and EAS operational L_s is shorter than optimal L_s . Furthermore, we hypothesized that excessive parturition-related strains cause sarcomere hyperelongation of EUS and EAS, leading to myofibrillar disruption and muscle dysfunction.

Materials and Methods

Study Groups

Institutional Animal Care and Use Committee approved all study procedures, conducted in 3-months-old nulligravid Sprague-Dawley female rats. Overall, 29 animals were utilized in the current study. Twenty two animals were used to establish the *in vivo* resting L_s length and to evaluate the impact of parturition-related strains on muscle functional units (uninjured controls n=4; animals subjected to SBI n=16; and intrapartum: n=2). For these L_s measurements, physiological and supraphysiological strains were induced in rats subjected to SBI via vaginal distention with 3 and 5 mL volumes, respectively.⁹ SBI was performed without (n=4/volume) and with (n=4/volume) disarticulation of the pubic symphysis to allow a more cephalad positioning of the distention balloon in order to impact EUS over its entire length, which is located more rostrally than the human EUS.^{14,15} To assess the effect of SBI on muscle ultrastructure, 2 additional animals were perfusion fixed, and EUS and EAS were examined using transmission electron microscopy (TEM) in 1 control and 1 injured rat. A separate group of animals (n=5) was used for the longitudinal functional studies, ascertained at baseline, immediately after and 4 weeks post-SBI.

Simulated Birth Injury

Animals were anesthetized with 2.5 % isoflurane in oxygen during the procedure. A 12-French transurethral catheter (Bard Medical, Covington, GA) with the tip cut off was inserted into the vagina, as previously described.⁹ The balloon was inflated to either 3 or 5 mL and a 130 g weight was attached to the end of the catheter, which was left in place for 2 hours to replicate circumferential and downward strains associated with parturition. For the pubic symphysis disarticulation group, a Cherney incision was used to expose the pubic symphysis, which was cut in the midline.

EUS and EAS Sarcomere Length

For the operational L_s , uninjured animals were euthanized, and the sphincteric muscles were fixed *in situ* in 10% buffered formaldehyde to preserve *in vivo* architecture. Rats subjected to SBI were sacrificed and fixed with balloon in place to assess the *in vivo* impact of muscle

strain. Urethra and anus were harvested and EUS and EAS were microdissected under a dissection stereomicroscope with an overall magnification of 6X (Leica M165C, Leica Microsystems, Wetzlar, Germany). Myofibers were procured from the rostral, middle, and caudal muscle regions. Samples were imaged with bright field microscopy at 10X magnification (Leica DM600B with Leica DFC 295 camera, Leica Microsystems, Wexlar, Germany). Sarcomeres were measured with ImageJ software at each region, as previously described.¹⁶

Muscle ultrastructure

To assess whether sarcomere elongation resulted in myofibrillar disruption, EUS and EAS were harvested from non-injured and injured animals after perfusion fixation with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15M sodium cacodylate buffer (pH 7.4). Samples were further prepared for TEM, as previously described.⁹

In vivo functional measurements

Leak point pressure (LPP) represents the intravesical pressure required to overcome urethral resistance, resulting in urinary leakage. LPP is a reliable measure of the urethral sphincter function, as it has been shown to correlate well with the maximal urethral closure pressure, the strongest predictor of urinary incontinence.^{5,17} LPP was measured using a standard approach.¹¹ Briefly, a water-filled pressure transducer (P23XL, Spectramed, Statham, Singapore) was calibrated at the level of the pubic symphysis and connected to 1) a custom-made bridge amplifier, 2) a syringe pump (SP220i, WPI, Sarasota, Florida), and 3) a digital acquisition system (4/35 Power Lab, ADInstruments, Sydney, Australia). LabChart software (v8.1.3, ADInstruments, Sydney, Australia) was used to record the pressure signal. Transurethral catheter (PE-50) was used to retrograde fill the bladder with 1 mL of room-temperature water at a rate of 5 mL/hour, and data were collected at a rate of 1000 samples/s. Upon completion of the retrograde fill, intravesical pressure was gradually increased via a Credé maneuver until leakage at the external urethral meatus was observed. The investigator performing Credé maneuver was blinded to the generated pressures. LPP was obtained by subtracting the baseline from the peak pressure, as previously described.^{11,18} LPP measurements were repeated 3 times at each time point in each animal.

