Vulnerability of continence structures to injury by simulated childbirth

Hardeep S. Phull,1 Hui Q. Pan,1 Robert S. Butler,2 Donna E. Hansel,3,4,5,6 and Margot S. Damaser1,6,7

1Department of Biomedical Engineering, Lerner Research Institute, 2Department of Quantitative Health Sciences, 3Pathology and Laboratory Medicine Institute, 4Taussig Cancer Institute, 5Genomic Medicine Institute, 6Glickman Urological and Kidney Institute, The Cleveland Clinic, Cleveland; and 7Research Service, Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, Ohio

Submitted 1 March 2011; accepted in final form 24 May 2011

Phull HS, Pan HQ, Butler RS, Hansel DE, Damaser MS. Vulnerability of continence structures to injury by simulated childbirth. Am J Physiol Renal Physiol 301: F641–F649, 2011. First published May 25, 2011; doi:10.1152/ajprenal.00120.2011.—The goal of this study was to examine acute morphological changes, edema, muscle damage, inflammation, and hypoxia in urethral and vaginal tissues with increasing duration of vaginal distension (VD) in a rat model. Twenty-nine virgin Sprague-Dawley rats underwent VD under anesthesia with the use of a modified Foley catheter inserted into the vagina and filled with saline for 0, 1, 4, or 6 h. Control animals were anesthetized for 4 h without catheter placement. Urogenital organs were harvested after intracardiac perfusion of fixative. Tissues were embedded, sectioned, and stained with Masson’s trichrome or hematoxylin and eosin stains. Regions of hypoxia were measured by hypoxyprobe-1 immunohistochemistry. Within 1 h of VD, the urethra became vertically elongated and displaced anteriorly. Edema was most prominent in the external urethral sphincter (EUS) and urethral/vaginal septum within 4 h of VD, while muscle disruption and fragmentation of the EUS occurred after 6 h. Inflammatory damage was characterized by the presence of polymorphonuclear leukocytes in vessels and tissues after 4 h of VD, with the greatest degree of infiltration occurring in the EUS. Hypoxia localized mostly to the vaginal lamina propria, urethral smooth muscle, and EUS within 4 h of VD. Increasing duration of VD caused progressively greater tissue edema, muscle damage, and morphological changes in the urethra and vagina. The EUS underwent the greatest insult, demonstrating its vulnerability to childbirth injury.

stress urinary incontinence; parturition; rat; female; histology

STRESS URINARY INCONTINENCE (SUI) is a medical condition affecting 35% of women over the age of 40 in the United States (29). It is characterized by involuntary leakage occurring when intra-abdominal pressure exceeds urethral resistance in the absence of a detrusor contraction, such as during coughing or sneezing (1). The maternal injuries of childbirth are the dominant risk factor and are likely responsible for nearly two-thirds of SUI cases (13). Trauma from vaginal delivery results in damage to pelvic floor structures in addition to nerve damage to the vagina and urethra (6). Moreover, hypoxia of pelvic organ tissues in the second stage of labor has been theorized to play a role in childbirth-associated SUI due to pressure-induced ischemia (30). However, the exact mechanism of this injury and its correlation with SUI are not known.

Human childbirth is the most traumatic of all mammals and delivery in no other animal, including nonhuman primates, can be utilized as equivalent to human for investigative purposes (48). Nonetheless, a rat model of vaginal distension (VD), which simulates childbirth-induced injury by inflating a balloon catheter in the vagina, enables investigation of the injuries of childbirth in an animal model (18, 28). This model also reliably reproduces urodynamics and cystometry consistent with SUI (37). We previously determined that VD in rats causes a significant reduction in blood flow and increased levels of hypoxic injury to genitourinary organs (12). However, the acute effects of VD on edema, inflammation, and morphometry have not previously been studied.

