Effect of extended-term estrogen on voiding in a postpartum ovariectomized rat model

Narihiko Hayashi, MD; Anthony J. Bella, MD; Guifang Wang, MD; Guiting Lin, MD, PhD; Donna Y. Deng, MD; Lora Nunes; Tom F. Lue, MD

ABSTRACT

Introduction: We tested the hypothesis that extended-term (5-week) estrogen therapy would negatively impact voiding function in a postpartum, ovariectomized rat model.

Methods: Immediately after delivery, 30 primiparous Sprague–Dawley rats underwent intravaginal balloon dilation, followed by ovariectomy 1 week later. Cystometry at postpartum week 2 determined normal or abnormal voiding patterns. After randomization, one-half the normal and abnormal voiding rats received 5 weeks of estrogen therapy, while the remainder received placebo. Estrogen effect was determined by repeat cystometry and immunohistochemical analysis of the urethra and vagina.

Results: Abnormal voiding increased from 60.0% to 73.3% in the estrogen-treated group and declined from 60% to 33% for the placebo group. Rats were then divided into 4 groups for comparison: normal voiding versus placebo (group 1), abnormal voiding versus placebo (group 2), normal voiding versus estrogen (group 3) and abnormal voiding versus estrogen (group 4). Bladder capacity, leak point pressure and maximum voiding pressure were most depressed in group 4. Estrogen treatment was associated with a significant downregulation of $\alpha_{1A}$ and $\alpha_{1D}$-adrenoceptors in the urethral submucosa but an upregulation of nNOS in the urethral smooth muscle.

Conclusion: Extended-term estrogen therapy in a rat model of simulated birth trauma and ovariectomy resulted in a higher rate of incontinence. Immunohistochemical examination demonstrated significant downregulation of urethral $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptors and upregulation of neuronal nitric oxide synthase (nNOS) in the urethra of estrogen-treated groups. These studies question the use of hormone replacement therapy in the treatment of postmenopausal incontinence.

Copyright © 2007 Canadian Urological Association
women who received the drug had a marked increase in urinary incontinence, compared with those who took placebo (17% v. 4%) as well as an increase in voiding frequency, endometrial thickness and uterine enlargement. Secondary to the number and severity of adverse events, the trial was prematurely halted after 10 months.\(^8\)

Over the past 7 years, our laboratory has investigated the influence of simulated birth trauma and hormonal deficiency on urinary continence in a postpartum, ovariectomized female rat model.\(^9\)\(^-\)\(^11\) We determined that prolonged labour and ovariectomy induced higher rates of SUI versus ovariectomy alone. Further, our results confirmed that muscular and neurological alterations in the urethra and pelvic floor likely play a role in the development of SUI.\(^11\)

Although scientific data are available regarding the effects of female hormones on the vagina, uterus and bladder, examination of the urethra has largely been neglected to date. The objectives of this study were to evaluate the effect of estrogen replacement on voiding function in our postpartum, ovariectomized rat model and identify the immunohistochemical alterations of the urethra and vagina.

**Materials and Methods**

**Experimental design**

This study was approved by the local ethical committee for experimentation (University of California, San Francisco, Institutional and Animal Care Use Committee) and complied with National Institutes of Health (NIH) regulations for the care and use of laboratory animals. Thirty primiparous pregnant 2-month-old Sprague–Dawley female rats at gestational day 16 (230–280 g) were housed at a constant 16°C room temperature and 47% humidity, with a 12-hour light-dark cycle. Immediately postpartum, animals underwent intravaginal balloon dilation under anesthesia and 1 week later underwent bilateral ovariectomy (Fig. 1). Two weeks postpartum, initial transvesical cystometry was performed. Based on the urodynamic findings, each rat was classified as having normal (NV) or abnormal voiding (AV). After cystometry, AV animals outnumbered the NV group (18:12), and we felt that the comparison would be difficult after estrogen treatment. Therefore, the 2 groups (NV v. AV) were randomly divided in half and were cross-mixed, establishing 2 equal groups of 15 animals each. At the 3-week mark, 1 group underwent dorsal subcutaneous implantation of a 0.5-mg 17β-estradiol pellet, while the other group received placebo. Estrogen or placebo pellet placement was repeated at 6 weeks to maintain constant drug bioavailability. Repeat cystometric results were obtained 7.5 weeks after balloon dilation, and the rats were again evaluated with urodynamic studies. For final outcome analyses, animals were grouped as follows: 1) NV plus placebo; 2) AV plus placebo; 3) NV plus estrogen; 4) AV plus estrogen (Fig. 1). At sacrifice, the pelvic tissues, midurethra and vagina were harvested. The midurethra was chosen because it represents the thickest region of the urethra with both striated and smooth muscle components and has demonstrated the least anatomic variation in our previous studies.