Anorectal manometry (ARM) measures the anal sphincter complex pressure, where the resting anal pressure is attributed to tonic contraction of the internal and external anal sphincters, and the increase in pressure, in response to the rectal balloon, indicates an active contraction of EAS.¹⁹ The change in pressure (δ pressure), the active contribution of the EAS to the anal canal pressure, was measured.¹⁹ A latex balloon (size 4, Harvard Apparatus, Holliston, Massachusetts) contiguous with syringe pump was placed at the level of the anus and advanced 4 mm to access EAS. Pressure sensor was connected to a custom-made bridge amplifier, data acquisition system and LabChart. Data were acquired at 10 samples/s. Resting anorectal pressure was determined, after which the balloon was inflated with 1 mL of room-temperature water. Data were acquired over a 30-minute period and changes between resting and peak pressures, interval between contractions (duration of refractory period), time to peak contraction, and number of peaks per contraction were determined, as previously described.¹²

Statistical analysis

Based on the experimental EUS and EAS L_s variability (coefficient of variation: 4%) in the control group obtained a priori, 4 animals/group/distention volume were needed to achieve 80% power at a significance level set to 0.05 to detect a 10% difference. Comparisons of operational and a known optimal rat L_s were made using a one sample Student's t-test. Data are presented as mean $\pm$ SD. L_s was compared between the following groups: 1) uninjured and injured animals, 2) different distention volumes, and 3) SBI with and without disarticulation of pubic symphysis. For the *in vivo* functional measurements, 5 rats were required to detect a 30% difference with 80% power and significance level of 0.05, based on published coefficient of variation of 18%.²⁰ Data were compared using repeated measures one-way ANOVA followed by Tukey's post-hoc pairwise comparisons, using GraphPad Prism v7.0, San Diego, CA, USA.

Results

EUS and EAS Sarcomere Length

The resting operational L_s was $2.09 \pm 0.07 \mu\text{m}$ in EUS and $2.02 \pm 0.03 \mu\text{m}$ in EAS. For both sphincters, L_s was significantly shorter compared to the rat optimal L_s of $2.4 \mu\text{m}$ ¹⁶ (EUS: $P=0.04$, EAS: $P=0.02$). This indicates that the rat pelvic striated sphincters operate on the ascending limb of the length-tension curve (Figure 1), similar to the human EAS.¹³

Mechanical Impact of Parturition-Related Strains on the EUS and EAS Functional Units

SBI with 3 mL distention volume resulted in significant L_s increase compared to non-injured animals (EUS: $2.91 \pm 0.09 \mu\text{m}$ vs. 2.09 ± 0.07 , $P<0.01$; EAS: $3.23 \pm 0.03 \mu\text{m}$ vs. 2.02 ± 0.03 , $P<0.01$), Figure 2A,B. Increasing distention volume to 5 mL resulted in further elongation of the sarcomeres, but did not reach statistically significant difference relative to the outcomes of 3 mL distention (EUS: $3.09 \pm 0.11 \mu\text{m}$ vs. $2.91 \pm 0.09 \mu\text{m}$; EAS: $3.37 \pm 0.09 \mu\text{m}$ vs. $3.23 \pm 0.03 \mu\text{m}$, $P>0.5$), Figure 2A,B.

With the pubic symphysis disarticulated, a 3 mL volume resulted in L_s comparable to SBI without disarticulation (EUS: $3.03 \pm 0.3 \mu\text{m}$ vs. $2.91 \pm 0.09 \mu\text{m}$, $P=0.9$; EAS: $3.06 \pm 0.03 \mu\text{m}$ vs. $3.23 \pm 0.03 \mu\text{m}$, $P=0.2$), Figure 2C,D. At 5 mL, EUS L_s was significantly longer with, compared to without, pubic symphysis disarticulation ($3.79 \pm 0.42 \mu\text{m}$ vs. $3.09 \pm 0.11 \mu\text{m}$, $P<0.05$), Figure 2C. EAS L_s , on the other hand, significantly decreased when the pubic symphysis was disarticulated ($3.0 \mu\text{m}$ vs. $3.37 \pm 0.09 \mu\text{m}$, $P<0.01$), due to a more rostrally located balloon (Figure 2D). Furthermore, L_s of both sphincters in the intrapartum group, was analogous to the values observed in response to vaginal distention with intact pubic symphysis (EUS: $2.94 \pm 0.09 \mu\text{m}$; EAS: $3.22 \pm 0.09 \mu\text{m}$, $P=0.9$), further validating our experimental approach (Figure 2C,D).

The examination of EUS and EAS with TEM demonstrated a normal striation pattern of the myofibers in the control group (Figure 3A,C). In response to 5 ml SBI, disruption of the muscle ultrastructure was evident especially in EUS, with misalignment of Z-lines, which border individual sarcomeres (Figure 3B,D).

Impact of Parturition-Related Strains on the EUS and EAS Function

Immediately following SBI, LPP was dramatically decreased compared to baseline (21.5 ± 1.0 cm H₂O vs 43.4 ± 8.5 cm H₂O, $P < 0.05$), Figure 4A-C. After a 4-week recovery period, EUS function improved relative to acutely post-injury (27.7 ± 1.3 cm H₂O vs 21.5 ± 1.0 cm H₂O, $P < 0.01$); however, LPP remained significantly lower than baseline ($P < 0.05$), indicating persistent EUS dysfunction (Figure 4A,B,D).

