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ARTICLE

Validation of a new rat model of urethral sphincter injury and leak point pressure measurements

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ABSTRACT

Aims: *In vivo* experiments were performed to establish and validate a rat model of urethral sphincter injury and to develop a method for leak point pressure (LPP) measurements performed repeatedly in the same animal.

Methods: Twenty-four Sprague-Dawley female rats underwent bladder and epidural catheter implantation. Five days later, cystometry was performed using continuous infusion. Anesthesia with isoflurane, ketamine-xylazine (KX) or fentanyl-fluanisone-midazolam (FFM) was used. After three micturition cycles, intrathecal bupivacaine was administered leading to the suppression of reflex bladder contractions. LPP measurements were performed using vertical tilt. After the initial LPP measurement, animals underwent partial resection of the striated urethral sphincter. The effect was evaluated 6 weeks after surgery, by repeating the LPP measurement in the same animal.

Results: Ten out of 19 animals showed full micturition cycles under isoflurane, and all 9 animals under KX anesthesia. No significant difference in micturition pressures (Mean ± SEM; 30.1 ± 2.3 vs. 26.8 ± 1.6 mmHg) and LPP (31.0 ± 2.4 vs. 28.0 ± 0.9 mmHg) was observed between isoflurane and KX groups, respectively. Reflex micturition was suppressed with FFM. Bupivacaine led to overflow incontinence in all cases. Sphincter injury caused fibrotic changes and a significant increase in LPP (26.4 ± 2.3 before vs. 46.9 ± 4.6 mmHg after injury, $p < 0.05$).

Conclusions: KX anesthesia preserves bladder contractions. Intrathecal bupivacaine eliminates reflex micturition, allowing for repeated LPP measurements in the same animal. Resection of striated sphincter resulted in increased LPP 6 weeks post injury. The site of urethral sphincter resection healed with fibrosis.

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Urinary incontinence; animal model; rat; leak point pressure; anesthesia

Introduction

Stress urinary incontinence (SUI), the involuntary leakage of urine on effort or exertion, affects millions of individuals worldwide and is severely debilitating [1]. Treatment of SUI includes conservative management which often fails, and pharmacological therapy which has limited effectiveness. Surgical therapies wherein the surgeon uses non-degradable synthetic materials to create a sub-urethral hammock or mechanically obstruct the urethra, can lead to serious complications [2,3]. Alternative treatments that restore natural continence mechanisms have been broadly investigated in animal models [4,5].

Rodent models of SUI have been most widely used in both basic research and preclinical studies, evaluating new treatment strategies. They were designed with the intent to mimic known pathophysiological mechanisms that have been linked to SUI [6]. The main limitations of currently available rat animal models include failure to develop stable chronic incontinence and lack of a testing procedure that can reliably quantify the degree of incontinence repeatedly in the same animal [7]. The degree of urethral sphincter dysfunction is quantified by measuring leak point pressure (LPP), defined as intravesical pressure leading to urine leakage. LPP measurements in rodents require the elimination of reflex micturition [8]. To date, elimination of the supraspinal micturition reflex has been achieved by spinal cord transection, which does not allow for repeated measurements of LPP in the same animal [9,10]. In addition, the LPP measurement cannot be performed in awake animals. All available anesthetic agents affect urodynamic parameters and therefore the anesthesia or sedation with least effects on the urethral sphincter must be selected [11,12]. Our study aimed to address these limitations by developing a method, where urinary sphincter pathology is present six weeks after injury and LPP can be measured repeatedly in the same animal. In addition, we compared the effects of three anesthetics most broadly used in lower urinary tract function protocols.