The goal of this study was to determine the acute effects of VD duration on hypoxia, inflammation, edema, muscle damage, and morphometry of the urethra and vagina in a rat model. We hypothesized that increasing duration of VD would result in proportionally increased severity of injury and could suggest cellular mechanisms of injury during childbirth that contribute to SUI.

MATERIALS AND METHODS

All protocols were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. Twenty-nine virgin female Sprague-Dawley rats (180–220 g body wt; 8–10 wk old) were anesthetized with 80 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally. VD was performed as previously described (8). Briefly, the vagina was accommodated to a larger capacity by inserting and removing increasing sizes of urethral dilators lubricated with surgilube (E. Fougera, Melville, NY). A modified 10 Fr. Foley catheter was then inserted into the vagina and secured with a 3–0 vicryl suture. The balloon was distended with 3 ml saline, the capacity of the vagina in these rats (8), for 0 h (n = 5), 1 h (n = 6), 4 h (n = 6), or 6 h (n = 7). Control animals (n = 5) were anesthetized for 4 h without vaginal accommodation or catheter placement.

One hour before euthanasia, the rats were injected intraperitoneally with hypoxyprobe-1 (60 mg/kg), which distributes throughout the body and forms adducts with intracellular proteins having oxygen tension <10 mmHg (Pimonidazole, Natural Pharmacca International, Research Triangle Park, NC). Before deflating the distending balloon, the rats were euthanized by intracardiac perfusion at the end of the VD period with heparinized saline (1.0 ml/s) for 5 min or until the liver appeared pale. Perfusion continued with 4% paraformaldehyde (0.7 ml/s) for 5 min followed by 10% paraformaldehyde (0.5 ml/s) for 15 min.

Urogenital organs were dissected immediately after perfusion for morphometric assessment in most animals as follows. A ventral midline incision was made, the symphysis pubis was cut longitudinally, and the distending balloon was deflated. The urethra, vagina, and bladder were exposed, harvested, pinned en bloc on a cork, and immersed in formalin overnight at 4°C. The urethra and anterior vagina were then dissected en bloc 6 mm distal from the ureteral orifice in the bladder, an area previously determined to contain the highest number of nerve fascicles and striated muscle (24). An additional two animals at each VD duration (n = 10) were perfused as described above and refrigerated overnight with the inflated balloon in place before dissection to fully perfuse the fixative and retain the

http://www.ajprenal.org
morphology of distension. These specimens demonstrated extreme contraction, making it impossible to quantitatively analyze the various tissue regions accurately and, as a result, they were not included in the quantitative morphometric analysis. Dissected tissues were transferred to a tissue-processing cassette and stored in 70% ethanol at 4°C until processed and embedded in paraffin. Tissues were sectioned serially (5 μm) and consecutive sections were used for analysis. For immunohistochemistry, immunostained sections were deparaffinized in xylene and rehydrated through graded ethanol steps. All immunohistochemical stains were developed using an avidin-biotin peroxidase method with diaminobenzidine as the substrate chromagen for visualization. Immunostained sections were then rehydrated through graded ethanol steps and lightly counterstained with hematoxylin.

For hypoxprobe staining, sections were treated with Protease I solution (Ventana Medical Systems, Tucson, AZ) for antigen retrieval and blocked with serum-free protein (DAKO, Carpinteria, CA). Primary mouse monoclonal hypoxprobe antibody (100 μl at 1:50 dilution in PBS) supplied with the hypoxprobe-1 kit (Natural Pharmacica International) was applied for 30 min followed by incubation with secondary goat anti-mouse antibody (Lab Vision, Fremont, CA). Myeloperoxidase immunohistochemistry staining was performed on adjacent sections from each rat without antigen retrieval. Primary myeloperoxidase rabbit polyclonal antibody (100 μl at 1:1 dilution in PBS; Lab Vision) was applied for 30 min followed by incubation with secondary goat anti-rabbit antibody (Lab Vision). Counterstaining and dehydration were performed as above. Tissue positive controls were used for each antibody according to the manufacturer’s instructions: kidney for hypoxprobe (Natural Pharmacica International) and spleen for myeloperoxidase (Lab Vision). Reactions without primary antibody were used as negative controls. Adjacent sections were stained with Masson’s trichrome or hematoxylin and eosin (H&E).

