Neuroanatomical and behavioral correlates of urinary dysfunction induced by vaginal distension in rats

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Running head: Urinary dysfunction in awake rats after vaginal distension

Keywords: external urethral sphincter; dorsal nerve of the clitoris, major pelvic ganglion, micturition
The aim of the present study was to use a model of simulated human childbirth in rats to determine the damage to genitourinary structures and behavioral signs of urinary dysfunction induced by VD in female rats. In Experiment 1, the length of the genitourinary tract and the nerves associated with it were measured immediately after simulated human delivery induced by vaginal distension (VD) or sham (SH) procedure. Electroneurograms of the dorsal nerve of the clitoris (DNC) was also recorded. In Experiment 2, histological characteristics of the bladder and major pelvic ganglion (MPG) of VD and SH rats were evaluated. In Experiment 3, urinary parameters were determined in conscious animals, during 6 h of dark and 6 h of light, before and three days after VD or SH procedure. VD significantly increased distal vagina width (p<0.001) and length of the motor branch of the sacral plexus (p<0.05), the DNC (p<0.05) and vesical nerves (p<0.01), and decreased DNC frequency and amplitude of firing. VD occluded the pelvic urethra inducing urinary retention, hematomas in the bladder and thinness of the epithelial (p<0.05) and detrusor (p<0.01) layers of the bladder. MPG parameters were not modified after VD. Rats dripped urine in unusual places to void, without the stereotyped behavior of micturition after VD. The neuroanatomical injuries after VD occur alongside behavioral signs of urinary incontinence as determined by a new behavioral tool for assessing micturition in conscious animals.
Introduction

Micturition consists of two phases, storage and urine expulsion. During storage the detrusor is relaxed while the bladder neck and urethra are activated, preventing involuntary bladder emptying (11). Extrinsic elements such as the levator ani muscle also contribute to maintenance of continence (3). When the bladder reaches its threshold volume, spinal and supraspinal reflexes are triggered to induce bladder contraction and urethral relaxation, and urine flows through the urethra (11). Damage to the lower urinary tract and/or its innervation can induce urinary dysfunction (3, 29, 41).

Urinary dysfunction affects the health of many women (60). Stress urinary incontinence has been described as involuntary loss of urine during effort, and is the most prevalent urinary disorder related to vaginal childbirth, which is known to injure the pudendal nerve and denervate the external urethral sphincter (EUS) (3, 15, 57).

Maternal pelvic viscera and nerve damage results from the difficulty of human childbirth due the large fetal head and brain relative to maternal pelvis size. Neonates at birth have heads that are close to the size of the maternal birth canal through which they must pass during the second stage of parturition (48). Births of fetuses over 4 kg or fetal malposition often prolong parturition (30), retaining the fetus in the pelvic cavity, the main anatomic resistance to fetal expulsion. Prolonged second stage of parturition is not uncommon in primigravid women, with an incidence of 37% (30, 55).
Simulated delivery injury models, including vaginal distension with a balloon (VD) in rats, have been created to better understand the injury process during parturition of women (20, 33). The diameter of the balloon and the duration of the distention can be adjusted to mimic difficulty of parturition in women. A prolonged parturition can be modeled with VD of longer duration compared duration of parturition in intact rats (19, 34). Employing the VD model, investigators have demonstrated bladder, urethral and vaginal hypoxia (9), anatomical and functional damage to the EUS and its innervation (4, 21), and decrease in urethral resistance (26). Whether these VD-induced structural and functional changes are sufficient to cause signs of voiding dysfunction in awake animals is unknown.

Most of the studies using simulated delivery injury models have focused on urethral neuromuscular injury (4, 21, 58). However, in addition to the pudendal nerve, there are other somatic and autonomic nerves running over, or adjacent to, the vaginal wall that could also be stretched during childbirth (40). Nonetheless, injury to pelvic, perineal or bladder nerves have not been investigated in the rat VD model of simulated delivery. Moreover, no behavioral correlate of urinary dysfunction has been investigated after VD in conscious rats.