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Materials and methods

Animals

Twenty-four ($n = 24$) female Sprague-Dawley rats (weighed 250–350 g, aged 10–20 weeks) were housed at the University of Southern Denmark Central Animal Care Facility as per institutional guidelines. The Ethics Committee of the Danish Animal Experiments Inspectorate approved the study procedures (Protocol No. 2016-15-0201-01042). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical procedures

The initial surgical procedures involved the implantation of an intravesical catheter and the insertion of an intrathecal catheter in the lumbar region. The LPP was measured 5 days later. Spinal anesthesia was used to eliminate the micturition reflex. The initial LPP measurement was followed by the resection of approximately 30% of the striated urethral sphincter. The cystometry and LPP measurement was repeated in the same animal six weeks following sphincter injury. The surgeries were performed under sterile conditions and the animals were followed closely. No signs of bladder or wound infection were detected. All signs of discomfort were resolved prior to cytometric studies.

Bladder catheterization

A lower midline abdominal incision was performed to expose the dome of the urinary bladder and a second incision was made between the shoulder blades. Turning the animal onto its side, a hemostat was advanced subcutaneously through the incision between the shoulder blades and tunneled obliquely towards the abdominal incision. The tip of the instrument was advanced until it pierced through the abdominal muscles and entered the abdominal cavity. PE-50 tubing with a flare created on one end was then pulled out through to the incision between the shoulder blades. A purse-string suture was loosely placed on the dome of the bladder. A 21-G needle was used to create a cystotomy in the bladder dome at the center of the purse-string suture (Figure 1). Through this cystotomy, the flared end of the PE-50 tubing was inserted into the bladder, secured in place by tying the purse-string suture around the tubing and pulling on the tubing until the flared end was positioned in the bladder dome.

Intrathecal catheterization

With the animal in a prone position, a 20-mL syringe was placed under the abdomen to elevate the lumbar spine. A skin and fascial incision was made in the lumbosacral area. A modified 22-G needle with an open groove was inserted into the spinal canal between the L6 and S1 vertebrae. A tail twitch confirmed the correct placement of the needle tip before the insertion of a 32-G intrathecal catheter (Instech, Plymouth Meeting, PA, USA) with its tip advanced to the level of T12 (Figure 1(B)). The catheter was then secured in place by sutures through the lumbar fascia and connected to PE-10 tubing. This tubing was then tunneled subcutaneously and externalized at the skin incision between the shoulder blades. Distal ends of both the intravesical and intrathecal tubes were plugged and coiled in a subcutaneous pocket for easy retrieval. Both skin incisions were closed with running sutures.

Sphincter injury

The sphincter injury was created on day 5 post bladder catheterization, immediately after the recording baseline LPP. Access was gained to the urethral sphincter muscle through a lower midline abdominal incision. The bladder was pulled cephalad and blunt dissection was used to expose the urethra behind the pubic symphysis. Two vertical incisions in the striated urethral sphincter were made using microforceps at 2- and 10-o'clock positions. Striated muscle was bluntly dissected from the urethra and removed (Figure 2). The abdominal incision was closed in two layers. The excised tissue was confirmed to be striated muscle by histological evaluation.

Postoperative care

For pain relief, all rats received subcutaneous injections of buprenorphine (0.05 mg/kg every 8 h) 24 h after surgery and carprofen (5 mg/kg every 24 h) 2–3 postoperative days as needed based on clinical signs.

Cystometry and LPP measurement using three anesthetic protocols

Filling cystometry and LPP measurements were performed using three anesthetic protocols. A dose which provided sufficient sedation while preserving toe pinch reflexes was used. We assessed the feasibility and compared cystometric parameters under inhaled isoflurane (Attane Vet® 1000 mg/g, Piramal Critical Care, Netherlands) with oxygen as the carrier; intraperitoneal ketamine/xylazine (KX – Ketaminol® 50 mg/mL, MSD Animal Health, Netherlands; Rompun® 20 mg/mL, Bayer, Germany), and subcutaneous fentanyl-fluanisone-midazolam (FFM – Hypnorm®-fentanyl 0.315 mg/mL and fluanisone 10 mg/mL, Janssen-Cilag, UK; Dormicum®-midazolam, 5 mg/mL, Roche AB, Sweden). For intravesical filling, a syringe with 0.9% NaCl was placed in the infusion pump connected in series to a Digitimer/NeuroLog system pressure transducer (Digitimer Ltd., UK) and intravesical catheter. The infusion rate was set at 5 mL/hour. A Spike-2 software (CED, UK) was used to record three reproducible micturition cycles. Subsequently, spinal anesthesia was administered using repeated intrathecal injections of bupivacaine (50 μg) in 6-minute intervals and LPP was recorded. LPP was defined as intravesical pressure at the time when the first drop of urine appeared at the urethral orifice.