To maintain consistency between vaginal and urethral areas, we focused assessment in the anterior vagina on an area whose width was contiguous with the outer diameter of the urethra in the midvagina. The lamina propria (VLP) and smooth muscle (VSM) of the vagina, urethral/vaginal septum (UVS), external urethral sphincter (EUS), and urethral smooth muscle (USM) were evaluated as distinct regions in cross-sections of the vagina and urethra in each animal by light microscopy. Tissue edema was characterized on H&E and Masson’s trichrome stains as widened spaces between connective tissue with less dense, pale-staining regions (31, 44). Striated muscle disruption in the EUS was determined by the degree of striated muscle bundle alignment and fragmentation of muscle bundles. Each of these parameters was evaluated independently using a semiquantitative graded histological scale (0 = none; 1 = mild; 2 = moderate; 3 = severe) by an investigator blinded to the experimental group.

Inflammation was assessed on H&E-stained slides by a blinded investigator counting extravasated polymorphonuclear neutrophils (PMNs) within each tissue region. Myeloperoxidase, an abundant enzyme marker present in neutrophil granules (35), was used to qualitatively confirm identification of PMNs. Hypoxia was evaluated by quantifying the intensity of hypoxprobes staining in each region using a semiquantitative graded scale (0 = none; 1 = light; 2 = medium; 3 = dark) by a blinded observer.

For statistical comparison of PMN results, VLP and VSM data were combined and classified as vagina since their responses were similar. For each VD duration, the amount of edema, muscle disruption, hypoxia, and number of PMNs were tested for significant correlation between duration, tissue region, and their interaction using mixed models statistical analysis. The method of mixed models enables an assessment of the outcome as a function of continuous and categorical variables that can be treated as fixed and/or random (51). As a result, we chose to use this method to assess tissue type, distension time, and the interaction of these two variables. Dunnett’s post hoc test was used to compare each region to that of controls and a Tukey-Kramer post hoc test was used to make pairwise comparisons of different regions to each other at the same VD duration. In all cases, \( P < 0.05 \) was used to indicate significant differences. Parametric methods were appropriately performed to analyze the ordinal data since the scales meet the criteria of reporting discrete and observational data (9). Quantitative data are presented as means ± SE.

RESULTS

Control specimens showed well-defined borders between specific tissue regions including a clear UVS space and a circumferential EUS around the urethra with the urethral lumen centered within the urethra (Fig. 1). After 0 h of VD, the tissue was compressed but the tissue regions were nonetheless discernible and the urethral lumen remained centered in the urethra. Between 1 and 6 h of distension, the urethra was displaced anteriorly (away from the balloon distension) and the UVS and EUS lost their well-defined borders due to edema and muscle disruption, respectively (Fig. 1). The urethra appeared distorted into a crescent-moon shape after 1 h, but it became more vertically elongated with increasing duration of distension. After 4 or 6 h of VD, undulating waves were observed in the vaginal epithelium (Fig. 1).

After VD, smooth muscle (VSM, USM) and striated muscle (EUS) fibers became elongated, while the urethral lumen became flattened and demonstrated a loss of mucosal infoldings of the urothelium. Blood vessels also became elongated and the endothelium was no longer visible, making it difficult to differentiate the vessel lumen from newly created gaps between connective tissue. This morphometry was preserved when the specimens were refrigerated overnight before dissection (Fig. 2).

