Ultrasonography of the adult male urinary tract for urinary functional testing

Teresa T Liu^{1,2,*}, Allison C Rodgers^{3,*}, Tristan M Nicholson⁴, Jill A Macoska^{5,6}, Paul C Marker^{6,7}, Chad M Vezina^{6,8}, Dale E Bjorling^{6,8}, Alejandro Roldan-Alzate^{2,9,10}, Diego Hernando^{10,11}, Granville L Lloyd¹², Timothy A Hacker³, William A Ricke^{1,6}

¹Department of Urology, University of Wisconsin-Madison, Madison, WI, USA

²K12 Kure, University of Wisconsin-Madison, Madison, WI, USA

³Cardiovascular Research Center, Department of Medicine, University of Wisconsin-Madison, Madison, WI, USA

⁴Department of Urology, University of Washington, Seattle, WA, USA

⁵University of Massachusetts Boston, Boston, MA, USA

⁶U54 George M. O'Brien Center, University of Wisconsin-Madison, Madison, WI, USA

⁷College of Pharmacy, University of Wisconsin-Madison, Madison, WI, USA

⁸School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI, USA

⁹Department of Mechanical Engineering, University of Wisconsin-Madison, WI, USA

¹⁰Department of Radiology, University of Wisconsin-Madison, WI, USA

¹¹Department of Medical Physics, University of Wisconsin-Madison, WI, USA

¹²Rocky Mountain Regional VA Medical Center/ University of Colorado, Aurora, CO, USA

Abstract

The incidence of clinical benign prostatic hyperplasia (BPH) and lower urinary tract symptoms (LUTS) is increasing due to the aging population, resulting in a significant economic and quality of life burden. Transgenic and other mouse models have been developed to recreate various aspects of this multifactorial disease; however, methods to accurately quantitate urinary dysfunction and the effectiveness of new therapeutic options are lacking. Here we describe a method that can be used to measure bladder volume and detrusor wall thickness, urinary velocity, void volume and void duration, and urethral diameter. This would allow for the evaluation of disease progression and treatment efficacy over time. Mice were anesthetized with isoflurane, and the bladder was visualized by ultrasound. For non-contrast imaging, a 3D image was taken of the bladder to calculate volume and evaluate shape; the bladder wall thickness was measured from this image. For contrast-enhanced imaging, a catheter was placed through the dome of the bladder

Corresponding Author: William A. Ricke, rickew@urology.wisc.edu, Tel: (608) 265-3202.

* contributed equally to this work

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The authors have nothing to disclose

using a 27-gauge needle connected to a syringe by PE50 tubing. A bolus of 0.5ml of contrast (Perflutren Lipid Microsphere, DEFINITY) was infused into the bladder until a urination event occurred. Urethral diameter was determined at the point of the Doppler velocity sample window during the first voiding event. Velocity was measured for each subsequent event yielding a flow rate. In conclusion, high frequency ultrasound proved to be an effective method for assessing bladder and urethral measurements during urinary function in mice. This technique may be useful in the assessment of novel therapies for BPH/LUTS in an experimental setting.

SUMMARY:

We describe the use of high frequency ultrasound with contrast imaging as a method to measure bladder volume, bladder wall thickness, urine velocity, void volume, void duration, and urethral diameter. This strategy can be used to assess voiding dysfunction and treatment efficacy in various mouse models of lower urinary tract dysfunction (LUTD).

Keywords

Ultrasound; benign prostatic hyperplasia; mouse models; urinary flow; aging; lower urinary tract dysfunction

INTRODUCTION:

Benign prostatic hyperplasia (BPH) is a disease that develops in men as they age and affects nearly 90% of men over 80 years of age^{1,2}. Although the development of BPH is generally associated with aging, other factors including obesity and metabolic syndrome can lead to BPH in relatively younger men^{3,4}. Many men with BPH develop lower urinary tract symptoms (LUTS) that significantly decrease their quality of life, and some experience complications that may include bleeding, infection, bladder outlet obstruction (BOO), bladder stones, and renal failure. The cost of treatment for BPH exceeds \$4 billion annually⁵⁻⁷. Diagnosis of LUTS caused by BPH generally relies on the use of the AUA symptom index (AUASI) score, uroflowmetry, and assessment of prostate size⁸. The etiology of BPH/LUTS is complex and multifactorial, and disease development and progression has been associated with prostatic hyperplasia (prostate proliferation), smooth muscle contractility, and fibrosis. Current treatments include the use of α -adrenergic blockers to regulate smooth muscle tone within the bladder and prostate to alleviate LUTS and/or 5 α -reductase inhibitors to decrease androgen metabolism and decrease prostate size. Better disease models, murine and other, to allow the accurate study of the effects of varied causative and therapeutic factors in this disease process over time is highly desirable⁹.