Twenty-four hours after the first LPP measurement, under isoflurane anesthesia, the experiment was repeated in the

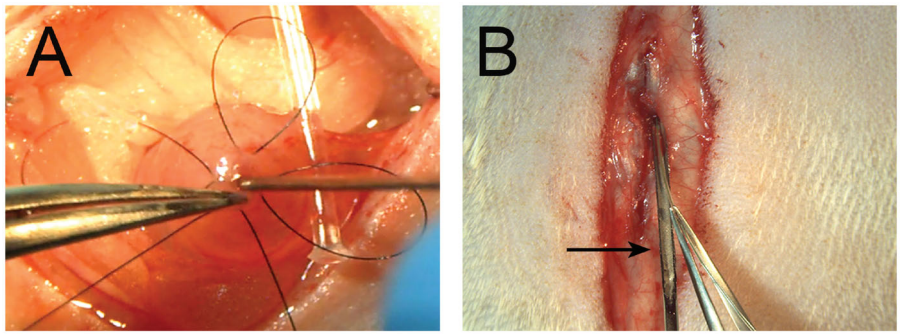

Figure 1. (A) Purse-string suture placed at the dome of the bladder and opening made for insertion of a flared end of the PE-50 tubing. (B) Skin and fascial incision over the lumbosacral spine. A modified 22-G needle with an open groove inserted into the spinal canal between L6 and S1 (marked by arrow) vertebrae for intrathecal catheter insertion.

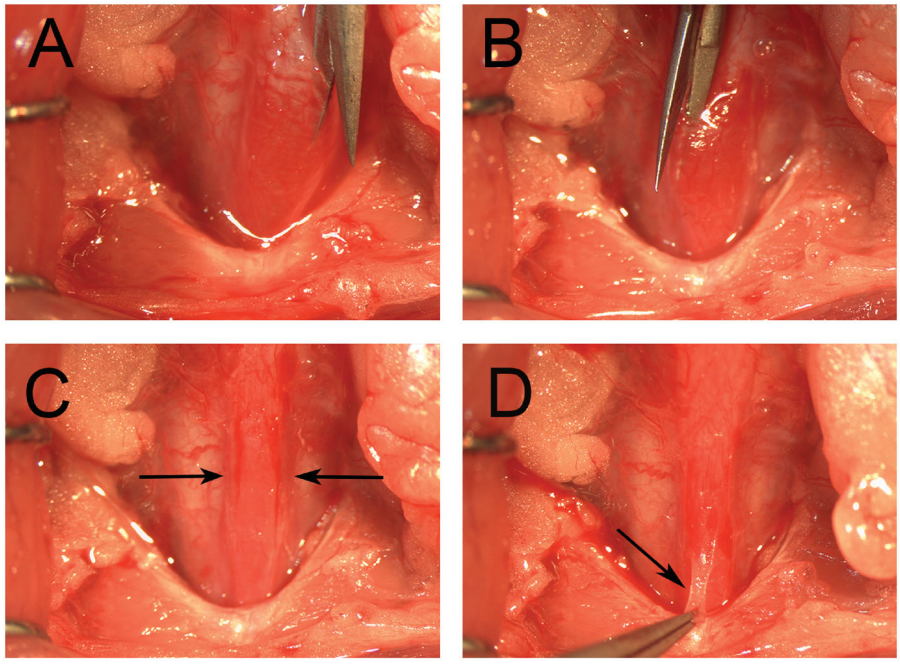

Figure 2. Depiction of the microsurgical technique of external sphincter resection. (A–C) Two longitudinal striated sphincter incisions approximately at 10- and 2-o'clock position (arrows). (D) Dissection of the striated muscle (arrow).