The aim of the present study was to determine in anesthetized rats, the VD-induced damage to genitourinary structures and nerves, and in conscious animals, the behavioral signs of urinary dysfunction.

Materials and Methods
Experimental design

Thirty four adult nulliparous Wistar female rats (250-300 g body weight) were housed with water and food ad libitum, and maintained on a 12/12 h light/dark cycle. The experimental protocol was approved by Tlaxcala University Committee on Laboratory Animals, according to the guidelines of the Mexican Council on Laboratory Animals Care (NOM-062-Z00-1999).

Animals were randomized to undergo 4 h of VD (n=19) or sham VD (SH, n=13). In order to simulate the difficulty of the fetus passing though the pelvic cavity and the injury induced by a prolonged second stage of human childbirth, the balloon was filled with 4 ml water, resulting in a balloon diameter of 19 mm, ~15% greater than the diameter of the cranium of newborn pups (16.5 mm from occipital to nose). To simulate prolonged human 2nd stage of parturition, the duration of VD was 4 h, 45% greater than the average duration of parturition of intact rats (2.5-3.0 h, from bleeding to whole pups expulsion) (19, 34). Considering that during a second stage dystocic parturition, the fetus stays in the pelvic cavity trying to be expelled, the VD was provided tonically for four hours.

In Experiment 1, immediately after VD (n=6) or SH procedure (n=6), the length of the genitourinary tract and the nerves associated with it were determined, and electroneurograms (ENGs) of the dorsal nerve of the clitoris (DNC) were recorded in 4 animals per group. In Experiment 2, histological characteristics of the bladder and MPG of VD (n=4) and SH (n=4) rats were evaluated. In Experiment 3, urinary behavior was characterized in conscious animals, during 6 h of dark and 6
h of light, before and three days after VD (n=9) or SH procedure (n=3). All analysis was performed in a blinded manner.

Vaginal distension (VD)

Rats were anesthetized with a mixture of intraperitoneal ketamine (60 mg/kg) and xylazine (7.5 mg/kg), and additional doses were used as needed. In VD animals, for catheter accommodation, a cotton swab with mineral oil was introduced into the vagina. Then, a modified 10F Foley balloon catheter was inserted into the vagina and inflated with 4 ml water for 4 h. In SH animals the catheter was placed into the vagina but was not inflated. The catheter was secured with a double silk suture at the skin of the perivaginal orifice.

Gross anatomy

In Experiment 1, immediately after VD or SH procedure, with the catheter in place and before balloon deflation, a longitudinal skin incision was made at the midline of the abdominal and pelvic region. Using a stereomicroscope (MZ6 Leica), a laparotomy was performed and the pelvic bones were uncovered. The width of the distal vagina and the length of the bladder were determined. The volume of urine in the bladder was measured with an insulin syringe. Then, a portion of the right ilium, ischium and pubic bones were dissected and in an antero-posterior direction, the length of the vesical nerves, of the DNC and of nerves running over the ventral wall of the vagina (1-3 in Fig. 1) were measured (4 in Fig. 1). The length
of the motor branch of the sacral plexus (MBSP) was measured in a ventro-
dorsolateral direction. To measure the length of the nerves, a thread was placed
over each one (in the anatomical direction described above) and cut with the
endings aligned with anatomical reference points showed in Fig. 1. The length of
the cut thread was then measured with a caliper. Drawings were made and digital
photographs were taken.

Electroneurograms

The DNC was dissected at the level of the ischiatic arch. The right DNC was
transected and bipolar platinum hook electrodes were placed on the distal portion
of the sectioned nerve. The electrodes were connected through a Grass 7P511
amplifier (bandpass filtered at 100 Hz to 3 kHz) to an electrophysiological recording
system (Digidata 1440A, 10 kHz sampling connected to a computer running
AxoScope software) to store and print the action potentials. Warm mineral oil was
applied. To stimulate activity of sensory fibers, ENGs were recorded before,
during, and after gently squeezing the clitoral hood with forceps for approximately
one second (4.8” stainless steel Adson forceps without teeth). This was performed
at least 3 times.