There was no evidence of edema in any region in control animals or after 0 h of VD (0.0 ± 0.0 for all regions). Edema was most prominent in the EUS and UVS, particularly after 4 h (1.2 ± 0.3 and 1.5 ± 0.3, respectively) or 6 h (1.6 ± 0.2 and 2.6 ± 0.2, respectively) of VD compared with controls (\( P < 0.01 \); Fig. 3). Although edema was greater in the UVS and EUS after 6 h of VD compared with 4 h of VD, these differences were not statistically significant due to the number of comparisons made in the statistical analysis. Nonetheless, edema in the UVS after 6 h of VD was significantly greater than that after 0 and 1 h of VD (\( P < 0.001 \)), whereas edema in the UVS after 4 h was significantly greater than that after 0 h of VD (\( P < 0.001 \)). The prominent edema in the UVS after 6 h of VD was significantly greater (\( P < 0.001 \)) than in the vagina (VLP: 0.0 ± 0.0; VSM: 0.4 ± 0.2) and USM (0.4 ± 0.2). In the EUS, after 6 h of VD, edema was significantly greater than control (0.0 ± 0.0) and 0 h (0.0 ± 0.0; \( P < 0.01 \)). Edema in the EUS after 6 h of VD was also significantly greater than edema in the VLP and VSM after 6 h (\( P < 0.001 \); Fig. 3).

Normal EUS striated muscle fibers are parallel, contiguous, and evenly spaced, with a framework of connective tissue providing a surrounding meshwork (Fig. 4). Immediately after 6 h of VD, the striated muscle was dramatically altered with gross muscle disruption and fragmentation (Fig. 4). Fragmentation led to the appearance of clumps of muscle fibers, remnants of the original strands. Thinning and stretching of the striated muscle fibers also occurred, along with a distinctive waviness. Significantly more muscle disruption occurred in the EUS after 6 h of VD (2.2 ± 0.2) compared with control (0.0 ± 0.0) and all other durations of VD (0 h: 0.0 ± 0.0; 1 h: 0.8 ± 0.3; 4 h: 1.0 ± 0.4; \( P < 0.01 \); Fig. 4). Muscle disruption was...
not significantly greater after 1 or 4 h of VD compared with 0 h or controls.

After 4 or 6 h of VD, blood vessels in the UVS showed a marked presence of PMNs with evidence of adhesion to the vascular endothelium, transmigration, and extravasation into the tissue (Fig. 5). This process was also apparent in EUS vessels and tissue (Fig. 5). High-magnification images of a prominent UVS vessel after 6 h of VD confirmed the neutrophil infiltrate, characterized by eosinophilic cells with multilobed nuclei (Fig. 5). Myeloperoxidase stain of the same region in a near section validated identification of neutrophils on H&E-stained specimens with diffuse brown staining within neutrophil granules (Fig. 5).

The total number of PMNs was maximal in the UVS after 4 h (156 ± 34.8) or 6 h (106 ± 11.4) of VD, and both were significantly higher than in controls (2.0 ± 0.3) and after 0 h (2.0 ± 1.3) or 1 h (2.0 ± 0.9) of VD (P < 0.001; Fig. 5). PMNs in the UVS after 4 or 6 h of VD were also significantly increased compared with all other regions after 4 h (vagina: 20 ± 2.1; EUS: 27 ± 7.6; USM: 42.7 ± 4.4) or 6 h (vagina: 20 ± 4.0; EUS: 26 ± 5.4; USM: 40 ± 7.8) of VD, respectively (P < 0.001), paralleling the edema results. The total PMNs in the USM were not significantly different between 4 h (42.7 ± 4.4) and 6 h (40.2 ± 7.8), but both were significantly higher than after 0 h (2.2 ± 0.8; P < 0.01), 1 h (4.8 ± 2.0; P < 0.02), and in controls (0.0 ± 0.0; P < 0.01) in the same region.