same animal under anesthesia with KX. FFM anesthesia was used in five animals. In the final group of experiments, under KX anesthesia, sphincter injury was performed after the first LPP measurement. A second LPP measurement was repeated six weeks later (Figure 3).

Histopathological analysis

After the second LPP measurement, the urinary bladder and proximal urethra were resected, and the animal was euthanized. The tissue was fixed for histological analysis using hematoxylin and eosin (H&E) and Sirius Red stains.

Statistical analysis

The values were reported as mean ± standard error of the mean (SEM). A student's paired *t*-test was used to compare LPP before and six weeks after the sphincter injury in the same animal. A two-sample *t*-test was used to compare differences between the cystometric parameters under three different anesthetics. The following parameters were compared: maximal micturition pressures (MP) and LPP. Differences between groups were considered statistically significant at p-value <0.05.

Results

LPP measurement under isoflurane anesthesia

The success rate of intrathecal catheter insertion was ~90%. In the initial experiments, the position of the intrathecal catheter was confirmed by an observation that injection of bupivacaine caused transient paraplegia in the awake rat. Cystometry under isoflurane anesthesia produced full micturition cycles in 10 out of 19 animals tested. The concentration of isoflurane necessary for sufficient sedation ranged from 1.2 to 2% with oxygen administered as the carrier at 0.8 L/min. The mean MP was 30.1 ± 2.3 mmHg. Intrathecal administration of bupivacaine completely blocked micturition contractions resulting in overflow incontinence in all animals.

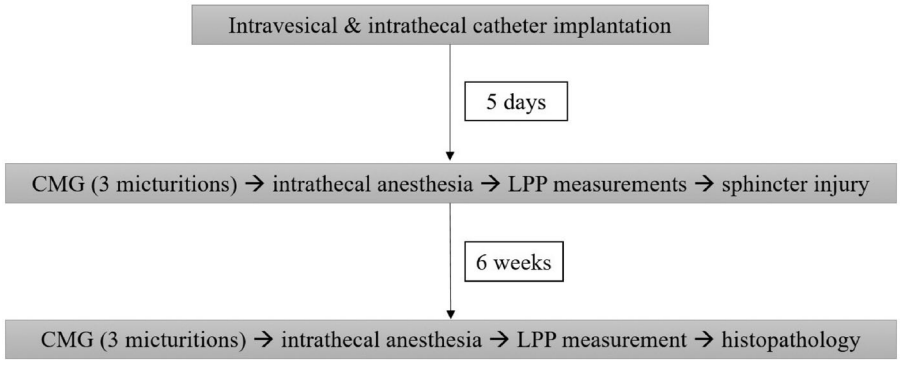

Figure 3. Diagram of the model validation experiments. CMG: cystometrography; LPP: leak point pressure.

Table 1. Cytometric parameters of 3-cycle micturition pressure (MP) and leak point pressure (LPP) in isoflurane vs. ketamine-xylazine anesthetized rat models.