Rodent models have been extensively used to study urodynamics; however, most studies are focused on female micturition and disease¹⁰. In order to fully examine all aspects of male LUTS, rodent models have been developed and used to study different aspects of BPH including changes in cellular proliferation, smooth muscle function, collagen deposition, and inflammation¹¹⁻¹⁴. However, rodent and human prostate anatomy differ. While the human prostate is compact and encased by a condensed fibromuscular layer, the rodent prostate is lobular; and these differences complicate direct comparisons of disease progression and

treatment efficacy. Additionally, LUTS are difficult to assess in mice, since it is not possible to directly measure bother. Instead, current methods for studying disease correlate histological features with physiological features (i.e. bladder volume and wall thickness with uroflowmetry, void spot assays, and cystometry endpoint data) that compare the level of urinary dysfunction between BPH model and control animals^{12,15–18}. Physiological features are frequently evaluated as post-mortem necropsy endpoints, and there is an inability within the same animal to observe BOO across time. Recently, we have identified a subdivision of the pelvic urethra (the prostatic urethra) where exogenous hormone implants cause a narrowing based on post-mortem necropsy assessments¹². Current methods do not allow for the direct, *in vivo* assessment of urethral narrowing during voiding.

Ultrasound is a non-invasive diagnostic and evaluation technique that has successfully been used in other disease models. It is used to quantify organ volume and assess vascular flow^{19–21}. Ultrasound is also used to visualize and guide microinjections, allowing for targeted injections of stem cells or other drugs, and to evaluate systolic and diastolic cardiac function.

This protocol describes the use of high frequency ultrasound to evaluate lower urinary tract anatomy and assess urinary physiology in anesthetized mice. We describe the use of ultrasound for measuring bladder volume and wall thickness. We also describe the use of contrast-enhanced ultrasound (Perflutren Lipid Microsphere, DEFINITY) to measure urine velocity, urine volume, void duration, and urethra diameter. The use of ultrasound provides a more comprehensive understanding of the lower urinary tract *in vivo*, determines how disease alters normal voiding function, and gives us the tools to better evaluate the effectiveness of new therapeutic options. Currently, the non-contrast imaging protocol is non-terminal, while the current contrast-enhanced imaging protocol is a terminal procedure.

PROTOCOL:

NOTE: All procedures were performed in mice in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols.

1. Animal preparation

1. Place a 24 month old, C57Bl6/J male mouse in a pre-charged chamber with 3–5% isoflurane until righting reflex is lost and breathing rate slows.
2. If necessary, use clippers to shave the abdominal hair from the animal for surgery and/or imaging. All remaining hair is removed using a depilatory cream.
3. Place the mouse in a supine position in a nose cone with 2% isoflurane on a heated platform to maintain anesthesia. Depth of anesthesia is confirmed by loss of motion from the animal in response to a pedal-withdrawal reflex (Figure 1B).

2. Ultrasound set-up

1. Connect a MV707 probe with center frequency of 30 MHz to the active-port, with the application preset to “abdominal imaging” (Figure 1A).

2. Position the ultrasound probe parallel to the long axis of the bladder (Figure 1C).
3. Long and short axis images of the bladder, the prostate, and the urethra are made in B-Mode (Figure 1D).
4. Use the “xy” micro-manipulators to move the mouse.

3. Non-contrast imaging protocol

1. The bladder wall thickness is measured using the linear distance measurement tool and tracing the outside edge to inside edge of the bladder wall b-mode post acquisition.
2. The bladder 3D volume is measured with the volumetric tool on the 3D mode acquisition by tracing the inside of the bladder walls to create a contour. Multiple contours are then generated through the thickness of the bladder to calculate volume.

4. Microbubble contrast resuspension/activation

1. Activate DEFINITY by shaking in the vortex mixer for 45 seconds to encapsulate the microbubbles in solution. This step is critical for optimum contrast enhancement.

