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Long-Term Effects of Simulated Childbirth Injury on Function and Innervation of the Urethra

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Abstract

Aims—Pudendal nerve and external urethral sphincter (EUS) injury during vaginal delivery are risk factors for stress urinary incontinence (SUI). Although most patients with short-term postpartum SUI regain continence within 1 year, they have a higher predisposition to develop recurrent SUI years later, suggesting a possible mechanistic relationship. In contrast, animal models generally recover spontaneously and have not been studied much in the long term. The aim of this study was to investigate the long-term effects of simulated childbirth injury in rats.

Methods—Thirty-four Sprague–Dawley female rats underwent sham injury or pudendal nerve crush and vaginal distension (PNC + VD), a simulated childbirth injury. Nine weeks later, leak point pressure (LPP) and EUS electromyography (EMG) were recorded simultaneously. The pudendal nerve was harvested for histological analysis. EUS neuromuscular junctions (NMJs) and their innervation were qualitatively assessed using immunofluorescence. A *t*-test was used to compare quantitative outcomes between groups, with *P* < 0.05 indicating a significant difference.

Results—There was no significant difference in LPP or EUS EMG amplitude or firing rate between the two groups. Nonetheless after PNC + VD, NMJs in the EUS were diffuse and were innervated by tortuous and multiple axons, demonstrating that reinnervation of the EUS was still in progress.

Conclusions—Although continence function recovered 9 weeks after simulated childbirth injury, innervation of EUS was not complete at this time point, suggestive of persistent neurogenic

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deficiency which when compounded by the effects of aging may lead to a delayed recurrence of SUI in this animal model with increased age.

Keywords

electromyography; morphology; neuromuscular junction; pudendal nerve; rat; urethra; urinary incontinence

INTRODUCTION

Stress urinary incontinence (SUI), the complaint of involuntary loss of urine in the absence of a bladder contraction, affects quality of life at all ages, particularly among women.¹ Vaginal delivery is correlated with development of SUI, although women often do not become symptomatic until years later.² During the process of childbirth, the vaginal wall and muscles, ligaments and connective tissue of the pelvic floor can be compressed and overstretched.³ In addition, the pudendal nerve in Alcock's canal is vulnerable to trauma during delivery, particularly with increased duration of second stage of labor, forceps delivery, or large birthweight baby.¹ A prospective study demonstrated that among women with SUI during delivery or 3 months after it, only 3% exhibited SUI symptoms 1 year postpartum.⁴ However, among women with SUI postpartum, 92% had recurrent SUI 5 years later.⁴ These latter findings suggest that even with functional recovery from postpartum SUI, there are latent pathophysiologic changes in the continence mechanism, which could lead to later development of recurrent SUI.

Several animal models have been developed to replicate the injuries during human vaginal delivery. Vaginal distension (VD) leads to hypoxia and disruption of external urethral sphincter (EUS) muscle fibers, simulating dilation of the vagina during the second stage of labor.⁵ Bilateral pudendal nerve crush (PNC) results in EUS dysfunction, causing a denervation injury to the EUS.⁵ A simulated childbirth injury rat model, consisting of both PNC and VD has been demonstrated to cause a more severe and durable damage to EUS, pudendal nerve and connective tissue than either injury alone.^{6,7} Although urethral resistance to leakage, as measured by leak point pressure (LPP), recovers 4–6 weeks after PNC + VD, EUS electromyography (EMG) does not recover at this time.⁶

Although clinically, SUI in most women does not become symptomatic until years, often decades, after vaginal delivery,² few long term studies have been performed on animal models of simulated childbirth injury. We have previously found that the EUS EMG was not fully recovered at 6 weeks time point. We therefore chose 9 weeks to investigate longer-term effects of simulated childbirth injury on the EUS function and neuromuscular junction (NMJ) morphology.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic. Thirty-four 8-week-old, female, virgin, age-matched Sprague–Dawley rats (200–250 g) underwent PNC + VD or sham injury. Nine weeks later, LPP with simultaneous EUS EMG recordings were performed simultaneously in six rats in each group. Since

measurement of EUS EMG requires extensive dissection, additional animals were used to harvest tissue for NMJ immunostaining in the EUS (five per group) and qualitative pudendal nerve morphology (six per group).