Histology of bladder and MPG

In Experiment 2, immediately after VD or SH procedure, using a
stereoscopic microscope, the bladder and the right MPG were harvested, fixed in
formalin (4%), embedded in paraffin and sectioned with a microtome at 7 µm
thickness. The sections were stained with hematoxylin & eosin (H&E) or Masson’s
trichrome, examined with an optical microscope (Axio Imager A1, Carl Zeiss, Thornwood, NY), and photographed with a digital camera (Cannon PowerShot S50, Canon USA, Lake Success, NY).

Thickness of bladder layers were determined in three cross sections of the middle region, using AxioVision digital image processing software, version 4.6 (Carl Zeiss). Two values were taken per section: at the midline of the dorsal and ventral walls. The two values were averaged for each animal and the mean was utilized to create a group average.

The area of the MPG was determined measuring three sections of the middle of the ganglion per rat and averaging the data. From these sections, the area of the soma of 20 neurons localized in the middle of the MPG was also determined. Neurons with a visible nucleus were chosen. The area was measured by tracing the outline of the MPG or cell body and calculating the enclosed area using AxioVision. MPG thickness was determined by adding the thickness of 7µm sections where neurons were found.

Micturition recording

Micturition was recorded as previously described (24). The recordings took place in the 6 last h of dark and the 6 first h of light. The rats were habituated in an observational cage during the pre-test prior to VD. The observational cage consisted of a large (43 x 53 x 20 cm) transparent cage, with water and food in the middle of the cage, and the plexiglas floor replaced with a wire grid supported 4 cm above a glass-topped stand. A camera was placed in front of the cage. A urine
A closed circuit video with infrared cameras was used to record urinary parameters and voiding behavior. The rats were observed via digital video, with the monitor in a room adjacent to the animal room. When urine was observed in the collector plate, the observer entered the animal room and measured the urine volume using an insulin syringe and wiped the urine collector. The urinary behavior parameters recorded were voiding frequency, defined as number of voids during 6h (dark or light) and voided volume, defined as the mean volume collected per voiding in a 6h period. The videos were replayed to characterize the female’s behavior during urine expulsion. In previous studies it has been described that male rats void at the edges of the cage, mostly in the corners (24, 25). To urinate, the rat moves to the edge of the cage, places the rump toward the wall, raises the tail and expels the urine (25). In the present study, the criteria to classify a urine expulsion as a void was the stereotyped behavior of micturition: placing the rump facing the wall of the cage to expel urine (in any edge of the cage). Any urine expelled in another posture was considered to be a result of urinary dysfunction or urinary leakage.

**Data Analysis**

Results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Sigma Plot Software (version 12, Systat Software, Inc.). In Experiments 1 and 2, means among VD and SH groups were compared using a Students t test.
For the ENGs, a 1-second recording sample during clitoral hood stimulation was segmented and archived using AxoScope (Axon instruments). Quantitative assessment of ENG signals was performed by determining the mean rectified amplitude and firing rate or mean frequency of firing. Mean value from 3 samples in each rat were calculated. Values for each animal were used to calculate the mean and standard error. Students t test was used to determine significant differences between groups.

In Experiment 3 the number of urine expulsions (voids and leaks) per rat were counted per light/dark phase (6 h dark or light). From those values the mean and standard error were calculated. ANOVA for repeated measures was used to compare data of urinary behavior parameters, before (dark and light phases) and after (dark and light phases) SH or VD. Urinary parameters were: voiding frequency, defined as number of voidings during the 6h; voiding interval, defined as time in minutes between one voiding and the next; voided volume, defined as the volume collected per voiding; percentage of voids and percentage of leaks. The Tukey posthoc test was used to compare individual groups. P<0.05 indicated a statistically significant difference for all statistical comparisons.

