High-Frequency Micro-Ultrasound: A Novel Method to Assess External Urethral Sphincter Function in Rats Following Simulated Birth Injury

Lukman Hakim,1,2* Masayuki Endo,3,4,5 Andrew Feola,4,5 Doddy M. Soebadi,2 Jan Deprest,3,4,5 Dirk De Ridder,1,3,5 Maarten Albersen,5 and Frank Van der Aa1,3

1Laboratory of Experimental Urology, Department of Development and Regeneration, KU Leuven, Leuven, Belgium
2Department of Urology, Airlangga University School of Medicine/Dr. Soetomo General Hospital, Surabaya, Indonesia
3Pelvic Floor Unit, Department of Development and Regeneration, KU Leuven, Leuven, Belgium
4Research Unit Bio-Implants, Bio-Mechanics and Tissue Engineering, Department Development and Regeneration, KU Leuven, Leuven, Belgium
5Faculty of Medicine, Interdepartmental Center for Surgical Technologies, KU Leuven, Leuven, Belgium

Aims: We evaluated external urethral sphincter (EUS) function using high-frequency micro-ultrasound (US) in rats that were either uninjured (Control, C) or underwent vaginal distension (VD) as a substitute for vaginal birth injury induced stress urinary incontinence (SUI). Methods: Thirteen female nulliparous Sprague–Dawley rats of 12 weeks were divided into two groups, either C (n = 6) or VD (n = 7). Vaginal balloon distension was performed under pentobarbital anesthesia for 4 hours. Five days after the injury, all animals underwent US assessment of the urethra during high-rate bladder filling and urine leakage/voiding. Urinary leakage, the presence, absence, and pattern of EUS bursting during the voiding phase were registered, and pre-determined parameters of intercontraction interval (ICI), length of contraction (LOC), and rate of contraction (ROC) were registered. Results: Our ultrasound findings consistently showed the presence of rhythmic EUS bursting in all of the C rats (6/6), which were absent in all VD rats (0/7). The mean of ROC, ICI, and LOC in C group were 3.02 ± 0.12 contractions/sec, 471.43 ± 17.9 msec, and 103.41 ± 3.28 msec, respectively. Conclusions: Pre-determined parameters of LOC, ICI, and ROC during US provide objective and measurable data on EUS function. US showed the total disappearance of EUS bursting in the VD group as compared to the C group. These results indicate that ultrasound testing may become a valuable non-invasive tool in future translational studies to investigate SUI/urethral function in rat models. Neurourol. Urodynam. © 2014 Wiley Periodicals, Inc.

Key words: external urethral sphincter; high-frequency ultrasound; stress urinary incontinence; vaginal distension

INTRODUCTION

Urinary incontinence (UI) remains a significant problem, and affects more than 200 million people worldwide.1,2 A national survey in the US conducted between 2001 and 2004 claimed that 49.6% of women have self-reported UI symptoms, of which 49.8% reported pure stress incontinence, 34.3% had mixed and 15.9% had urge.3 The underlying mechanism of stress urinary incontinence (SUI) in humans is poorly understood and considered multifactorial. A controlled-experiment in humans would face many ethical issues. Therefore, preclinical animal models are important and may support in understanding the pathophysiology of SUI. A reproducible preclinical model is a prerequisite to develop new treatments.

Among the risk factors for SUI that have been studied, vaginal delivery has been shown to cause urethral injury, while multiparity may lead to urethral dysfunction.4 An animal model that closely simulates urethral and pelvic floor injuries may be advantageous for studying the pathophysiology of SUI.4,5 The effects of vaginal birth were simulated in rodents by vaginal distension (VD), including that on urinary incontinence.5 There is no clear cut universally accepted method to measure urethral resistance and/or function. Among all methods to determine SUI, leak point pressure (LPP) has been generally accepted and widely used to quantify changes in urethra and bladder function in rats.4,6,7 The method (in general pushing the index finger to the rodents abdomen to gradually increase the pressure) is subject to inter-operator variability and requires a learning curve to obtain stable results. The VD model alters LPP not only by effects on the external urethral sphincter (EUS), but also by direct urethral, pelvic ganglia, and levator muscles injury.5

Other studies focusing on the urethral function in rodents have been conducted using an electrophysiology approach.8,9 The electromyogram of the external urethral sphincter (EUS EMG) shows an interrupted bursting of EUS during voiding.8 This bursting phenomenon reflects a series of opening and closing of the urethra in order to void urine, which may disappear acutely following injury of the nerve and recover at a later time point.10 Apart from its invasive nature, the issues related to different sites and types of electrodes and the need for correction of signal collected (noise) are difficult to standardize.