5. Catheter insertion

1. With the mouse anesthetized and taped to the heated platform, expose the bladder with a midline incision using straight sharp/blunt scissors through the skin and abdominal wall.
2. Insert a 27-gauge needle connected to a syringe by flexible polyethylene tubing (PE 50) into the bladder. The tubing is prefilled with saline to ensure no air bubbles are injected into the bladder.

6. Contrast-enhanced imaging protocol

1. To confirm needle placement, 10 μ L of saline is instilled into the bladder while being observed via ultrasound.
2. The saline syringe is replaced with a syringe containing contrast to improve visualization of urethral walls and voiding events, because the urethra is normally collapsed. Once a complete long axis view of the urethra is obtained and an image saved, the probe is rotated 90 degrees to obtain a short axis view and an M-Mode image.
3. A bolus of microbubbles is instilled at 0.5ml/3seconds into the bladder until a urination event occurs.
4. During the first voiding event, the urethral diameter is measured at the point of the Doppler velocity sample window using the linear distance tool and measuring edge to edge.

5. With the urethra properly located, the probe is angled in relation to the urethra to become more parallel to the urine flow.
6. A second bolus of microbubbles is instilled into the bladder, the event velocity is measured using the velocity time integral (VTI) tool.
7. After data collection, the mouse is euthanized with cervical dislocation.

7. Data Calculation and Analysis

1. The VTI tool was selected to measure velocity by tracing the recorded images.
2. The diameter of the urethra was measured from the b-mode or m-mode image using the leading edge to leading edge convention.
3. The cross-sectional area (CSA) was calculated using the following formula ($CSA = \pi r^2$) using image measurements obtained above.
4. Estimated void volume is calculated using the CSA of the urethra and multiplying that by the area under the Doppler trace (velocity time integral)($CSA \times VTI = \text{volume}$).
5. Actual voided urine volume is calculated assuming **one gram per cubic centimeter** density.

REPRESENTATIVE RESULTS:

Ultrasound can be used with or without contrast enhancement depending on the experimental design and endpoint measurement. Mice are anesthetized with isoflurane and shaved and all traces of hair removed with a depilatory cream. Anesthetized animals are placed on a heated platform with the ultrasound probe positioned along the long axis of the bladder (Figure 1).

Figure 2 shows representative ultrasound images of a mouse bladder acquired without contrast agent. The bladder wall is hyperechoic (white), and the bladder wall thickness is measured using the Vevo Lab[®] software measurement package. A bladder surface projection can be rendered to determine bladder volume, wall thickness, and wall volume (Table 1).

For contrast enhanced bladder and lower urinary tract imaging, a catheter must be inserted into the bladder and microbubbles injected. It is important to activate the microbubbles per manufacturer's protocol for optimal imaging. Figure 3 shows a representative image of a microbubble-filled bladder. The bladder is hyperechoic (appears white in the image). A low frequency ultrasonic burst destroys the bubbles and the bladder becomes transiently hypoechoic (appears black), before the bubbles reform, confirming this structure as bladder. During the destruction pulse, the transmit power goes to 100% and the transmit frequency drops to 10MHz. A bolus of microbubbles (0.5ml/3seconds) triggers a voiding event and makes it possible to visualize the urethra. The urethra is confirmed by applying a low frequency ultrasonic burst during the urination event and observing destruction and reformation of microbubbles. Several measurements can be made during the urination event. The urethral lumen diameter can be acquired pre- and post-urination along with the flow

velocity of urine passing through that region of the urethra and total outflow (Figure 4). Using contrast imaging, measurements were made through the entire length of the urethra (Table 2). From these measurements, further calculations are made to examine urinary flow and bladder compliance (Table 3).

DISCUSSION:

Current techniques for evaluating the lower urinary tract of rodents are limited by their ability to directly correlate changes in voiding physiology with changes in prostatic histology consequent to disease progression. Void spot assays and uroflowmetry can be used to assess spontaneous urination events in rodents, and these techniques can be used to evaluate changes over a period of time^{15–17}. However, for both techniques, bladder fullness cannot be assessed prior to the start of the test. Additionally, changes in urination may occur due to behavior rather than a direct result of disease progression, making it difficult to determine the impact of disease on urination. Cystometry, another technique for evaluating bladder dysfunction in rodents, can provide an *in vivo* assessment of bladder function¹⁶. However, the dynamic effect of the rodent prostate on voiding function is not clear. Previous studies have documented mouse urethral histological changes in mice associated with altered voiding function¹². However, these studies can only look at one discrete time point, and bladder, prostate, and urethral anatomy are not assessed at the same time as functional testing. Other methods of evaluating changes within the bladder (i.e. mass, volume) occurs at time of euthanasia^{11,12}, rendering it impossible to observe evolution of a disease process over time. Because there is the risk of urination at time of sacrifice as well as the inability to regulate the water intake prior to sacrifice, the variability of measured bladder volume increases even within treatment groups. This paper describes the use of ultrasound to image the lower urinary tract of mice with or without a contrast agent. This technology allows for the visualization of changes in bladder size in an intact animal, as well as assessment of functional changes of bladder volume, bladder wall thickness, urethral lumen diameter, and velocity of contrast passing through the urethra. Specifically, contrast-enhanced ultrasound allows for the visualization of the urethral lumen, specifically in the prostatic region, during voiding in a fashion that pinpoints a region of potential dysfunction.