With respect to EAS function, active EAS contraction, indicated by change in pressure (δ pressure), significantly decreased immediately after SBI, compared to baseline (5.1 ± 2.3 cm H₂O vs 8.2 ± 2.06 cm H₂O, $P < 0.01$), Figure 5A-C. In contrast to EUS, EAS function further deteriorated 4 weeks post-SBI, with significantly lower δ pressure of 2.5 ± 1.0 cm H₂O relative to δ pressure observed acutely post injury ($P < 0.05$), Figure 5A,C,D. Interestingly, SBI did not significantly impact the other functional EAS parameters (Figure 5E-G).

Discussion

Our data demonstrate that the rat EUS and EAS operational resting L_s is significantly shorter than the optimal L_s . Such design allows these muscles to increase active and passive force production in response to small physiological strains. For the EAS, our results correlate with previous human studies, demonstrating that the human EAS resting L_s is also on the ascending limb of the length-tension curve.¹³ Operational L_s of human EUS has not been determined. Given the analogous design of the human and rat EAS, as well as rat EAS and EUS, we anticipate that the design of the human EUS is also similar, making the rat a suitable and practical model to study the mechanics of pelvic sphincteric muscles.

Published investigations, mainly conducted in the rat SBI model, identified several mechanisms of striated sphincter injury. Specifically, SBI has been shown to cause hypoxia and ischemic changes, due to decreased blood flow to EUS.¹⁰ Also, pudendal neuropathy, experimentally induced by the crush nerve injury, has been shown to cause EUS and EAS dysfunction.^{11,20,21} These important mechanisms are centered on the factors extrinsic to the sphincters, however, it is imperative to also consider how parturition-related strains effect the intrinsic structural muscle components.

It is established that excessive strains in the limb muscles lead to significant L_s increase with the associated decline in muscle force production.⁸ Analogous to the limb muscles⁸ and the rat pelvic floor muscles⁹, parturition-related strains resulted in EUS and EAS sarcomere elongation (Figure 2), with the corresponding shift from the ascending to the descending limb of the length-tension curve (Figure 1). Also analogous to the other skeletal muscles, sarcomere hyperelongation caused by SBI led to myofibrillar disruption (Figure 3). Importantly, the mechanical events associated with SBI led to acute and long-term urethral and anal sphincter dysfunction, determined by *in vivo* functional testing (Figure 4, 5).

Similar to the direct relationship between a larger fetal head size and a higher degree of muscle stretching observed in women²², and increasing stretch ratios with rising distention volumes observed in the rat pelvic floor muscles⁹, we expected to see progressive sarcomere

elongation of EUS and EAS with increasing distention volumes. However, EUS L_s did not differ significantly in response to vaginal distention with 3 mL vs 5 mL. Initially, we were surprised by this finding. We hypothesized that the above is due to the position of the rat EUS relative to the balloon. To test this assumption, we repeated the experiment in a group of animals, in whom pubic symphysis was disarticulated prior to vaginal distention, which allowed a more rostral position of the large balloon. Imposing strains on EUS along its entire length resulted in significantly greater L_s , compared to vaginal distention with intact pubis. Not surprisingly, a more rostral position of the balloon had an opposite effect on EAS, with significantly less impact on L_s with disarticulated, as compared to intact pubic symphysis. Taken together, the above confirms the direct relationship between parturition-related strains and sarcomere hyperelongation of the pelvic sphincteric muscles, adding to the strengths of this study.

For the functional measurements, the baseline LPP were comparable to the published values.^{11,20} However, our results differ with respect to LPP 4 weeks post-SBI, which were significantly lower than the baseline pressures, as opposed to the normalization of LPP 3 weeks after SBI demonstrated in previous studies.¹¹ One of the important differences between this and the published studies lies in the study design. While the other investigators utilized a cross-sectional study design, we performed a longitudinal study, with LPP data gathered from the same animals repeatedly over the study period. This structure is the strength of the current work, as tracking changes in the experimental group that serves as its own control enables the determination of cause and effect relationships.