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*Correspondence to: Lukman Hakim, M.D., M.H.A., Laboratory of Experimental Urology, Department of Development and Regeneration, KU Leuven, Leuven, Belgium. E-mail: lukman0908@yahoo.com

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We aimed to test the utility of a new technique, standardized, non-invasive and easy to use evaluation tool for EUS using a high-frequency micro-ultrasound (US) in control rats and rats exposed to VD.

MATERIALS AND METHODS

Vaginal Distension Model Establishment

Nulliparous female Sprague–Dawley rats of 12 weeks old weighing 200–250 g were obtained from the animalium of the KU Leuven. All animals were housed at a constant temperature and humidity level with a 12-hr light-dark cycle, and free access to food and water. Thirteen rats were randomly distributed into two groups, consisting of six uninjured (Control, C) rats and seven undergoing VD.

In this study, a modified-VD model based on Lin et al. and Sievert et al. was used. A virgin-female SD rat of 12 weeks underwent intravaginal balloon distention using a 14 F catheter (Rusch, Teleflex Medical) filled with 5 ml of saline for 4 hr, under pentobarbital anesthesia (40 mg/kg, i.p; Nembutal, Lundbeck Inc., Copenhagen, Denmark). A 130 g weight was hanged from the catheter in a supine position. In our study, the asymmetrical balloon was situated with the area of maximum pressure directed antero-laterally. Buprenorphine (Temgesic®, 0.01 mg/kg) was given following the injury.

A flared-tip transperitoneal suprapubic catheter (PE-50, IntramedicTM) was implanted at 4 days following the injury, 0.01 mg/kg) was given following the injury. Two layers. Leakage and infection. Skin and muscle incision was closed in two groups, consisting of six uninjured (Control, C) rats and seven undergoing VD.

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A flared-tip transperitoneal suprapubic catheter (PE-50, IntramedicTM) was implanted at 4 days following the injury, under 2% isoflurane inhalational anesthesia. A purse-string suture was applied to the bladder dome and tunneled subcutaneously through the back of the rat’s neck where it externalized the skin. We also included an uninjured rats group (C) as control; these animals did not undergo vaginal distention, but underwent catheter implantation. The external catheter tip was capped until further evaluations to prevent urinary leakage and infection. Skin and muscle incision was closed in two layers.

High-Frequency Micro-Ultrasound (US) Assessment

The Vevo 2100 system (VisualSonics Inc., Toronto, Canada) is a micro-ultrasound platform using high-frequency transducers specifically designed for use in small animals which is equipped with B (brightness)-mode and M (motion)-mode features. It displays high temporal resolution images (1,000 fps) either in two or three-dimensions. For this study we opted for the MS 400 (30 MHz) probe which is commonly used for abdominal and cardiovascular imaging in rats and rabbits. Rats were scanned at 5 days following the injury. US was set up to acquire 28 dB, transmit power 100%, sweep speed 200 Hz, and S/ V gate: depth 10.04 mm, length 2.5 mm, angle 0° while depth and focused-depth were adjusted to generate the best image quality of EUS.

The abdominal hair was first removed to ensure good visualization using a chemical hair remover (Nair, Church & Dwight, Princeton, NJ). The US examination was done under urethane anesthesia (1.2 g/kg) to image the urethra during bladder filling and voiding at a saline infusion rate of 60 ml/hr. Rats were placed in the supine-position and a hypoallergenic US gel (Aquasonic, Parker Laboratories, Fairfield, NJ) was applied onto the abdomen (Fig. 1A). To improve anatomical evaluation of the pelvis, 0.2 ml of US gel was injected into the vagina, using a 1.0 ml-syringe, creating contrast around the urethra and bladder neck. First, the bladder was emptied manually which was confirmed by ultrasound, prior to saline infusion for each measurement. Saline infusion into the bladder was performed using a transvesical catheter connected to a syringe pump (Perfusorsecura FT, B-Braun) at a fixed rate of 60 ml/hr to assure accuracy and objectivity until one cycle of voiding was visualized under US, which was reflected by the EUS bursting. Manual compression of the bladder was performed to empty the bladder as confirmed by the US, prior to the 2nd and 3rd measurements. The ultrasound transducer was fixed and placed onto the abdomen just above the bladder during saline infusion to identify the EUS bursting. During both the filling and voiding phases and during urinary leakage, specific attention was given to the presence or absence of EUS bursting. We defined several quantifiable parameters which we could obtain under our standardized setting using motion mode (M-mode) observation: (1) length of contraction (LOC), defined as the average time for each contraction of the EUS (msec), (2) inter-contraction interval (ICI), defined as the average interval time for EUS to contract (msec), and (3) rate of contraction (ROC), defined as the average number of contraction (number of contraction/sec). During saline infusion, the LOC and ICI were measured three times for each rat and means were calculated, meanwhile a ROC was generated from the first 3 sec of bursting measurements. All these three parameters were measured using the Vevo2100 US software feature with a magnification that allowed us to point out the starting and ending point of measurement in an accurate way.