Animal	1st MP (mmHg)	2nd MP (mmHg)	3rd MP (mmHg)	Mean MP (mmHg)	LPP (mmHg)
Isoflurane					
Rat 1	23.5	26.8	27.4	25.9	29.3
Rat 2	32.0	36.9	35.6	34.8	31.0
Rat 3	19.6	18.2	17.9	18.6	24.4
Rat 4	38.6	34.6	34.9	36.0	42.2
Rat 5	33.9	36.9	37.1	36.0	43.8
Rat 6	25.0	25.3	24.5	24.9	27.2
Rat 7	18.6	29.6	33.0	27.1	24.1
Rat 8	27.6	31.5	39.0	32.7	30.3
Rat 9	24.3	20.6	22.7	22.5	21.2
Rat 10	36.5	42.7	48.0	42.4	36.8
Summary				30.1 ± 2.3	31.0 ± 2.4
Ketamine-xylazine					
Rat 11	26.3	24.5	23.7	24.8	30.5
Rat 12	22.7	23.4	23.0	23.1	26.0
Rat 13	31.5	25.8	22.9	26.7	31.8
Rat 14	23.6	16.8	16.1	18.8	23.9
Rat 15	27.8	27.5	28.1	27.8	27.3
Rat 16	29.6	30.5	28.9	29.7	31.5
Rat 17	20.7	26.8	25.7	24.4	25.8
Rat 18	30.4	28.9	29.6	29.6	29.2
Rat 19	32.6	36.0	40.9	36.5	26.0
Summary				26.8 ± 1.6	28.0 ± 0.9

The mean LPP was 31.0 ± 2.4 mmHg (Table 1). LPP in the 9 animals that did not void was significantly lower (25.9 ± 1.1 mmHg).

LPP measurement under ketamine-xylazine anesthesia

KX – 60 mg/kg and 6 mg/kg intraperitoneal respectively – which represents 60% of the dose recommended for surgical anesthesia – proved sufficient for cystometry and for the LPP measurement. Cystometry under KX anesthesia preserved full micturition cycles in all 9 animals. The mean MP was 26.8 ± 1.6 mmHg. The mean LPP was 28.0 ± 0.9 mmHg (Table 1).

LPP measurement under fentanyl-fluanisone-midazolam anesthesia

In all animals ($n = 5$), subcutaneous FFM (80 μg/kg, 2.5 mg/kg, 1.25 mg/kg, respectively) – a dose equivalent to one third of that recommended for surgical anesthesia – resulted in sufficient depth of anesthesia for intravesical infusion and pressure recording. This reduced dose eliminated micturitions and induced urine leak under low intravesical pressures (18.3 ± 0.98 mmHg).

Comparison of LPP before and 6 weeks after the sphincter injury

A LPP of 29.8 ± 1.7 mmHg was recorded before and 46.9 ± 4.6 mmHg after sphincter injury. The successful experiments were performed in 10 out of 12 animals. In two animals, measurement failed due to an obstruction of the vesical catheter. An increase in LPP six weeks post injury was recorded in all animals where the two LPP measurements were completed successfully (Figure 4).

Histopathological analysis

Histological examination of the urethral sphincter region six weeks after surgical resection showed a defect in the sphincter with a fibrotic scar involving approximately 1/6 of the circumference. At six weeks, only a slight inflammatory reaction persisted in the adventitia (Figure 5).

