Vulnerability of continence structures to injury by simulated childbirth

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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 polymophonuclear 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.

Address for reprint requests and other correspondence: M. S. Damaser, Dept. of Biomedical Engineering, The Cleveland Clinic, 9500 Euclid Ave. ND20, Cleveland, OH 44195 (e-mail: damasem@ccf.org).
morphology of distension. These specimens demonstrated extreme compression, 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, 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 hypoxpyrobe 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 hypoxpyrobe antibody (100 μl at 1:50 dilution in PBS) supplied with the hypoxpyrobe-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 hypoxpyrobe (Natural Pharmacica International) and spleen for myeloperoxidase (Lab Vision). Reactions without primary antibody were used as negative controls. Adjacent sections were stained with Mason’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 Mason’s trichrome stains as widened spaces between connective tissue with less dense, pale-staining regions (31, 44). Striated muscle disruption was 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 Mason’s trichrome stains as widened spaces between connective tissue with less dense, pale-staining regions (31, 44). Striated muscle disruption was evaluated as distinct regions in cross-sections of the vagina and urethra in each animal by light microscopy.

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
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 (0.7 ± 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 ± 2.0).
In the VLP, in which the stratum spinosum predominated, brown immunostaining of the hypoxyprobe reagent, most no-
31% in the vagina, 63% in the UVS, 78% in the EUS, and 0%
infiltration occurred after 6 h of VD, but to a greater extent:
infiltration was 27% in the vagina, 46% in the UVS, 69% in the
became nearly zero in the USM. After 4 h of VD, tissue
creased as regions closer to the EUS were considered, but then
infiltration of inflammatory cells and an
increased inflammatory response (54), we represented the in-
degree of tissue infiltration of inflammatory cells and an
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 in-
travascular 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 in-
creased 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 hypoxyprobe reagent, most not-
ably 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
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 under-
lying 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 accom-
modated to increased length during distension and then col-
lapsed upon balloon deflation. When specimens were refriger-
ated overnight before dissection, vaginal tissue was able to
fully fix in position and the undulating waves were not ob-
erved, 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 eutha-
nized by perfusion fixation, which euthanized them and termi-
nated 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-
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-
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 physiological 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 implicate the vulnerability of this region to childbirth-associated injury and the subsequent development of SUI.
REFERENCES