To ensure collection of consistent and accurate ultrasound data, it is crucial that a single trained sonographer collects and reads ultrasounds throughout the course of the study. For contrast enhanced imaging, it is important to activate DEFINITY microbubbles according to manufacturer protocol. Activated microbubbles should be diluted with 0.9% saline solution. Undiluted microbubbles are so concentrated that they prevent ultrasound wave penetration and will shadow structures lying below them. Microbubble dilution also reduces experimental costs. Microbubble dilutions can be varied without negative experimental effects as needed by the user.

Evaluation of bladder volume and bladder wall thickness in an intact animal allows examination of disease progression and evaluation of treatment efficacy over time. Currently, treatments for BPH are administered in rodent models either as the disease is induced or at a time point previously determined to result in significant disease progression^{11,22,23}. Efficacy of treatment is typically determined after a single, discrete period of time, despite the fact

that biological variability may affect time required to respond to treatment. Using our novel technique, a rodent model for BPH can be evaluated from the induction of the disease phenotype through the entire treatment protocol.

A contrast agent enables the lower urinary tract to be visualized before, during, and after a urination event. We have previously examined urethral histology in a mouse model of BPH. We localized the prostatic urethra as the putative region giving rise to urinary dysfunction. This region contains more prostatic ducts, denser collagen, and a smaller lumen than in control mice¹². Additionally, the BPH-susceptible mice demonstrate voiding dysfunction as measured by uroflowmetry and void spot assays. Using ultrasound with microbubbles, we can directly evaluate the region of the prostatic urethra to measure flow velocity, duration, and luminal diameter (Figure 3 and 4). By identifying the region where flow is impeded using ultrasound, that specific region can then be further evaluated histologically to determine the main component of dysfunction.

This technique is reproducible across mouse strains and across a range of mouse ages and treatment conditions. In addition to aged, male mice, this technique can be used to evaluate younger mice with metabolic abnormalities that could lead to BPH/LUTD. The technique can also be used to evaluate female mouse voiding and lower urinary tract function. Although the ultrasound protocol with contrast described here is a terminal procedure, we can perform a suprapubic cystostomy, creating the potential for non-terminal contrast imaging of the lower urinary tract²⁴. Future experiments will optimize visualization of urinary tract functions to allow for repeated measures. Depending on the experimental questions, the techniques described here can be combined with other functional urinary testing techniques to gain more insight into disease progression and treatment efficacy.

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Figure 1. Ultrasound setup.

A. Overall imaging setup. **B.** Positioning of mouse on platform. **C.** Bladder is exposed and catheterized with the ultrasound probe lined up for long axis bladder imaging. **D.** Ultrasound gel and probe placed on exposed, catheterized bladder for long and short axis for imaging.

Figure 2. Non-contrast imaging of mouse bladder.

A. Image of mouse bladder without microbubbles. **B.** Measurements of bladder wall thickness from b-mode bladder image. **C.** 3D reconstruction of mouse bladder. **D.** Surface area and shape extrapolated from 3D image for further analysis.

Figure 3. Contrast-enhanced imaging of mouse bladder and urethra.

A. Full bladder with microbubbles. **B.** Full bladder with microbubbles after destruction event. **C.** Mouse urethra with microbubbles. **D.** Mouse urethra with microbubbles after destruction event. Urethra outlined in red.

Figure 4. Measurements taken from mouse urethra.

A. Urethral lumen diameters measured during a urinary event. **B.** Urinary flow velocity through the penile urethra during voiding.

Table 1.
Non-invasive ultrasound measurements of the bladder.

Bladder volume and bladder wall thickness measured by ultrasound.