One-sample Kolmogorov–Smirnov test was used to identify the data distribution and considered normal if P > 0.05. Normally distributed data were presented as mean ± SEM. Comparison between VD and C groups was performed using descriptive analysis. No statistical test could be performed since VD group did not show any value due to the absence of contraction.

RESULTS

Ultrasound allowed us to clearly visualize the different anatomical structures of the rat pelvic region (Fig. 1B). The bladder and vagina appeared as hypoechoic spaces. The pubic bone was hyperechoic with a clear acoustic shadow (Fig. 1B–D). In the absence of voiding it was difficult to identify the urethra. During voiding in the C rats and leakage in the VD rats however, the urethra could be seen as a hypoechoic tube-shaped structure connecting the bladder to the external meatus. Using longitudinal dynamic imaging, we saw the bladder neck moving distally during the filling phase and cranially during the voiding phase (Fig. 1C and D). A fixed distance between the starting point of EUS and the bladder neck was thus not obtainable.

In this study, the EUS bursting was clearly shown among all (6/6) of the C rats as a rhythmic pattern within a consistent ICI, as visualized by the M-mode (Fig. 2A). This bursting was not observed at all in the VD group (0/7) (Fig. 2B). We observed leakage of urine from the urethral meatus of all the VD rats in the absence of EUS bursting under US. In the Figure 2 B, this is showed by the narrowing of urethral lumen (UL) without evidence of EUS bursting. Conversely, the C rats showed consistent EUS bursting under US follow by micturition of urine from the urethral meatus (Fig. 2A).

Figure 2C shows how ROC, ICI, and LOC measurements were conducted. Using the US feature, the exact starting and ending point of measurements were determined under magnification. The ROC, ICI, and LOC of C rats in this study were 3.02 ± 0.12 contractions/sec, 471.43 ± 19.9 msec, and 103.41 ± 3.28 msec, respectively. Meanwhile in the VD group, no contraction was observed during saline infusion into the bladder.
DISCUSSION

In this study, we propose a novel and objective measurement of urethral function by characterizing the EUS in a real-time manner using high-frequency micro-ultrasound during high rate intravesical saline infusion. The EUS bursting during voiding was quantified using pre-determined parameters (LOC, ICI, and ROC) and the aim was to compare between VD and C rats. In comparison to the EUS EMG, this method is non-invasive. Unlike the manual LPP method, this novel method does not require operator’s active involvement to increase the bladder pressure.

Animal Models of SUI

Various models in rodent have been described to mimic human SUI, mainly the pudendal nerve injury model, the VD model or the combination of both.5,6,12–17 In this study, we used the VD model in comparison to a control group for several reasons. First, since it was introduced by Lin et al. to study the effect of vaginal trauma on adjacent structures and urinary continence mechanism, the VD model has been commonly used to simulate birth injury related SUI.4–6,11,18–20 Secondly, we believe that the VD model mimics the multifactorial etiology of SUI in human.

Despite its limitation, it is generally accepted that the VD model decreases urethral function and in the short-term leads to SUI. However, this injury model has been shown to recover overtime.21 Pan et al. studied the effect of increased duration of VD on cystometry, LPP and histology using an inflated balloon of either 1 or 4 hr in female rats. The author observed a significant decrease of LPP at 4 days and 10 days following the injury but did not see differences at 6 weeks following a distension of 4 hr, suggesting that the VD model recovered by time. Moreover, at 10 days following 1 hr of VD, no significant difference was observed in comparison to sham group. When combined, these results showed that increased duration of VD resulted in increased time of recovery.22 Similarly, Jiang et al. compared VD alone (4 hr of balloon inflated), pudendal nerve crush (PNC) alone or combined model to simulate childbirth injury. The author observed the absence of EUS bursting activity based on EMG study among all groups at 4 days following injury, little recovery at 3 weeks and significant recovery of the guarding reflex at 6 weeks following injury.16 There are various studies of the VD model in rats which used different sizes of catheters, different balloon volumes and