**Results**

*Experiment 1. Genitourinary tract and nerves stretching*

VD significantly increased distal vagina width (SH 9.8 ± 0.2 mm vs VD 17.0 ± 0.3 mm, p<0.0001, Fig. 2) and bladder length (SH 12.0 ± 0.5 mm vs VD 25.0 ±
1.1 mm, p<0.0001, Fig. 2) but not urethral length (SH 22.5 ± 0.5 mm vs VD 27.0 ± 0.6 mm, p=0.07). In VD animals the balloon pressed the middle urethra against the pelvic bone, inducing occlusion of that structure, which prevented micturition and in consequence produced temporary urinary retention during VD (urine volume; SH 0.40 ± 0.15 ml vs VD 1.67±0.25 ml, p<0.001). Hematomas were present only in bladders of VD rats (Fig. 2).

VD significantly increased the length of the genitourinary nerves. The vesical nerves were elongated by about 56%, the anastomotic branches that originate from MPG by 36%, and the MBSP and the DNC by 50% (Fig. 1, Table 1). ENG amplitude and frequency visibly increased during clitoral hood stimulation but both were significantly decreased after VD compared to SH (Fig. 3).

**Experiment 2. Bladder and MPG features**

VD overdistended the bladder and significantly decreased thickness of the epithelial (p<0.05) and detrusor layers (p<0.01), but not the submucosa (Fig. 4). Blood and leucocyte extravasation were observed in VD rat bladder tissue. MPG area (SH 0.397 ± 0.050 mm^2 vs VD 0.419 mm^2 ± 0.061 mm^2) and thickness (SH 653.0 ± 51.0 µm vs VD 808.0 ± 128.0 µm), and the area and diameter of MPG neurons (Fig. 5, p>0.05) did not change with VD.

**Experiment 3. Micturition behavior**

SH rats voided more frequently in the dark than the light phase (1.65±0.15 vs 0.8±0.06 voids/h, p<0.01; Tables 2, 3). Voiding interval (p<0.01) and voided volume (p<0.05) were higher in the light than the dark phase (Tables 2, 3). To void,
100% of the SH rats walked to the edges of the cage (urine was found in the four edges but mainly on the corners), placed the rump toward the wall and expelled urine, flowing in a stream. In 50% of the voids, the rats had a pronounced rise of the tail (Fig. 6A). SH rats did not drip urine in any body posture. The pattern of the urinary parameters in the dark-light cycle (Tables 2, 3) or the voiding behavior did not change three days after sham VD.

The pattern of urinary behavior parameters and the micturition behavior was similar to the SH rats prior to VD. However, three days after VD the animals dripped urine in the absence of the stereotyped voiding behavior during the dark (38±7 drips/6h) and light phases (12±3 drips/6h). In the dark phase, 72±7% of the urine expulsions were dripped in unusual places for voiding (Fig. 6); from this number ~69% were 1-3 drops (20-30 μl) expelled during behaviors implicating stress such as standing to reach food, sneezing, scratching, running, leaning the body to lick the vaginal orifice or standing on their hind legs for vertical exploration (Fig. 6C) and the other 3% were >7 drops expelled while the rats were rushing to the edge of the cage. Only 28±7% of the urine expelled were voids. Compared to the number of voids prior to VD, the number of voids during the dark phase decreased significantly (p<0.05) and the voiding interval increased after VD (Table 2).

In the light phase most of the time the rat was sleeping but periodically awoke to stretch its body, void, eat and/or drink. After VD the rats dripped urine while stretching the body, scratching the head or leaning the body to lick the vaginal orifice. Although ~50 % of the urine expulsions were drips (Fig. 6B) the
number of voids, voiding interval or voiding volume during the light phase did not significantly change after VD (Table 2). VD rats did not drippe d urine with every effort behavior but only occasionally, for example, 1 drip of six vertical explorations.
Discussion

Parturition is considered as an important risk factor for pelvic floor dysfunction in women (3, 47). To test the effect of vaginal delivery, an animal model with vaginal balloon dilatation has been proposed (33). VD induces hypoxia to the urogenital organs (9) and anatomical and functional damage of the urethra (4, 21, 26, 58).