Since intravascular neutrophils may not always indicate local activity, we separately quantified nonadherent intravascular neutrophils and those that had transmigrated into the surrounding tissue. Although there was no significant difference between VD for 4 h (74 ± 21) and 6 h (67 ± 4.7) in the UVS, both of these time points had significantly more tissue PMNs than in the USM after 0 h (0.3 ± 0.2), 1 h (1.3 ± 0.8), and in controls (0.0 ± 0.0; P < 0.001). As with previous comparisons, there was a significantly greater number of PMNs in the UVS compared with all other regions after 4 h of VD (vagina: 4.0 ± 1.0; EUS: 17 ± 2.5; USM: 3.3 ± 2.0) and 6 h of VD (vagina: 5.8 ± 2.0; EUS: 20 ± 2.7; USM: 0.0 ±
The number of tissue PMNs in the EUS after 4 h of VD (17 ± 2.5) was not significantly different from the number after 6 h of VD (20 ± 2.7), but it was still significantly greater than after 0 h (0.0 ± 0.0) or 1 h (0.8 ± 0.5) of VD or in control animals (0.0 ± 0.0; P < 0.02).

Since a higher percentage of tissue PMNs indicates a greater degree of tissue infiltration of inflammatory cells and an increased inflammatory response (54), we represented the intravascular and tissue PMNs as a percentage of total PMNs in each region after 4 and 6 h of VD (Fig. 5F). The EUS was found to have the highest degree of PMN infiltration into the tissue. The proportion of tissue infiltration progressively increased as regions closer to the EUS were considered, but then became nearly zero in the USM. After 4 h of VD, tissue infiltration was 27% in the vagina, 46% in the UVS, 69% in the EUS, and 8% in the USM (Fig. 5F). A similar pattern of tissue infiltration occurred after 6 h of VD, but to a greater extent: 31% in the vagina, 63% in the UVS, 78% in the EUS, and 0% in the USM.

Hypoxic regions of the urethra and vagina were observed as brown immunostaining of the hypoxapore reagent, most notably as focal spots in the EUS and USM and a continuous band in the VLP, in which the stratum spinosum predominated (Fig. 1). Overall, 0 h (1.1 ± 0.2), 1 h (1.2 ± 0.3), and 4 h (1.7 ± 0.3) of VD caused significantly increased hypoxia compared with controls (0.4 ± 0.1; P < 0.03). However, hypoxia after 6 h of VD (0.8 ± 0.2) was not significantly different than control.

Within the hypoxic regions (VLP, EUS, USM), peak levels of hypoxia occurred after 1 h of VD for the VLP (2.5 ± 0.3), 4 h for the EUS (2.7 ± 0.3), and both 1 and 4 h in the USM (1 h: 2.1 ± 0.7; 4 h: 2.7 ± 0.2; Fig. 6). Within each region, there were no statistical differences in hypoxia between different VD durations, except for the EUS that was significantly increased after 4 h compared with both controls (0.0 ± 0.0) and after 6 h of VD (0.4 ± 0.4; P < 0.02). Hypoxia in the EUS after 6 h of VD was not significantly different from controls.

DISCUSSION

The normal physiologic mechanism for opposing SUI occurs due to the coordinated effort of the urethra and surrounding support of pelvic floor tissues to resist increased abdominal pressure changes (6, 17, 32). Vaginal delivery can damage urethral and pelvic floor structures involved in continence by direct trauma or denervation of the pudendal nerve (10). Increased duration of the second stage of labor, large birth weight, and multiparity are key risk factors correlated with these poor outcomes (29, 50).

The development of childbirth simulation animal models has enabled the systematic investigation and evaluation of the mechanisms responsible for these clinical observations (18). The most commonly utilized model consists of VD with a Foley catheter balloon (8, 28). Longer-duration distension has been shown to have more severe outcomes with a longer recovery time (37, 52). In addition, hypoxia and reperfusion have been shown to be likely mechanisms for the injury to continence structures during VD (12, 46, 54).