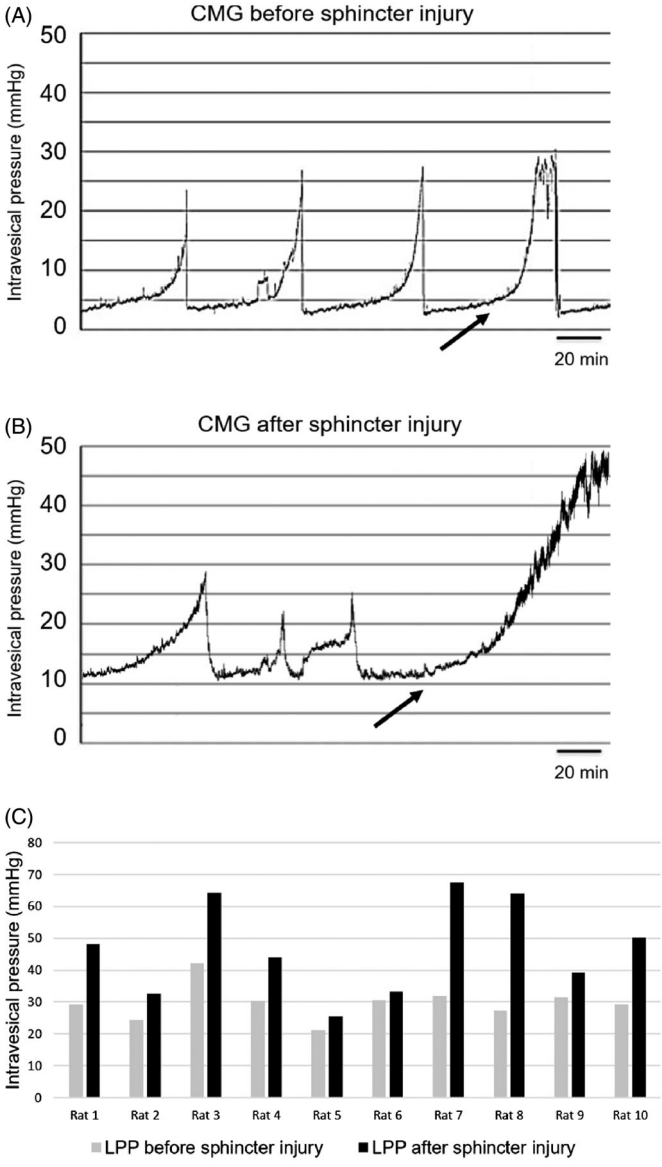

Figure 4. Representative cystometrograph (CMG) recording showing three reproducible micturition cycles followed by intrathecal anesthetic administration (marked by the arrow) before (A) and six weeks after sphincter injury (B). Leak point pressure (LPP) was recorded at the point when first drop of urine appeared at the urethral orifice. (C) Bar graph summarizing LPP before and six weeks after sphincter injury.

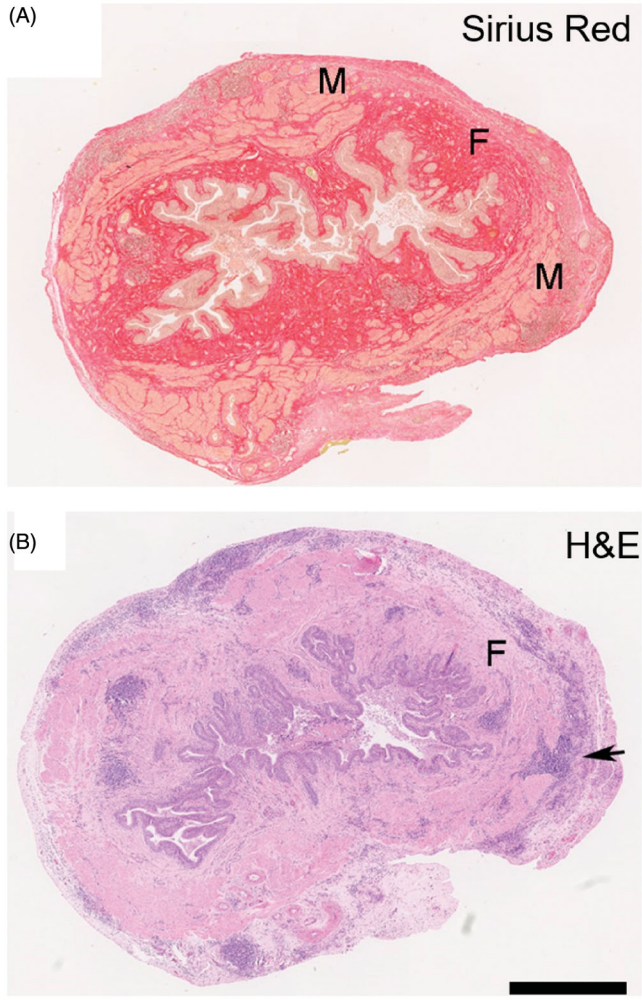

Figure 5. Histology of the lesioned urethral sphincter. (A) Sirius Red stain showing the fibrous scar tissue (F) in the gap between the striated muscle of the sphincter (M). (B) Hematoxylin and eosin (H&E) stain showing low cellularity in the damaged area indicating a mature scar. Some inflammation persists in the adventitia (arrow). Bar scale = 1 mm.