PNC and VD were performed as done previously.⁶ In short, after anesthesia with isoflurane (2% dosage), a 2–3 cm longitudinal midline dorsal incision was made. The pudendal canal was located in the space between the ischium and spine bilaterally. After separation of muscle and fat, the bilateral pudendal neurovascular bundle was crushed twice (30 sec each) with a Castroviejo needle holder. VD was performed immediately after PNC. Increasing sizes of Otis Bougie a Boule urethral dilators (24–32 F) were inserted into the vagina to prevent rupture and bleeding. A modified 10 F Foley catheter was then inserted into the vagina and the balloon was filled with 3 ml water. A single stitch kept the catheter and balloon in place for 4 hr while isoflurane anesthesia was maintained. The same procedures were performed on sham injury animals except the pudendal nerve was not separated during PNC and the Foley catheter balloon in the vagina was not filled during VD. Buprenorphine (0.1 mg/kg, subcutaneous) was given subcutaneously twice daily after PNC + VD or sham injury for 2 days for analgesia.

LPP and EUS EMG were recorded simultaneously by an investigator blinded to the experimental group, as previously described.⁶ Under urethane anesthesia (1.2 g/kg, intraperitoneal), a polyethylene catheter (PE-50) was inserted into the bladder transurethrally and was connected to a pressure transducer (Model P300, Grass Instruments, West Warwick, RI) and a syringe pump (Model 200, KD Scientific, New Hope, PA) to fill the bladder (5 ml/hr). A 3–4 cm midline abdominal incision was made followed by pubic symphysectomy to expose the urethra. A parallel platinum bipolar electrode (tip diameter: 125 μ m diameter, PBSA1075, FHC, Bowdoin, ME), connected to a recording system (Dash 8X, Astro-Med, Inc., West Warwick, RI), was placed under stereomicroscopy at the mid-urethra approximately 5 mm distal to the bladder neck for EMG recording.

The bladder was filled for 3–4 voiding cycles and mean filling time was calculated as the interval between the end of each void until the beginning of next void. The bladder was then manually emptied and refilled until approximately half mean filling time, when a slowly increasing external pressure was applied directly to the bladder using a cotton-tipped swap. This externally applied pressure was rapidly removed at the first sign of leakage at the urethral meatus. We have previously shown that LPP is not highly sensitive to bladder volume in rats, except at very low volumes.⁸ Therefore, this estimate of $\frac{1}{2}$ capacity was sufficient to ensure consistent results. This LPP test was repeated three to four times in each rat. LPP was calculated by subtracting baseline bladder pressure from peak bladder pressure during LPP testing (AstroVIEW X, Astro-Med, Inc.).

At the end of each LPP test, the increased EMG activity did not immediately disappear when the external pressure on the bladder was removed, but continued for a few more seconds, indicating that the increased signal was not caused directly by movement of the bladder (Fig. 1). One second segments of EUS EMG data were selected at baseline bladder pressure during filling just after emptying when intravesical pressure was stable and in the absence of non-voiding contractions. EUS EMG data during LPP testing was analyzed from

a 1 sec segment of data chosen to end at the moment of peak bladder pressure (Fig. 1). The raw signal was smoothed and band-pass filtered at 60 and 120 Hz (LabChart 7, ADInstruments, Dunedin, NZ). Mean EMG amplitude and firing rate at both baseline and peak were calculated for each LPP test in each rat with a 15 μ v threshold as we have done previously.⁹ A mean of LPP and EMG outcomes was calculated for each rat and utilized to create a mean and standard error of the mean for each experimental group.

Due to the extensive dissection required for EUS EMG recording, separate animals underwent PNC + VD or sham injury and were euthanized 9 weeks later for qualitative assessment of nerve morphology. To minimize injury to the EUS when dissecting the urethra in these rats, we performed symphysectomy carefully without splitting the pubic bone or stretching the urethra. After intracardiac perfusion of 0.2% heparinized saline, followed by a dilute fixative solution (1% paraformaldehyde and 1.25% glutaraldehyde) and then a concentrated fixative solution (4% paraformaldehyde and 5% glutaraldehyde),¹⁰ the pudendal nerve was harvested from the ischiorectal fossa 5 mm distal to the injury site. The nerve was postfixed, osmicated in 2% osmium tetroxide, and embedded in epon-araldite.¹⁰