The present study adds information about the regions of the genitourinary tract that are distended by the rodent VD model, the grade of elongation of the genitourinary nerves during VD, the effect of VD on urinary retention and bladder layers and the consequence of this procedure on micturition in awake animals. In fact, this is the first study that describes correlation of VD induced genitourinary neuroanatomy with behavioral signs of urinary dysfunction.

During VD, the inflated balloon did not stay completely in the pelvic cavity but protruded to the distal vagina, which made it necessary to suture the vaginal orifice to avoid balloon expulsion. In that position the balloon compressed the organs of the pelvic area (pelvic vagina, pelvic urethra, rectum, pelvic floor muscles, and its vessels and nerves) against the pelvic bone and distended the perineal genitourinary tract (distal region of the vagina and urethra, and the vessels and nerves related to them). Thus, the VD model in rats produces direct mechanical damage to the pelvic urethra and distal genitourinary tract, which may explain the resultant disorganization and thinness of the EUS (4, 39), and the physiological impairment observed after VD, such as hypoxia of the genitourinary organs, indicating a reduction in blood flow (9), abolished EUS activity (21), decreased leak point pressure (LPP), and/or urine leakage with effort, suggesting
urethral closure incompetence (26, 33, 56). VD-induced physiological urethral impairment may result from damage of the EUS muscle fibers, urethral smooth muscle and their innervation, since these factors contribute to urinary continence (23).

VD distends the distal vagina and the somatic nerves running along it, such as the DNC and the MPSP. These nerves were elongated by 50%, greater than that reported in a simulation model of pudendal nerve stretching during second stage of parturition in women (32). This nerve elongation may denervate the clitoris and the EUS. Stretching nerve injury may also decrease microtubules and tau protein, important elements in axonal function (51). DNC and MBSP stretching due to VD may induce sexual and urinary dysfunction, since denervation of the clitoris induces signs of coital urinary incontinence (5) and damage of the EUS innervation induced signs of urinary incontinence in rats (6, 29, 42).

Manual stretching of the motor branch of the pudendal nerve, about 74% of its length, abolished temporarily the activity of the EUS, which returned 30 min after the stretching (52). However, this procedure was performed at the level of the Alcock’s canal, and stretched the complete nerve, before branching. In contrast, VD may also stretch terminal nerves, those thin fascicles arriving at their targets that may get easily broken and disrupt motor endplates.

We observed urinary retention and bladder overdistension after VD, presumably as an indirect effect of the pelvic urethra occlusion while the balloon compressed the urethra against the pubic bone, preventing micturition. The hematomas in the bladder, thinness of the epithelium and detrusor layers, as well as stretching of vesical nerves may result in sensory and motor bladder
dysfunction. Sensory receptors localized in the epithelium and muscular layers (27) may decrease, affecting the transference of bladder information to the central nervous system. Motor nerves also may diminish and lead to an underactive bladder. Decrease in the adrenergic and cholinergic innervation of the bladder has been observed after induced urinary retention and bladder overdistension in rats (31, 59). Whether women suffer neuroanatomical changes in the bladder with labor is unknown, although it is possible, since urinary retention is a well-known condition after childbirth, with epidural analgesia, prolonged labor, episiotomy, and high birth weight as risk factors (28, 37, 38). The epidural analgesia commonly used to avoid pain during labor causes bladder hypotonia and eliminates the normal sensation to void, leading to bladder overdistension (62), which may damage the detrusor, and consequently induce long term voiding difficulties (64). Thus, for physiological or pharmacological reasons, bladder overdistension is not uncommon during labor (5) and because it is dangerous for women’s urinary health, physicians try to avoid bladder overdistension by catheterizing the urethra at some stage in labor (14). The fact that the VD model also induces bladder overdistension is positive for the model because it mimics what happen clinically.

There are also some reports of bladder rupture during labor (63), which can lead to underactive bladder. Thus, impaired detrusor contraction is greater in women with a history of urinary retention (1). Symptoms of underactive bladder are common in both older men and women (7, 61), however, its prevalence in people <65 years is higher in women than men (20.6 % vs 9.3%) (61).