With respect to EAS, it was previously demonstrated that the EAS function is not altered by vaginal distention.²¹ In contrast, we observed a significant decrease in EAS contraction. This discrepancy is likely due to the fact that a 2mL distention volume, which is lower than the average fetal rat size⁹, was used in the other studies.²¹

The limitations of the current study include the use of fixed tissue samples, with potential differences in the topographical appearance of the sphincters *in vivo* due to the loss of muscle tone and possible shortening of L_s . Fortunately, the differences in the topology do not appear to affect L_s . We have previously demonstrated that L_s does not differ between fixed and fresh human EAS muscles.¹³ Another limitation is inherent to the use of the experimental model, with a variable position of the rat vs human EUS. However, the rat model is valuable for establishing the precise intrinsic alterations that occur in the pelvic sphincters due to birth injury. Furthermore, we performed SBI with and without pubic symphysis disarticulation to ensure that we capture the full extent of mechanical impact on EUS. We are also conscious that pudendal nerve injury can lead to the EUS and EAS dysfunction. Even though we did not directly test the pudendal nerve, the refractory period, which is altered in the other skeletal muscles as a result of neuropathic injury²³, was unchanged by SBI. Thus, we opine that the deterioration in muscle function observed in the current study is myogenic in origin.

Our results confirm our hypothesis that excessive strains placed on the pelvic sphincteric muscles, cause sarcomere hyperelongation and disruption, leading to muscle dysfunction. The current findings provide data necessary for the development of therapeutic strategies to

counteract SUI and FI by targeting these pathophysiological events. One of the fruitful directions for future investigations includes correlating the extent of myofibrillar lattice disruption with the function of pelvic sphincters.

Conclusions

Resting operational L_s of the rat EUS and EAS is shorter than optimal, which allows these muscles to increase active force as well as passive tension in response to physiological strains. Larger strains in response to SBI, result in pathological sarcomere hyperelongation and disruption of muscle ultrastructure, in turn, leading to dramatic alterations in EUS and EAS function. The above provides a mechanistic link between birth injury and pelvic striated sphincter dysfunction.

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Figure 1. Length-tension curve of the rat skeletal muscle.

A sarcomere is represented schematically by the thick (myosin) and thin (actin) horizontal lines, and vertical lines (Z-disks). The maximum active force is produced at the optimal L_s (2.4 μm in the rat), marked by asterisk. For both external urethral (white rectangle) and anal (black circle) sphincters, the operational resting L_s is shorter than the optimal L_s , placing them on the ascending limb of the length-tension curve. Such muscle design allows a higher active and passive force production in response to small strains.

Figure 2. Mechanical response of the intrinsic structural components of external urethral (EUS) and external anal (EAS) sphincters to parturition-related strains.

Simulated birth injury using vaginal distention with 3 and 5 mL volumes resulted in substantial sarcomere hyperelongation of both sphincters, compared to sarcomere length (L_s) in uninjured controls (A,B). Intrapartum (IP) L_s was analogous to intact pubic symphysis, validating our experimental approach (C,D). Simulated birth injury performed in rats with disarticulated pubis symphysis (SP), resulted in progressive EUS sarcomere hyperelongation with rising distention volume due to strains imposed along the entire length of EUS (C). Conversely, EAS L_s decreased when pubis symphysis was disarticulated (SP) due to a more rostral position of the distention balloon (D). Results are presented as mean $\pm$ standard deviation. N=4/group.

*Significantly different P values derived from one-way analysis of variance (ANOVA), followed by Tukey's post-hoc testing with significance level set to 5%.

Figure 3. Transmission electron microscopy images of external urethral (EUS) and external anal (EAS) sphincters.

Normal striation pattern with aligned sarcomeres is observed in the control muscles (A,C).

Disruption of normal muscle microstructure is observed in response to simulated birth injury, as evident from distortion of Z-lines and misalignment of adjacent sarcomeres outlined by black ovals (B,D). Scale bar is 5 μ m.

Figure 4. *In vivo* measurement of external urethral sphincter (EUS) function.

Leak point pressures (LPP), determined longitudinally, demonstrate substantial decrease caused by simulated birth injury, compared to uninjured values. LPPs were obtained at baseline (A,B); acutely following simulated birth injury (A,C); and after a 4-week recovery period (A,D).

Individual animals are represented by differently shaped and pattern symbols (N=5). The acute and 4-week time points are significantly different compared to baseline ($P < 0.05$). P -values derived from repeated measures one-way analysis of variance (ANOVA), followed by Tukey's post-hoc testing with significance level set to 5%.

Figure 5. *In vivo* measurement of external anal sphincter (EAS) function.

Anorectal manometry, determined longitudinally, demonstrates substantial decrease in pressures generated by EAS contraction, following simulated birth injury relative to uninjured values. Anorectal manometry was performed at baseline (A,B); acutely following simulated birth injury (A,C); and after a 4-week recovery period (A,D). The other functional EAS parameters measured by anorectal manometry did not change (E-G).

Individual animals are represented by differently shaped and pattern symbols (N=5). The acute and 4-week time points are significantly different compared to baseline and each other ($P < 0.05$). P -values derived from repeated measures one-way analysis of variance (ANOVA), followed by Tukey's post-hoc testing with significance level set to 5%.