Since different tissue fixation methods are needed for immunofluorescence and neuromorphology, additional animals were euthanized 9 weeks after PNC + VD or sham injury for qualitative assessment of EUS NMJs by immunofluorescence. Following intracardiac perfusion of heparinized saline, a 1 cm piece of urethra (~5 mm from the urethral orifice) was dissected. The specimens were held straight and embedded in room temperature Tissue-Tek® O.C.T. (Optimal Cutting Temperature) compound (Sakura Finetek Inc., Torrance, CA). After freezing in liquid nitrogen, the specimens were sectioned longitudinally using a cryostat (CM 1850, Leica, Buffalo Grove, IL) at 15 μ m thickness. Slides were stored at -80°C until staining.

For NMJ immunostaining, slides were first washed with PBS followed by blocking solution (consisting of 5% donkey serum, 2% Bovine serum albumin stock solution, 1% fish skin gelatin, 0.1% triton X-100, and 0.05% Tween® 20). The samples were incubated in the primary antibody (1:100, mouse anti-neurofilament 68 and 200 kDa monoclonal antibodies, Sigma, St. Louis, MO) overnight at 4°C and then secondary antibody (1:400, FITC labeled donkey anti-mouse secondary antibodies, Invitrogen, Eugene, OR) for 1 hr in room temperature.¹¹ Four microgram per milliliter of tetramethylrhodamine-conjugated α -bungarotoxin (Rh- α -BTX; Invitrogen, Eugene, OR), was applied for 1 hr incubation to stain acetylcholine receptors (AChRs) in the motor end-plates of EUS NMJs.^{11,12} Slides were incubated in 20 μ g of Alexa Fluor conjugated phalloidin (0.5%, Invitrogen) in 400 μ l PBS for 45 min to stain EUS muscle fibers.¹³ The slides were then mounted with ProLong® Gold Anti-fade Reagent (Invitrogen). Using a fluorescence microscope, two investigators, blinded to the groups, qualitatively characterized and categorized the innervation of EUS according to the morphology of neurofilaments and motor end-plates.

Quantitative data were analyzed in a blinded manner and are presented as mean $\pm$ standard error of the mean for each group. Since LPP, EMG amplitude and EMG firing rate data were normally distributed, a two way ANOVA plus Student-Newman-Keuls post hoc test was

used to compare injury and sham injury groups as well as baseline and peak bladder pressures, with $P < 0.05$ indicating a statistically significant difference between groups.

RESULTS

Both sham and injured rats demonstrated typical LPP and EUS EMG data with a statistically significant increase in EUS EMG firing rate during LPP testing, considered a guarding reflex to try to maintain continence despite the increase in bladder pressure with the externally applied pressure (Fig. 1).⁶ There was no significant difference in LPP between sham and injury groups, consistent with our previous investigation with this injury model 6 weeks after injury.⁶ There were likewise no significant differences in EUS EMG outcomes between the dual injured and sham injured groups (Fig. 1).

Light osmication of the pudendal nerve motor branch distal to the injury site demonstrated splaying of regenerating axons coursing over the pudendal artery and Wallerian degeneration which would appear as clumps of myelin debris was not detected 9 weeks after PNC + VD (Fig. 2).⁷ On immunofluorescence, sham injured rats demonstrated organized motor endplates at the NMJs of the EUS co-localizing with thick innervating axons (Fig. 3A). Nine weeks after PNC + VD, though with a few normally innervated NMJs, animals demonstrated tortuous innervating axons, consisting of multiple collateral fibers (Fig. 3B upper half) and diffusion of AChRs at motor end-plates (Fig. 3B lower half), suggesting that neuroregeneration was still ongoing. A blinded assessment of immunofluorescence results enabled 100% accurate categorization into the two experimental groups.