The MPG is sexually dimorphic in rats: bigger in males than in females (44). The MPG in female rats is within adipose tissue attached on the rostral vaginal
tract, almost lateral to the cervix (36). It has been estimated to contain around 5000 neurons (13, 17).

Considering that the vagina is a large hollow organ, during VD the balloon could pull the rostral region of the vaginal tract down, stretching and damaging the MPG. However, our results did not show any significant differences in the MPG or area of neurons between SH and VD animals. This data suggest that VD does not significantly affect the morphometry of MPG neurons.

In contrast, a previous study reported a decreased number of MPG neurons in VD rats (33), from 3-8 neurons in SH animals to 0-1 neurons in VD rats. The controversy can be related to the method used to obtain the tissue to be analyzed. As described in methods, we cut the complete ganglion and analyzed three sections of the middle of it, and found ~100 neurons per section but analyzed only those with a visible nucleus. The previous study reported that the genitourinary tract was collected and analyzed. Considering that the female MPG is very thin and hard to visualize, it is possible that they did not collect the complete MPG, since they reported very few neurons (3-8) in the sham animals.

The female rats in this study demonstrated a stereotyped behavior of micturition, similar to that described previously in male rats (24, 25). The design of the top of the cage with the food and water container in the center of the jumbo cage let us to determine that normal rats do not void in the center of the cage. To void, the rats walked to an edge of the cage and placed the rump facing the wall of the cage, sometimes just walking backward until reaching the wall, and expelling urine. Expression of this behavior suggests rats feel bladder fullness and volition to expel urine the move to the edges of the cage. Urine marking behavior has been
described in rodents (35, 43). To mark, the rat placed the urinary meatus close to
the object to be marked (35). Considering that the cage of our rats did not contain
objects for marking, any urine expelled without the stereotyped behavior of
micturition was considered as a sign of micturition dysfunction or leakage.

Other methods used to analyze micturition in normal and experimental rats
have analyzed voiding place preference by placing paper below the grid of the
cage to collect the urine (16, 29). Marks in the front of the cage, after pudendal
nerve injury, are considered to indicate urinary incontinence (29). However, in the
present study we noticed that rats voided in the four edges of the cage. So,
although a detailed study is necessary to determine whether the rats void more
frequently in a special edge of the cage, urine in the front of the cage seems to not
be an accurate method to determine urinary incontinence.

The metabolic cage has also been widely used to study micturition in awake
animals (8, 53). In this cage the urine slips on the wall of the cage to the urine
container. If the container is placed on a force transducer connected to a computer,
voiding frequency and voided volume may be automatically recorded (10, 29).
Certainly this system seems to be good to study micturition in control animals but
not leakage. Indeed, finding that frequency of voiding in intact rats varied in relation
to day-night corroborate prior studies using the metabolic cage (~60 % of the voids
are in dark phase, active period of the rats) (29), indicating that our findings are not
an artifact of our methods. However, 1-3 drops alone do not slip along the wall to
reach the urine funnel of the metabolic cage, so, they are not recorded. Thus,
although it requires more work, the analysis of the behavior of micturition seems to
be reliable to determine signs of urinary dysfunction in conscious rats.
Considering that the stereotyped behavior of micturition suggests that sensory information of bladder fullness reaches the somatosensory cortex of the brain and the rat maintains urinary continence until reaching the stereotyped posture for voiding, similar to people, expulsion of urine without that posture can indicate urinary dysfunction. Dripping small volumes of urine in unusual places to void correlated with stress behaviors (sneezing, stretching the body, standing in hind limbs, etc) and may be an indicator of induced stress urinary incontinence. Expulsion of large amount of urine before reaching the edge to perform the stereotyped behavior may suggest urgency and overactive bladder. Increased number of voids during the rest period (light) may indicate signs of nocturia. According to this behavioral correlation, most (69%) of the urine drips in VD rats are considered signs of stress urinary incontinence (they expelled 1-3 drops of 20-30 μl released during effort behavior) and 3% of urgency (200-300 μl of urine released during daily activities or while walking to the edge). Since we observed bladder overdistension, it would be important in future studies to discern the effect of direct damage from VD vs the effect of VD-induced bladder overdistension on the physiology of micturition of these awake animals.