**Intravaginal balloon inflation**

Immediately postpartum, all rats underwent anesthesia using intraperitoneal injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). To simulate prolonged labour, a modified distal-tip shortened 22 Fr Foley catheter was inserted transvaginally and inflated (3 mL sterile water). A 130-g weight was attached to the catheter’s proximal end. The rat was placed in a fixed prone position at the edge of the mini operating table, leaving the caudal end of the animal hanging downward without touching the table. This allowed the weighted catheter to hang freely against gravity. The rat’s colour, warmth and respirations were monitored without positional disturbance for 3 hours. This mechanism simulates the pressure effects of a large fetus on the pelvic floor during the second stage of labour and has been previously shown to create a consistent model of SUI.\(^11\) We used postpartum instead of virgin rats because it is more representative of the clinical situation for which most SUI occurs (after vaginal birth).

**Bilateral ovariectomy**

One week after balloon dilation, the rats were anesthetized as above. Using a small vertical lower
midline abdominal incision, vascular pedicles were suture ligated and ovaries removed, followed by a 2-layer abdominal closure. We performed ovariectomy 1 week after vaginal dilation because performing both procedures at the same time increased the morbidity and mortality of the rats in our previous experiments.

**Transvesical cystometry**

Two weeks after delivery, under sedation with ketamine (100 mg/kg), a repeat lower midline incision exposed the bladder. A 27-gauge butterfly needle was inserted into the bladder dome, and all urine was evacuated. Using a 3-way stopcock, the

---

**Fig. 1.** Experimental design. AV = abnormal voiding.
needle was connected to both a Baxter Uniflow
pressure transducer (Baxter Healthcare Corp.,
Irvine, Calif.) and a Harvard Model 22 infusion
pump (Harvard, Millis, Mass.). Saline at 37°C was
infused at 0.1 mL per minute after calibrating the
pressure transducer to zero atmospheres. A cus-
tomized Macintosh Quadra 800 (Apple Computer
Inc., Cupertino, Calif.) with LabView 4.0 software
(National Instruments Corp., Austin, Tex.) collect-
ed the data.

Voiding was considered normal if a detru-
sor contraction was followed by expulsion of
saline from the meatus and with bladder empty-
ing. The modified leak point pressure was deter-
mined at this point, and the instilled intravesi-
cal volume represented bladder capacity. A
pattern of moderate incontinence was identified
on cystometry if intermittent small leakage
occurred with or without bladder contraction
during filling, followed by a sustained detrusor
contraction and voiding. Cystometric studies
in animals with severe incontinence displayed
a continued small amount of saline leakage at
low detrusor pressures, without the bladder fill-
ing to capacity (Fig. 2). Urodynamics were
repeated at 7.5 weeks postpartum. Because these
studies were performed under ketamine seda-
tion, we could not reliably differentiate over-
active bladder from sphincter deficiency, as in
the case of awake cystometry and therefore

grouped them together as the AV group.

**Estrogen or placebo pellet implantation**

One week after initial cystometry and randomiza-
tion, a 0.5-mg 17 β-estradiol (21 d timed-release
formula) or placebo pellet (Innovative Research of
America, Sarasota, Fla.) was implanted in a sub-
cutaneous pouch. After ketamine sedation, a
5-mm incision in the dorsal skin at shoulder height
allowed pellet deposition. This procedure was
repeated in all groups 3 weeks later to main-
tain constant estrogen or placebo levels. In our
pilot studies, we did not notice any changes with
shorter-term estrogen therapy, thus we implanted
a second pellet at 3 weeks, when the first pellet’s
effects had terminated.

Upon completing the second cystometry at 7.5
weeks after delivery, animals were euthanized.
The pelvic floor was exposed, and samples of the
midurethra and vagina were removed.