Fig. 1. The use of high-frequency micro-ultrasound (US) to identify the EUS function in female rats. A: US using a 30 MHz probe. The probe is fixed by an arm; the anesthesia table with the rat can be moved to obtain maximal quality images. B: Normal pelvic structure of a rat under micro-ultrasound in a longitudinal view. (1) bladder, (2) vagina, (3) urethra, (4) pubic bone, and (5) cervix. To the right of the screen a bar displays the dimension scale. C and D: Micro-ultrasound longitudinal view of (C) Control group and (D) VD group, during the voiding phase. To the left of the image the tubular structure (1) is the urethra in the external urethral sphincter area, (2) corresponds to the bladder neck, (3) is the bladder. The white line (4) corresponds to the pubic bone, with its acoustic shadow.
different types of animals (i.e., nulliparous or breeder rats). Ranges varied between 8 and 22 F of catheter size with 1.5–5 ml of balloon volume. We used 5 ml for balloon dilatation in virgin-female rats to assure it damaged the pelvic floor muscles including the pudendal nerve axons.4–6 In this study, 4 hr of distension with 130 g weight was implemented to assure that functional alteration still exist by the time of measurement at 5 days following the injury.

Methods to Determine SUI

Clinical urodynamics cannot simply be implemented in animals. Most attention has been focused to the measuring of urethral resistance in animals and previous studies have described various methods to determine SUI: (1) sneeze-testing, (2) manual LPP testing, (3) vertical tilt Table LPP testing, electrical stimulation LPP testing, and (4) urethral closure pressure testing. Most of the current methods are operator dependent and subject to inter-observer variability.23 Moreover, it is not always clear whether these methods are used to detect SUI or whether they were used to detect overflow incontinence since none of these methods allows to determine bladder filling status during urine leakage.

Among these methods, manual LPP testing has been advocated to simulate clinical SUI. In this test, an operator’s index finger is placed on the abdomen just above the bladder to gradually increase bladder pressure until leakage of urine is observed from the external urethral meatus. Manual LPP testing has a learning curve to achieve stable results and is subject to inter-observer variability. To address this shortage, Lee et al. has invented the vertical tilt Table LPP method by positioning a female-rat in vertical position while connecting

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**Fig. 2.** External urethral sphincter (EUS) functional measurement results using US. A and B. M (motion)-mode images of the (A) control group and (B) VD group. The lower part of the image displays the motion visualized in the cross section at the level of the EUS, marked by the line M. The motion pattern suggests EUS bursting compatible with opening and closing of the sphincter during pressure induced voiding. This feature was absent following VD. On cross-section opening of the urethral lumen (UL) suggests urinary leakage process. C. M (motion)-mode image of a control rat. To the right of the screen a bar displays the dimension scale, while numbers at the bottom show time of measurement in sec. (1) shows the time of contraction (TOC), which was measured in 3 sec in this study, showing a result of 3,025 msec. The motion pattern suggests EUS bursting, with a total of right contractions in 3 sec measurement. Combining these results, it shows a rate of contraction (ROC) of 8/3 or 2.67 contractions/sec. (2) The length of contraction (LOC) in ms, and a mean was counted from three cycles for each rat. (3) Inter-contraction interval (ICI) in ms, and mean was also generated from three cycles for each rat. The black area (4 to 4I) shows the opening of urethral lumen during voiding.
the bladder to a reservoir of saline using a transvesical catheter.24 This method is more likely objective than manual determination of LPP, since a step-wise height increased of reservoir (1–3 cmH₂O) was performed instead of using operator’s index-finger. However, this method is invasive and eliminates the spontaneous bladder activity in response to the increasing bladder pressure, by transecting the spinal cord at the level of T9.23

Shoffstall et al. developed a device to standardize LPP experiments in rats. A soft-tipped force applicator with a force sensor which can move in vertical direction by a linear actuator and completed with a wired remote control system was used by either an expert or novice experimenter to measure LPP at different speeds. These results were then compared to manual LPP measurement performed by the same experimenters. Though mean values did not differ among experimenters and among methods of measurements, the use of this novel device significantly reduced variability in comparison to the manual LPP method. The device also detected leaks 385 ± 187 msec earlier than the manual method, which may lead to a significant potential impact on LPP outcomes.25

Since it was introduced by Maggi et al.,8 the use of EUS EMG has been increasingly used as a method to assess the EUS function. The voiding pattern in normal rats has been characterized as an interrupted bursting which may disappear acutely after injury, and recovered by time.4-10,26,27 The absence of EUS bursting based on US among injured-VD group in our study support these studies.

Apart from its invasive nature, the EUS EMG results will depend on the location of electrode placement, the type of electrode, the EUS injury that might have happened during the placement of electrode and the access of EUS for electrode placement. Opening the symphysis pubis using a forceps as proposed by liang et al.9 to access the pudendal nerve, might injure the pudendal nerve itself which innervates the EUS from the lateral side of urethra. Moreover, the need for correction of signal collected (noise) needs to be standardized in order to minimize variability among studies. High-frequency micro-ultrasound in this study may be used as an novel technique to address this issue.