Bladder overdistension, and acute urinary retention, has been modeled with different techniques: acute complete bladder outlet obstruction with a silk ligature of the urethra, middle bladder neck obstruction with ligature and catheter, bladder overdistension with an inflated balloon into the bladder, and bladder infusion after clamping the distal urethra (12, 49, 50). In these models, structural changes and bladder dysfunction has been reported. Thus, partial outlet obstruction increased
voiding frequency and reduced voiding volume, lasting 2 weeks. On the other hand, direct urethral VD damage on the physiology of micturition has been modeled by denervating the EUS in rats (22). Lesion of the pudendal nerve in anesthetized rats decreases the urethral pressure and the leak point pressure, suggesting stress urinary incontinence. Considering that in our study the rats had the two VD effects: urethral damage and bladder acute urinary retention-overdistension together, we would expect increased voiding frequency as well as urine leakage during effort. However, our rats dripped urine but the voiding frequency did not increase, a sign of urethral dysfunction by weakness of urethral closure. To determine whether urethral dysfunction was more prominent than bladder dysfunction, masking the bladder dysfunction, or the bladder overdistension was not enough to affect the function requires further studies out of the scope of the current study.

Some limitations to be considered are: 1) micturition behavior does not indicate whether the bladder concurrently contracted with each urine expulsion. Cystometry in conscious animals could add important information; 2) the hormonal condition of the rats was unknown as the phase of the estrous cycle was not determined, although the estrous cycle did not previously affect VD-induced stress urinary incontinence in mice (18); 3) the rats were not pregnant, which means that the anatomical and physiological parameters could be different than postpartum since relaxation of pelvic ligaments, cervical ripening, estrogen peak preceding parturition (54), or the hypoestrogenic state of nursing could diminish or increase trauma and recovery from vaginal delivery. It has been proposed that estrogen treatment at the time of pudendal nerve injury facilitates nerve regeneration (2).
Even though there are differences between multiparous and nulliparous rats, VD is not innocuous in multiparous animals. It increases the percent of rats with urine leakage and the damage to urethral striated muscle and nerve fibers (45, 46), from which we conclude that the VD model in nulliparous animals is suitable to mimic prolonged vaginal parturition, with the consideration that in some variables the magnitude of the VD-induced damage may differ between nulliparous vs delivery groups.

Conclusions
The present study demonstrated that VD produces direct and indirect injuries on the genitourinary organs and their innervation in female rats. Direct mechanical damage occurs as the balloon compresses the pelvic region of the genitourinary tract and distends the distal region, including the DNC and MBSP nerves. Indirectly, the inflated balloon damages the bladder likely as a consequence of urinary retention. The neuroanatomical injuries occur alongside behavioral signs of stress urinary incontinence. The behavior of micturition may be an important tool in the studies of the physiology of micturition, as well as for long term studies of nerve plasticity and to test treatments that could facilitate urinary function recovery.
Acknowledgment

Funding: CONACyT: YCG 183446, JLPG 488223. SEP-SES-DEGESU: PADES 2016. We thank Julio Cuatecontzi and José Luis Tlachi for technical assistance and artwork. This work supported in part by the Cleveland Clinic and the Rehabilitation Research & Development Service of the United States Department of Veterans Affairs.


**Figure legends**

**Figure 1.** Drawing of the lower urogenital tract and its innervation of the female rat during vaginal distension. Note that beside the motor branch of the sacral plexus (3, MBSP) that innervates the striated urethral muscle, there are other nerves stretched during VD, such as the vesical nerves (1), the anastomotic branches (2), and the dorsal nerve of the clitoris (4, DNC). The lines associated with the numbers indicate the points of nerve measurement. MPG, major pelvic ganglia; Uh, uterine horn; Ub, bladder; Vg, vagina; Pg, preputial gland.