DISCUSSION

Vaginal delivery is an important risk factor for the development of SUI since primiparous women are 3.3 times more likely to develop SUI than nulliparous women.¹⁴ Although most women with postpartum SUI regain continence within 1 year, they have a greater predisposition to develop recurrent SUI several years later.⁴ Trauma to the pelvic floor nerves, muscles, and connective tissue during pregnancy and vaginal delivery may persist even in the absence of SUI symptoms, and could become an underlying risk factor for later development of SUI.¹⁵ As a result, transient antepartum or postpartum SUI is predictive of later development of recurrent SUI.¹⁶

Animal models have been used to better understand the mechanisms of postpartum SUI. Bilateral PNC produces denervation of the EUS and significantly decreases LPP, EUS EMG and pudendal nerve motor branch potentials.¹⁷ VD, another common model, damages the muscular and neurologic structures which maintain urinary continence.⁷ VD leads to attenuated LPP and EUS EMG but not pudendal nerve motor branch potentials.¹⁵ The combination of PNC and VD (simulated childbirth injury) can lead to synergetic effects causing more severe and long lasting damage than either PNC or VD alone.⁵

A few studies have investigated the long-term pathophysiological change in models of SUI. Resplande et al.¹⁸ demonstrated more apoptotic cells in the urethra and lower maximum urethral closure pressure 9 months after a model consisting of pup delivery, VD, and

ovariectomy. Electrocauterization, which produces intrinsic sphincteric deficiency in rats, results in significantly decreased LPP, disrupted EUS and reduced nerve fibers 16 weeks later.¹⁹ Twenty-four weeks after transabdominal urethrolisis in rats, both LPP and retrograde urethral perfusion pressure were significantly decreased.²⁰ More apoptotic cells and fewer neurons were identified in the urethra and bladder 12 weeks after urethrolisis.²⁰ The first model simulates not only childbirth injuries but also reduced hormone levels after ovariectomy. The latter two models are non-recoverable models of SUI that do not simulate the injuries of childbirth. None of these prior studies have focused on the long-term neuromuscular morphometry after simulated childbirth injury.

Similar to the clinical findings in patients with short-term postpartum SUI,⁴ our animal model demonstrates spontaneous functional recovery within several weeks.⁶ The present study revealed that the LPP was normal 9 weeks after PNC + VD, consistent with our earlier finding that continence returned 4–6 weeks after injury.⁶ Our previous studies indicate that the EUS EMG was not completely recovered 6 weeks after PNC + VD.⁶ Here we showed restored EMG amplitude and firing rate 9 weeks after injury, suggesting that EUS function was normal at this time.

The earlier recovery of LPP than EUS EMG illustrates the fact that urethral smooth muscle, pudendal nerve and spinal cord innervation, mucosal seal coaptation, and abdominal pressure transmission all contribute to LPP, in addition to EUS function.²¹ LPP tests urethral resistance to leakage rather than complete urethral function; thus, it can be overspecified by the various contributors such that they can each compensate for dysfunction of other contributing components to maintain normal LPP. On the other hand, EUS EMG is a more specific outcome, measuring innervation and neurological function of the EUS. Therefore, earlier recovery of LPP could occur if other contributors to urethral function compensate for more slowly recovering EUS function.

We chose to use a transurethral catheter to measure bladder pressure in this study to be consistent with our previous shorter term studies with this animal model.⁶ Although urethral catheterization has been shown to increase LPP clinically,²² a review of our previous studies using transvesical or trans-urethral catheters for LPP measurement in rats demonstrates no such trends.^{6,23}

To obtain a more global measure of EUS function from EMG measurement, we used parallel bipolar electrodes placed on the outside of the urethra instead of concentric needle electrodes inserted into the urethra, which would have given a measure of local function of the EUS near the needle tip location.⁹ Application of the externally placed parallel electrodes does not cause excessive damage to the urethra as demonstrated by a comparison of recently published papers, one with urethral histology after EUS EMG measurement with this method and one with urethral histology and LPP measurement without EMG.^{7,24}

Our previous pudendal nerve morphology results with this animal model demonstrated signs of Wallerian degeneration in the pudendal nerve motor branch 5 mm distal to the crush site as evidenced by the clumps of osmophilic ovoids 10 days after PNC + VD.⁷ In this study,

we observed injured nerve fibers spread across the pudendal artery, indicating recovery of the pudendal nerve motor branch near the crush site at this time.