Statistical analysis

There were no statistically significant differences in the MPs ($p = 0.271$) and LPPs ($p = 0.269$) between the two types of anesthesia. A statistically significant increase in LPP was recorded 6 weeks after sphincter injury ($p = 0.002$).

Discussion

This study described and validated three modifications to an existing rat model of SUI. We modified the urethral sphincter injury to achieve chronicity of the sphincter pathology and long-lasting functional impairment, replaced spinal cord transection with spinal anesthesia to allow for repeated LPP measurements in the same animal, and three types of general anesthesia were tested, and the protocol which best preserves sphincter function was identified.

Current SUI rodent models use vaginal distension and injury to the urinary sphincter, its innervation and/or its vascular supply [6,13]. The limitations of currently used rat models stem from the fact that the rat's urethral tissue, as well as its innervation and vascular supply have a strong capacity to regenerate. This is evident from the recovery of LPP to normal values after the initial drop, or absence of chronic urine leakage during behavioral studies [14]. Long-lasting incontinence in rodent models was achieved, when extensive sphincter injury (e.g. urethrolysis) or simultaneous injury to various mechanisms involved in rat continence (e.g. pudendal and hypogastric nerve transection or pubourethral ligament and pudendal nerve transection) were used. These models, however, have been criticized as being not entirely representative of etiological factors leading to clinical SUI [13]. The clinically relevant animal models of SUI should be stable, with a chronic quantifiable decrease in urethral closure pressure. Such an effect has been accomplished in a canine model which used resection of a quarter of the striated urethral sphincter [15]. In this study, we adapted this

technique in rats. We found the resection of ~30% of striated urethral sphincter feasible and reproducible. This intervention, however, has not achieved the desired effect – a decrease in LPP. An increase in LPP, suggestive of bladder outflow obstruction, developed 6 weeks post resection in all animals. As suggested by the pathology examination of the urethra, the bladder outflow obstruction possibly developed due to the fibrosis in the area of the sphincter resection. Even though this outcome did not fulfill our goal to create chronic SUI, it might prove to be a clinically relevant model of urethral fibrosis [16]. We also plan to examine its utility in the testing of stem cell therapy for urethral sphincter regeneration, post injury.

Measurement of LPP in experimental animals requires the elimination of reflex voiding. To date, this has been achieved in rats by spinal cord transection, which does not allow for repeated measurements (e.g. before and after therapy). Our study aimed to address this limitation and to develop a method where urinary sphincter function can be measured repeatedly in the same animal, thereby increasing the reproducibility and reducing the number of animals necessary to obtain significant data. Subarachnoid catheterization in a rat can be performed through the atlanto-occipital membrane or at the junction of the L5 and L6 lumbar vertebrae [17,18]. We used the latter technique and advanced the tip of the catheter to the level of the T12 spinal segment. The administration of a spinal anesthetic at this level blocked reflex detrusor contraction while preserving sphincter function [19]. Both lidocaine and bupivacaine could be used for this purpose. Bupivacaine has a lower neurotoxic effect and provides a longer duration of anesthesia, allowing for the use of a lower dose and increased time intervals between injections [20].