Among the limitations of these animal models are that urodynamic outcomes often quickly return to normal (26, 37, 41) and that urodynamic evaluation alone cannot detect underlying structural abnormalities. Therefore, a more sensitive analysis of the changes that occur immediately after VD in the acute phase of injury may serve to complement urodynamic data in the evaluation of SUI. In this work, we evaluated the acute effects of variable durations of VD on morphometry, edema, muscle damage, hypoxia, and inflammation in the urethra and vagina. To maintain tissues in their native state after VD, we relied on a perfusion fixation technique to preserve the acute structural and morphological changes in vivo.

Increased damage to tissues at greater duration of distension resulted in morphological changes such as elongation and progressive anterior displacement of the urethra. In addition, undulating waves were present in the vaginal epithelium after longer-duration distension, suggesting that this tissue accommodated to increased length during distension and then collapsed upon balloon deflation. When specimens were refrigerated overnight before dissection, vaginal tissue was able to fully fix in position and the undulating waves were not observed, supporting this mechanistic hypothesis. However, due to the distortion of these tissues, overnight refrigeration made it impossible to morphometrically analyze the specimen. Therefore, these specimens were not analyzed quantitatively. However, the cellular and molecular observations observed in these animals were the same as those not refrigerated overnight before dissection since all animals in this study were euthanized by perfusion fixation, which euthanized them and terminated molecular events (20, 49).

Acute edema increased with longer-duration distension and occurred predominantly in the UVS and EUS, suggesting that these tissues are preferentially damaged during VD. This is consistent with previous work showing marked edema after 4 h of VD associated with thinning of the UVS and interstitial swelling between striated muscle fibers (28). In addition, we previously showed that the urethra is the only organ demon-
strating a significant increase in hypoxia-inducible factor-1α after VD (52), indicative of more significant hypoxia in this organ than other pelvic organs. Therefore, the edema could have occurred as a consequence of tissue inflammation secondary to hypoxia (54). Alternatively, hypoxia could have resulted from compromised blood flow due to congestive edema.

In the current study, the EUS was disrupted acutely after VD, consistent with previous reports of EUS damage occurring after 4 h of VD (26, 28). The early thinning, stretching, and fragmentation of fibers we observed represent the initial stages of muscle atrophy in the striated muscle of the EUS (47). Preferential damage to the EUS may indicate why urodynamic dysfunction occurs after VD even of short duration (8).

Evidence of inflammatory damage was characterized by the marked presence of PMNs in vessels and tissues after 4 and 6 h of VD. The observation of maximal extravasation of PMNs into tissues at 4 and 6 h is consistent with the inflammatory process in other organs following damage (25, 33). Similar to our finding of higher PMN infiltration with increasing VD duration, Jordan et al. (21) found a pattern of progressively greater PMN infiltration occurring with time. Although there was a higher absolute number of PMNs in the UVS, the greatest degree of PMN penetration occurred in the EUS, suggesting that the EUS may have endured the greatest inflammatory damage of the anatomic regions studied, suggesting a mechanistic explanation for urethral edema and dysfunction after VD (38, 41).

Control animals had low levels of hypoxia, while animals that underwent as little as 0 h of VD showed evidence of some hypoxic injury, particularly in the USM and vagina, consistent with our previous work that likewise showed hypoxia in sham-distended animals (12). We previously observed a high proportion of fascicle degeneration in the USM after VD, even of short duration (11, 23). The vagina is closest to the site of balloon traction and, as a result, balloon dilation provides a constant force applied directly to this region from the initiation of balloon inflation. Therefore, it was not surprising that there was no statistically significant difference in levels of hypoxia over time in the VLP. Demonstration of greater hypoxic insult to the VLP than to the VSM could be a result of different oxygenation methods in the two tissues: the VLP is dependent on the less efficient process of diffusion for its nutrient and oxygen supply (31), whereas the VSM is part of the rich vascular plexus providing blood flow to the urethra (15). The prominent staining of the VLP is consistent with our previous study on hypoxia associated with VD (12).