Signal transduction in NMJs coordinates the interaction between muscle and its innervation.²⁵ Thus, intact NMJ components are important in sustaining muscle function. Using Rh- α -BTX to label AChRs, previous studies have measured the regulation of NMJs.¹¹ Under the regulation of intact neurotransmission, AChRs are restricted to the crest of folds in synapses and cannot move to the perisynaptic region.²⁶ Following denervation, AChRs migrate from the synapses into the perisynaptic space.²⁶ Our results demonstrate multiple tortuous neurofilaments and diffusion of AChRs at motor end-plates 9 weeks after PNC + VD, indicating incomplete reinnervation of NMJs in the EUS and persistent neurogenic deficiency as has been observed clinically with SUI.²⁷ Despite incomplete reinnervation of the EUS, EUS EMG amplitude and firing rate were normal, suggesting that this limited reinnervation is sufficient for EMG and continence as measured by LPP.

Insufficient innervation of the EUS may account in part for clinical development of recurrent SUI,¹⁵ since recurrent SUI occurs later in life when the continence threshold is reached due to the compounding effects of aging, such as reduction of the number of muscle fibers in the EUS, loss of elasticity of supportive tissues, and denervation of smooth and striated muscles.²⁸ Additional investigations at longer time points could be utilized to investigate if recurrent SUI occurs in this animal model.

Though these animal models provide valuable insight into pathophysiological mechanisms of postpartum SUI, there are several relevant differences between rodents and humans.⁵ Rats are quadruped rodents whose pelvic orientation is different from that of humans. As a result, the bladder is supported by the anterior abdominal wall, while in humans the bladder and urethra are supported by the pelvis and levator ani complex.²⁹ In addition, anesthesia was used during cystometry in rats, which may affect the neuromuscular mechanisms of continence.¹⁵ In the animal model, surgical procedures are performed to induce the pelvic nerve and muscle injury instead of vaginal delivery, leading to the absence of factors such as hormonal changes during gestation.³⁰ Nonetheless, these models can provide insight into possible mechanisms of SUI and can particularly serve to isolate the neuromuscular aspects of urethral insufficiency for further investigations.

CONCLUSIONS

The findings of this animal model demonstrate incomplete neuromuscular innervation of the EUS 9 weeks after PNC + VD despite functional recovery, suggesting that this model demonstrate the persistent neurogenic deficiency seen in SUI. Recurrent SUI may occur as a result of insufficient neuromuscular recovery compounded by the effects of aging bringing urethral closure capabilities below the threshold for continence. Further studies are needed to assess this mechanism clinically.

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Fig. 1.

Example of external urethral sphincter (EUS) electromyography (EMG) and leak point pressure (LPP) results 9 weeks after sham injury (A) showing the 1 sec segments used to quantitatively analyze the data as well as LPP (B), EUS EMG amplitude (C), and firing rate (D) both at baseline pressure prior to LPP testing and at peak pressure during LPP testing. LPP difference as well as EUS EMG amplitude and firing rate difference are calculated by subtracting the value of each outcome at baseline from the value of the same outcome at peak pressure during LPP testing. Each bar represents mean $\pm$ standard error of data from six animals. *Indicates a statistically significant difference compared to data at baseline in sham injury group. + Indicates a statistically significant difference compared to data at baseline in injury group.

Fig. 2.

Pudendal nerve motor branch distal to the injury site 9 weeks after pudendal nerve crush (PNC) and vaginal distension (VD). Light osmication of the pudendal nerve motor branch shows splaying of the nerve over the pudendal artery 5 mm distal to the injury site. The box in the left panel indicates the area magnified in the right. EAS, external anal sphincter; EUS, external urethral sphincter. Scale bar represents 200 μ m.

Fig. 3.

Examples of neuromuscular junctions (NMJs) and their innervation in the external urethral sphincter (EUS) 9 weeks after either sham injury (**A**) or pudendal nerve crush (PNC) and vaginal distension (VD). **B**: Immunofluorescence indicates acetylcholine receptors at the striated muscle motor end-plates (red); neurofilament of innervating axons (green) and striated muscle of the EUS (blue). Single axon innervation and organized acetylcholine receptors in NMJs are typical of sham-injured animals (**A**) while multiple collateral innervation and diffusion of acetylcholine receptors occur in the EUS of injured animals (**B**). Scale bars represent either 50 μm (**left**) or 20 μm (**right**).