**Immunohistochemistry and image analysis**

Tissue specimens were fixed in cold 2% formalde-
hyde and 0.002% saturated picric acid in
0.1 μmol/L phosphate buffer (pH 8.0) for 4 hours,
followed by overnight immersion in a buffer con-
taining 30% sucrose for cryoprotection. The spec-
imens were embedded in OCT Compound (Sakura
Finetic USA, Torrance, Calif.) and stored at –70°C
until use. Fixed frozen tissue specimens were cut
at 5 µm thickness, mounted to SuperFrost Plus
charged slides (Fisher Scientific, Pittsburgh, Pa.)
and air-dried for 5 minutes. Endogenous peroxi-
dase activity was blocked with 0.3% H₂O₂.
methanol for 10 minutes. After rinsing, sections were washed twice in phosphate-buffered saline (PBS) for 5 minutes and incubated with 3% horse serum in PBS with 0.3% Triton X-100 (Rohm and Haas Co., Spring House, Pa.) for 30 minutes (room temperature) to eliminate nonspecific protein binding. After draining, the slides were incubated overnight at 4°C with goat polyclonal antibodies to α1A- or α1D-adrenoceptors (Santa Cruz Biotechnology, Santa Cruz, Calif; 1:100) or mouse monoclonal antibody to neuronal nitric oxide synthase (nNOS) (BD Transduction Laboratories, San Jose, Calif; 1:500) to examine potential changes in contractile (α adrenergic) and relaxant (nitric oxide [NO]) properties of the urethral sphincter. Slides incubated without antibodies in a similar fashion served as negative controls. After washing with buffer sections, slides were immunostained with the avidin-biotin-peroxydase method (Elite ABC, Vector Laboratories, Burlingame, Calif.), using diaminobenzidine as the chromagen, followed by hematoxylin counterstaining. For image analysis, 5 randomly selected fields per tissue, per animal, for each treatment group were photographed and recorded with a Retiga Qimage digital camera and ACT-1 software (Nikon Instruments Inc., Melville, NY). Images were quantified with Image-Pro Plus software (Media Cybernetics, Silver Spring, Md.). The ratio of the α1A- or α1D-adrenoceptor or nNOS stained pixels to all pixels of the whole picture (equal to 100%) provided a percentage. Different team members performed the functional study and histological evaluation, and evaluations were blinded until after the final analyses.

All values are expressed as mean and standard error of the mean. We used the unpaired 2-tailed t test for statistical analysis, using YSTAT 2002 (Ikakutosho, Tokyo, Japan). For posttreatment continence status, we performed the chi-squared test. Comparisons were considered significant at p < 0.05.

Results

Urodynamics

Initial transvesical cystometric profiles performed 2 weeks after postpartum intravaginal balloon dilation and 1 week after ovariectomy identified 18 rats

<table>
<thead>
<tr>
<th>Table 2: Cystometric results after estrogen or placebo treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Resolution of AV, no.</td>
</tr>
<tr>
<td>Development of AV, no.</td>
</tr>
<tr>
<td>Bladder capacity, mL†</td>
</tr>
<tr>
<td>Maximum pressure, cm water‡§</td>
</tr>
<tr>
<td>Leak point pressure, cm water¶</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean; NV = normal voiding; AV = abnormal voiding; NA = not applicable.
†p < 0.05 for group 3 v. group 4, and groups 1 and 2 v. group 4.
¶p < 0.05 for group 1 and group 2 v. group 2 and group 4.
‡p < 0.05 for group 3 v. group 4, and groups 1 and 2 v. group 4.
§p < 0.05 for group 3 v. group 4, and groups 1 and 2 v. group 4.

<table>
<thead>
<tr>
<th>Table 3: Immunohistochemical analysis of the urethra and vagina</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urethral or vaginal region</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Urethral epithelium</td>
</tr>
<tr>
<td>Urethral submucosa</td>
</tr>
<tr>
<td>Urethral smooth muscle</td>
</tr>
<tr>
<td>Vaginal submucosa</td>
</tr>
</tbody>
</table>

SEM = standard error of the mean; 1A = alpha 1A-adrenoceptor; 1D = alpha 1D-adrenoceptor; nNOS = neuronal nitric oxide; ME = minimal expression
*Compared with matched placebo group; p < 0.05.
(60%) with AV patterns and 12 (40%) with normal voiding. Table 1 compares urodynamic findings. Repeat cystometry after estrogen treatment documented an increase in AV from 60% to 73.3% (from 9 to 11 rats), whereas the placebo group demonstrated a decline in AV from 60% to 33.3% (from 9 to 5 rats). The difference is statistically significant ($\chi^2$ of 4.821 and $p = 0.028$). In a subset analysis, estrogen-treated rats with abnormal voiding (group 4) more frequently displayed a higher severity of incontinence, compared with placebo-treated rats with AV (group 2), with continuous leakage hampering the ability to reach bladder capacity. Other parameters are summarized in Table 2, with statistically significant lower bladder capacity, maximal bladder pressure and modified leak point pressure noted in group 4.

**Immunohistochemistry**

We observed thickened epithelium and subepithelium, as well as increased amounts of smooth muscle fibres in the muscularis, for the estrogen-treated vaginal tissue, and we observed no general morphological differences for urethral specimens between estrogen- and placebo-treated groups.

Alpha adrenoceptors 1A and 1D were identified in both the urethra and the vagina. Estrogen therapy significantly downregulated $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor expression in the endothelium and subendothelial regions of the urethra while increasing levels of both receptors in the vagina (Table 3, Fig. 3 and Fig. 4).

nNOS staining was significantly elevated in the urethral smooth muscle and urothelium, as well as in the basal layer of the vaginal epithelium of rates treated with estrogen (Fig. 5 and Fig. 6).