High-Frequency Micro-Ultrasound

We used a two-dimensional B (brightness)-mode to visualize anatomical structures of the bladder, bladder neck and urethra at the time of urine leakage. A high-rate saline infusion of 60 ml/hr. was applied arbitrarily as forced-diuresis to induce SUI in VD rats and voiding in C rats. In the C group, saline infusion at 60 ml/hr. during the filling phase allowed the bladder to increase its capacity until a certain moment voiding started. The bladder neck position tended to move distally as bladder filling increased and vice versa as it decreased. The EUS, which appeared as a tube distal to the bladder neck, was clearly observed as a series of opening and closing during voiding (Fig. 1B-D). As soon as bladder pressure exceeded urethral pressure, the EUS showed a series of opening and closing activities while expelling urine. This bursting activity stopped as micturition ended. This phenomenon is remarkably identical to EUS bursting (milking) as previously described on EMG, which are believed to be associated with pulsatile flow of urine in male and female rats.28

The M (motion)-mode enables us to identify tissue motion with a single ultrasound beam. We observed an individual rhythmic interval pattern generated by EUS bursting in all C rats (Fig. 2A), showing a consistent force and time interval of the EUS in producing milking process of the urine.

Interestingly, we observed the total absence of EUS bursting in the VD group (0/7) at 5 days following injury, suggesting a complete alteration (absence) of urethral function (Fig. 2B). VD rats showed leakage of urine coming out from the urethral meatus in the absence of EUS bursting under US, during the intravesical infusion of saline, confirming SUI. This is the first report where US has been used to identify the presence and absence of EUS bursting in control rats and in a VD model. The absence of EUS bursting in our study suggests injury to the pelvic floor muscles and/or innervation of the EUS. Our study supports the results of Lin et al.5 that showed c-Fos immunoreactivity in neuron in the dorsal horn and around the central canal in spinal segments L6 and S1, indicating nerve injury or irritation in the area of simulated birth trauma following VD in rats. We also support the results of Cannon et al.4 who showed the evidence of pressure-induced ischemia and/or pelvic floor muscles injury in VD rats which lead to the dysfunction of urethral continence mechanism. Furthermore, Damaser et al. confirmed a higher percentage of degenerated nerve fascicles in the ventral region of the EUS than dorsal, suggesting more axons are injured near impact with a hard surface (pubic bone), rather than near the source of crushing injury (vagina). Meanwhile, more axons of the pudendal nerve course ventrally than dorsally in the urethra. Therefore, pudendal nerve axons are vulnerable to injury during VD.6

The mean ROC, ICI and LOC in the C group were 3.02 ± 0.12 contractions/sec, 471.43 ± 17.9 msec, and 103.41 ± 3.28 msec, respectively (Fig. 2C). We could not perform statistical test in this study for a comparison, since the VD group did not show any EUS bursting at all. These results were also similar to previous study by liang et al.9 who observed the elimination of EUS EMG activities in VD group at 4 days following injury.

LIMITATIONS OF STUDY

As other preclinical studies, this study has limitations. We used high-infusion rate of 60 ml/hr during the filling phase to induce urinary leakage in C and VD rats as a SUI model. To which extend this supra physiological filling rate influences the EUS bursting pattern, the bladder contraction pattern or both, is beyond our study. Future study comparing different rates of saline infusion should be addressed to identify the correlation between the rate of saline-infusion, the bladder contraction pattern and the pattern of EUS bursting based on US imaging.

Second, since the use of US to determine SUI in this study is a new technique, we focused more on the functional alteration results, rather than taking the inter-observer variability into account. In the clinical setting, the use of US has been shown to provide various intra or inter-observer variabilities, depending on the object of measurement. Therefore, future blinded study among groups involving more than 1 operator should be conducted to address this issue.

Thirdly, we did not compare our US findings with EMG findings in our rats. Our US findings show striking similarities to previously published EMG findings in similar rat studies. It would be interesting to perform both techniques simultaneously in a single experimental setup. We are currently setting up these experiments.

CONCLUSION

In control rats, high-frequency micro-ultrasound using predetermined parameters (LOC, ICI, and ROC) provides objective and measurable data on EUS function. After vaginal distention, which resulted in rat SUI, no EUS bursting was observed anymore. These results indicate that ultrasound imaging may
become a valuable objective and non-invasive tool in future translational studies to investigate urethral function and SUI in rat models.

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