Trial	Bladder volume (mm³)	Bladder wall thickness (mm)	Bladder wall volume (mm³)
164	47.44	0.82	12.14
166	87.54	0.83	29.84
167	100.94	0.58	51.53
163	152.12	0.7	74.61
165	116.39	0.61	59.28

Table 2.
Ultrasound measurements of the bladder and urethra.

Bladder and urethra measurements made by ultrasound during urinary voids.

Measurement Type	Image Required	Location	Measurement
Bladder volume (mm ³)	Bladder 3D Mode	Bladder	335
Bladder wall thickness (mm)	Bladder B-Mode	Distal to bladder neck	0.25
		Proximal to bladder neck	0.23
		Bladder neck	0.83
Urethra diameter (mm) *	Urethra B-Mode	Prostatic urethra	0.33
		Membranous urethra	0.5
		Penile urethra	0.25
		Bladder neck	3960
Urinary event time (ms) *	Urethra PW mode	Prostatic urethra	3740
		Membranous urethra	3530
		Penile urethra	4490
		Bladder neck	580
Acceleration time (ms) *	Urethra PW mode	Prostatic urethra	440
		Membranous urethra	240
		Penile urethra	180
		Bladder neck	1218.08
Acceleration (mm/s ²) *	Urethra PW mode	Prostatic urethra	3685.76
		Membranous urethra	3054.79
		Penile urethra	11031.4
		Bladder neck	184.2
Velocity time integral (cm) *	Urethra PW mode	Prostatic urethra	490.48
		Membranous urethra	157.55
		Penile urethra	676.93
		Bladder neck	494.09
Mean velocity (mm/s) *	Urethra PW mode	Prostatic urethra	1256.82
		Membranous urethra	467.04
		Penile urethra	1565
		Bladder neck	780.74
Peak velocity (mm/s) *	Urethra PW mode	Prostatic urethra	1655.85
		Membranous urethra	820.97
		Penile urethra	2190.94
		Bladder neck	0.98
Mean gradient (mmHg) *	Urethra PW mode	Prostatic urethra	6.32
		Membranous urethra	0.87
		Penile urethra	9.8
		Bladder neck	2.44
Peak gradient (mmHg) *	Urethra PW mode	Prostatic urethra	10.97
		Membranous urethra	2.7

Measurement Type	Image Required	Location	Measurement
		Penile urethra	19.2

* Measurements captured during urinary event

Table 3.
Calculations using ultrasound measurements.

Calculations and formulas applicable to ultrasound measurements to assess bladder function and urinary flow.

Calculation Type	Formula
Cross-sectional area (mm ²)	$CSA = \pi r^2$
Flow rate (mm ³ /s)	Flow rate = CSA × Mean Velocity
Estimated void volume (mL)	$V = (Flow\ Rate/1000) \times (Event\ Time\ in\ Seconds)$
Volumetric stretch	$Stretch = (V_{after} - V_{before})/V_{before}$

Name of Material/Equipment	Company	Catalog Number	Comments
21mm Clear Tubing	Supera Anesthesia Innov	301–150	
27 gauge needle	BD	Z192376	
4 port Manifold	Supera Anesthesia Innov	RES536	
DEFINITY	Lantheus Medical Imaging	DE4	
F/AIR Canister	Supera Anesthesia Innov	80120	
Graefe forceps (Serrated, Straight)	F.S.T.	11050–10	
Inlet/Outlet Fittings	Supera Anesthesia Innov	VAP203/4	
Isoflurane	Midwest Vet Supply	193.33161.3	
Isoflurane Vaporizer	Supera Anesthesia Innov	VAP3000	
MV707 probe	Fujifilm VisualSonics Inc		
Oxygen Flowmeter	Supera Anesthesia Innov	OXY660	
Polyethylene 50 tubing	BD	427516	
Pressure Reg/Gauge	Supera Anesthesia Innov	OXY508	
Rebreathing Circuits	Supera Anesthesia Innov	CIR529	
Small Mice Nose Cone	Supera Anesthesia Innov	ACC526	
Sterile saline	Midwest Vet Supply	193.74504.3	NaCl 0.9%, Injectable
Straight			
Sharp/Blunt	Fine Scientific Tools (F.S.T)	14054–13	
Scissors			
Syringe	BD	309646	5mL
Vevo 770	Fujifilm VisualSonics Inc		
VIALMIX	Lantheus Medical Imaging	VMIX	