Anesthetics affect urodynamic parameters through their selective effects on the nervous system and smooth and striated muscle [21,22]. In the case of LPP measurements, the use of general anesthesia or sedation cannot be avoided. Isoflurane is a widely used anesthetic that exerts its effect through the inhibition of neurotransmitter-gated ion channels in the CNS. It also has sites of action within the spinal cord that induce skeletal muscle relaxation through the inhibition of NMDA-type glutamate and glycine receptors [23]. Its major advantage is a short recovery time and good control of the depth of anesthesia. Isoflurane was shown to affect both the detrusor and the urinary sphincter [24]. Relevant to the LPP measurement, Chang and Havton documented that isoflurane has a suppressive effect on motoneuron function as recorded using sphincter electromyography [21]. Van Asselt et al. [25] proposed a model where LPP is measured under isoflurane anesthesia without performing spinal cord transection. The LPP they recorded was lower than that of previous studies. We used isoflurane and adjusted the level to the minimal dose that would provide sedation sufficient for cystometry. The goal was to see if reducing the dose would limit the suppressive effects. We documented that reflex micturition could be preserved only in approximately half of animals. The additional disadvantage of this anesthesia was that the dose needed for a successful LPP measurement differed between animals (range 1.2–2.0%). This inter-individual variability in the dose necessary to produce sedation and the effects of isoflurane on the lower urinary tract function suggest that isoflurane anesthesia is not appropriate for recording LPP.

Cannon and Damaser [11] had examined the effect of KX on the MP by comparing the values between awake cystometry and cystometry under urethane. They concluded that KX has no effect on MP. Their study used the Credé maneuver to induce urine leak, which could be considered a less accurate method for the measurement of LPP, due to urethral kinking [26]. When examining the use of KX, we started by determining the minimum sedation dose necessary for cystometry. We found that 60 mg/kg and 6 mg/kg of ketamine and xylazine respectively, representing approximately half of the dose recommended for surgery, was the lowest possible dose. This type of anesthesia preserved reflex micturition and allowed for the measurement of LPP in all cases. No statistically significant difference in MP and LPP was observed between isoflurane (the subgroup with preserved reflex micturition) and KX. This could have been due to the high variability in the isoflurane group. Fentanyl-fluanisone-midazolam is a preferred combination of anesthetic for surgery. When used for minor procedures (e.g. tail vein injection), it provides sufficient sedation and analgesia at a dose of 80 μg/kg, 2.5 mg/kg and 1.25 mg/kg respectively, which represents one third of that recommended for surgical anesthesia. The benzodiazepine, midazolam, is a muscle relaxant which, even at this significantly reduced dose, eliminated reflex bladder contractions resulting in continuous leak at a low intravesical pressure. In a rat study, Ceran et al. documented that midazolam caused a reduction in detrusor contractility *in vitro* [27]. Midazolam is, however, also a spinal cord-mediated skeletal muscle relaxant [28]. Therefore, urethral sphincter relaxation is likely the main reason for continuous leakage of urine at low intravesical pressures.

The administration of various types of anesthetics in a rat affected bladder capacity. In our study, bladder intercontractile intervals were longer in the isoflurane-anesthetized rats when compared with the KX-anesthetized rats. The other cystometric parameters did not exhibit significant differences between isoflurane and KX groups in this study. This is in contrast to previous studies using isoflurane for rat cystometry, which reported a suppression of the micturition reflex and inhibition of the urethral sphincter muscle activity [21,22]. A potential explanation for these variations between the studies may be attributed to the depth of anesthesia. A concentration of 2% isoflurane was used in the experiments by Matsuura and Downie [22], whereas in the present study lower levels of isoflurane anesthesia was used in the majority of experiments.

Conclusion

This study documented that striated sphincter resection creates dysfunction of the urethral sphincter, which persists 6 weeks post resection. Our results imply that the increase in LPP following resection of 30% of the striated sphincter is

possibly due to fibrosis. While this model does not induce chronic incontinence, we believe that it is suitable for testing therapies focusing on regeneration of damaged urethral tissue. Spinal anesthesia preserves urethral sphincter activity while eliminating reflex voiding. This model allows for repeated LPP measurements in the same animal. Sedation using a reduced dose of KX allows for reproducible cystometry.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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