In the urethra (EUS and USM), hypoxia was notably reduced after 6 h of VD compared with 4 h of VD, but inflammatory injury persisted and reached a maximal effect after 6 h of VD. Although previous work could suggest that a longer-duration VD would induce a greater amount of pressure-induced hypoxia (37), we believe these findings represent a mechanistic association between hypoxia and inflammation. Hypoxia likely occurs from compression of blood vessels during VD, potentially leading to cell death and eventual tissue

**Fig. 4.** Trichrome stain of example sections from the EUS of tissue from a control animal showing parallel, even alignment of muscle fibers (indicated by a yellow *; A) and intact surrounding connective tissue meshwork and from an animal that underwent VD for 6 h showing muscle disruption and fragmentation (indicated by a yellow arrow; B) as well as edema (indicated by a green arrow; B). Bar = 50 μm. Semiquantitative levels of muscle disruption in the EUS are shown for control animals and after 0, 1, 4, or 6 h of VD in C. Note that control and 0 h of VD had so little edema that these bars are not visible on the graph. Each bar represents means ± SE of the mean of data from 5–7 animals. These representative images show nonrefrigerated specimens only, from which the graphical data were derived.
necrosis (39); however, our previous work demonstrates that some blood flow is maintained to the urogenital organs during VD (12). As blood flow is restored, tissue undergoes reperfusion injury due to massive release of free radicals, prostaglandins, and cytokines from the injured tissue that recruits inflammatory cells such as PMNs into the region and increases vascular permeability to cause swelling and edema (21, 54). The possibility of reperfusion injury causing sustained inflammation in the setting of an initial hypoxic insult is supported by the fact that the blood flow to the female rat urethra is similar to that of women and is characterized by a rich collateral network of arterial and venous plexuses in this region (15). Moreover, reperfusion is highly likely since VD did not obstruct blood flow completely, as demonstrated by detection of hypoxyprobe reagent in all samples, which would not have gained access to tissues if there was complete ischemia. Reperfusion could also occur immediately after the release of VD, as blood flow has been found to triple during this time (12). Therefore, tissue regions with focal, rather than continuous hypoxyprobe staining such as the EUS and USM, likely represent areas of transient episodes of hypoxia followed by reperfusion, resulting in greater inflammation and tissue injury.

Our analysis of hypoxia in the current study was based on numerical grading of intensity, but not distribution, of the immunohistochemical stain. Consequently, a dark, focal spot of hypoxia in the EUS or USM received the same histologic score as a dark band within the vagina, even though the continuous staining within the vagina would seem to be greater. Therefore, this limitation precluded us from being able to directly compare hypoxia between tissue regions.

Although in this study we did not evaluate the acute effects of VD injury on tissue innervation, we previously correlated the location of nerve fascicle injury on the dorsal side of the urethra with areas of focal hypoxia in the EUS after VD, suggesting that an aspect of hypoxia may be involved in nerve injury (11, 12). Clinically, pressures in the vagina during delivery can exceed those that cause peripheral nerve damage (5), suggesting a possible ischemic or hypoxic mechanism to the nerve dysfunction observed after vaginal delivery (16, 34). The mechanistic relationship between hypoxia, reperfusion injury, inflammation, and innervation merits further investigation.

Although Sievert et al. (46) reported that oophorectomy in the presence of VD significantly increased the rate of inconti-
nence in rats, and our previous work suggests that estrogen may protect against pelvic floor damage (2, 22), we did not control for reproductive hormones in the current study. Instead, we relied on random animal selection into each of the study groups to balance out the effects of hormonal changes in our animals. Estrogen treatment in the clinical management of SUI has been reported with mixed results (36, 42), suggesting this as an area for further investigation.