**Figure 2.** Photos of the urogenital tract during sham distension (A) or vaginal distension (VD, B). Note that during VD the balloon distends the distal genitourinary tract and compresses the pelvic urethra against the pubic bone, leading to bladder overdistension as a consequence of urinary retention induced by the outlet obstruction (B). Compared to the bladder of sham animals (C), only bladders of VD rats presented with hematomas (D).

**Figure 3.** Example electrical activity recorded from the dorsal nerve of the clitoris of a sham (SH) or vaginal distension (VD) rat before or while the clitoris was being squeezed. The horizontal broken line below each ENG recording indicates the duration of the stimulus (squeezing). Values are mean ± SEM of data from 4 SH and 4 VD animals. ** represent significant differences vs SH with p<0.01.

**Figure 4.** Photos of transverse sections of the bladder (H&E stain) after sham distension (A, SH) and vaginal distension (B, VD) animals. Note that thickness of
the epithelium and detrusor layers are significantly decreased in VD animals (C).

Values are mean ± SEM of data from 4 animals. * and ** represent significant differences vs SH with p<0.05 and p<0.01, respectively.

**Figure 5.** Major pelvic ganglion (MPG) neurons. The drawing (A) represents the MPG and the square, the site where tissue for the microphotographs were taken. Representative postganglionic neurons of sham distension (B; SH) and vaginal distension are shown (VD, C). The graphs indicates the area (D) and diameter (E) of 20 neurons per rat. Values are mean ± SEM of data from 4 animals. Hg, hypogastric nerve; Pv, pelvic nerve. Vc, viscerocutaneous branch of the Pv. Sm, somatomotor branch of the Pv.

**Figure 6.** Photos of a female rat in the observational cage of micturition. Rats void at the edge of the cage (A). VD rats dripped urine during some behaviors implicating stress, such as standing up on the hind limbs during vertical exploration (C). The graph indicates the percentage of urine expelled as voids or drips before and 3 days after vaginal distension (VD, n = 9), or sham VD (SH, n = 3), during the last 6 h of the dark phase and the first 6 h of light phase. *** indicates p<0.0001 versus voids of the same phase. Values are mean ± SEM.
Fig. 3

- **SH**
- **VD**

![Waveform and Amplitude/Frequency Bar Graphs](Image)
Fig. 5
Fig. 6

A

B

![Graph showing data with labels VD Voids, SH Voids, VD Drips, SH Drips.](image)

C

expelling urine

dropping urine

No./6h

Before VD

Day 3 post VD

***

+
Table 1. Effect of vaginal distension on the length of genitourinary nerves

<table>
<thead>
<tr>
<th>Nerve length (mm)</th>
<th>SH</th>
<th>VD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesical branches</td>
<td>16 ± 0.26</td>
<td>25 ± 0.5**</td>
</tr>
<tr>
<td>Anastomotic branches</td>
<td>11 ± 0.45</td>
<td>15 ± 1.7*</td>
</tr>
<tr>
<td>MBSP</td>
<td>11.6 ± 1.67</td>
<td>17.5 ± 0.05*</td>
</tr>
<tr>
<td>DNC</td>
<td>10 ± 1.0</td>
<td>15 ± 1.0*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, *t* student *p*<0.05, **p*<0.01. MBSP, motor branch of the sacral plexus, DNC, dorsal nerve of the clitoris.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Voiding frequency (no / 6 h)</th>
<th>Voiding interval (Min)</th>
<th>Voiding volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>SH, n = 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.5 ± 1.8</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>± 1.2</td>
</tr>
<tr>
<td>VD, n = 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>7 ± 1.0</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.5</td>
<td>± 3.8</td>
</tr>
</tbody>
</table>

Values are means ± SEM, *p<0.05 vs before VD
Table 3. Urinary parameters of female rats with vaginal distension (VD) during 6 h of the light phase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Voiding frequency (no / 6 h)</th>
<th>Voiding interval Min</th>
<th>Voiding volume mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>SH, n = 5</td>
<td>4.5</td>
<td>± 0.6</td>
<td>5</td>
</tr>
<tr>
<td>VD, n= 9</td>
<td>6</td>
<td>± 0.3</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SEM