To maintain anesthesia in our animals, we used an anesthetic mixture that contains ketamine, which is known to cause vascular smooth muscle relaxation (3, 4). Therefore, it could be possible that ketamine caused vaginal or urethral smooth muscle relaxation, potentially affecting the results. However, the acute structural and morphological damage that occurred after VD was unlikely to be attenuated or exacerbated by the presence of ketamine alone since control animals also received this agent. Moreover, in contrast to previous studies that evaluated the effects of ketamine on smooth muscle on muscle strips ex vivo, our study was conducted in vivo, which would decrease tissue exposure to ketamine (7). Nevertheless, the potential for ketamine to affect smooth muscle in the urogenital tract merits future investigation, as previous studies only assessed vascular, cerebral, and tracheal smooth muscle (19, 43, 53).

When choosing duration of VD for this study, we took into account that the second stage of labor in women usually lasts 30–180 min (median of 50) in primigravid women and 5–30 min (median of 20) in multigravid women (14). In our experience, 30 min of VD was not sufficient to cause significant tissue injury and resulted in rapid recovery (8). Six hours of complete ischemia causes tissue necrosis (45), which led us to select 6 h as the upper limit of VD in this study, although VD does not cause complete ischemia (12).

We only assessed the anterior vagina in the midvaginal region to keep our observations in the vagina comparable with those in the urethra. The region for investigation was selected to enable optimal assessment of the urethra (24). However, the proximal and distal vaginal areas are likely affected by the VD as well and ought to be investigated in a future study.

We used a widely cited model of simulated childbirth injuries by VD that employs a 10 Fr. Foley catheter with 3-ml distension in young female rats, 8–10 wk old (8). In our experience, this method allows for reproducible measurement of functional parameters via urodynamics and also produces consistent tissue damage. Other researchers found that the volume of distension needed to produce symptoms of SUI depends on the weight of the rat. For example, Sievert et al. (46) used a 22 Fr. Foley catheter with 5-ml distension volume in rats weighing ~250 g. A study comparing induced stretch in the rat with that of a woman in labor was not within the scope of this study, and it has not been done previously, primarily because the pelvic structure of the rat is dramatically different than the pelvic bone in humans (27, 40), so that comparable stretch will cause different levels of damage due to differential impact of the pelvic bone.

Although we only evaluated the acute histological changes that occur after VD, other studies assessed longer-term outcomes after VD. Resplande et al. (41) demonstrated that 9 mo after VD urodynamic evaluation was normal but there was increased connective tissue in the vagina. Subsequent electron microscopy showed that urethral smooth muscle had altered shape, increased collagen deposition, and mitochondrial degeneration (41). A functional study of rats that underwent four separate VD procedures at 1-wk intervals demonstrated recovery of leak point pressure (LPP), a measure of urethral resistance, within 4 wk (26). Pan et al. (37) showed that animals undergoing 1 or 4 h of VD had significantly decreased LPP values 4 and 10 days after injury, but this normalized by 6 wk after injury with voided volume being the only urodynamic parameter remaining significantly decreased. They also observed an increase in smooth muscle density 6 wk after VD (37). None of these previous studies employed greater than 4 h of VD or quantitatively evaluated morphological, inflammatory, and hypoxic changes in the tissues. Therefore, it would be useful to further study the long-term morphological and physiologic recovery from different durations of VD in future investigations.

The current work sets the stage for future interventional studies to investigate causation of SUI after VD by identifying the acute mechanistic outcomes after VD. Future investigations should be performed to determine which of the pathological factors observed in this study are necessary and sufficient to lead to the dysfunction and slower recovery observed in functional studies. This could be accomplished by designing experiments that are able to either isolate the diffuse effects of VD identified by the current study into the individual components or to reverse one of them selectively.

In this study, we successfully used an in vivo fixation technique to evaluate the acute structural and histological changes after variable durations of VD without compromising resolution of the tissue regions. We conclude that increasing duration of VD resulted in progressively greater structural, edematous, myogenic, and inflammatory damage to urethral tissues, especially the EUS. Hypoxia and reperfusion likely contribute to EUS injury, the structure primarily responsible for the voluntary continence mechanism, which could imply the vulnerability of this region to childbirth-associated injury and the subsequent development of SUI.
REFERENCES