**Discussion**

Several recent publications have identified adverse effects of HRT in a diverse group of disease processes, including stroke, breast cancer, osteoporosis and urinary incontinence. Further, the Women’s Health Initiative (WHI) trial, evaluating the effects of postmenopausal estrogen plus progesterone therapy was prematurely halted secondary to multiple reported adverse trends in the treatment arm. The results of our experiment align with these aforementioned studies, indicating that extended term estrogen therapy (> 5 wk) worsened voiding dysfunction in our postpartum intravaginal balloon-dilated, ovariectomized rat model.

Using this model, we previously reported that birth trauma simulated by weighted ballooning and ovariectomy appears to contribute to SUI. We have also demonstrated that these rats rarely have UI during pregnancy or after ovariectomy alone without vaginal distension. Although the scenario does not create a true replica of prolonged labour as seen in humans, we believe this model is suitable for studying the urinary continence mechanism in the rat.

In our study, cystometric curve and the observation of urine (saline) leakage pattern clearly iden-
tified rats with normal voiding patterns and those with abnormal leakage. Since the cystometry was performed under ketamine sedation, we could not determine whether the AV pattern was due to pure sphincter insufficiency or mixed with overactive bladder, we included all rats with abnormal voiding in the AV group. Future studies with conscious cystometry will be required to further study the AV patterns.

Using immunohistochemistry, we observed a significant downregulation of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor expression in the endothelium and subendothelial regions of the urethral submucosa in rats treated with estrogen. Conversely, there was an increase in $\alpha_{1A}$- and $\alpha_{1D}$-receptor immunostaining in vaginal tissues. This differential regulation of adrenoceptor in the urethra and vagina is intriguing and warrants further investigation (Table 3).

Urinary continence is believed to be maintained by sympathetic impulses through $\alpha_1$-adrenoceptors in the bladder neck and urethral smooth muscle.\textsuperscript{15,16} Binding and molecular biological studies established the $\alpha_{1A}$-adrenoceptor to be the predominant subtype in the urethra, responsible for the contractile responses of the urethra via elevated intracellular calcium levels to adrenergic agonists.\textsuperscript{17–19} Taki and colleagues\textsuperscript{20} demonstrated that $\alpha_1$-adrenoceptors play an important role in maintaining the resting urethral tonus of the female urethra. They divided the urethra into segments and administered noradrenaline (NA), an $\alpha_1$ agonist, demonstrating a concentration-dependent contraction with a peak amplitude in the middle to proximal urethra, while acetylcholine produced contractions only in the proximal urethra and bladder neck. Applying these results to our experiment, a downregulation of $\alpha_1$-adrenoceptors would contribute to a significant decrease in contraction and tone of urethral smooth muscle.

The 4 reported $\alpha_1$-adrenoceptor subtypes include $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$ and $\alpha_{1L}$.\textsuperscript{21} In the proximal female urethra, the expression of $\alpha_1$-adrenoceptor subtype mRNA has a reported ratio of 90:0:10 for $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$, respectively.\textsuperscript{22} However, Nishimatsu and colleagues\textsuperscript{23} reported that phenylephrine, a nonselective $\alpha_1$-adrenoceptor agonist, induced contractions through the $\alpha_{1A}$ receptor, and not through the $\alpha_{1A}$ subtype. The role of each of these $\alpha_1$-adrenoceptor subtypes in relation to urethral contractile function has yet to be delineated. Regardless, our results suggest that downregulation of both $\alpha_{1A}$ and $\alpha_{1D}$ adrenoceptors may represent one mechanism by which impaired contraction of the urethra can lead to incontinence.

NO, a neurotransmitter that causes relaxation of urethral smooth muscle, is produced by NOS, an enzyme located at several sites, including neurons.\textsuperscript{24,25} Recent studies suggest that adrenergic and NO-mediated nerves may function in a complex interactive manner and not as independent agents.\textsuperscript{17} In the rabbit urethra, released NO suppresses NA release from adrenergic nerve terminals; the reverse has also been reported.\textsuperscript{26} Perhaps our finding of upregulated urethral nNOS in the estrogen group results in smooth muscle relaxation.
by both local effect and α₁-adrenoceptor down-regulation with a net decrease in NA release.

Further investigation is needed to determine the intricate mechanisms causing SUI. Both molecular study and physiological animal experiments are needed to advance our knowledge and improve clinical management. Identifying the mechanism by which estrogen modulates the α₁ adrenoceptor will be valuable not only to incontinence research but also to a broad spectrum of diseases.

Conclusion

Extended term estrogen therapy in a rat model of simulated birth trauma and ovariectomy resulted in a higher rate of incontinence. Immunohistochemical examination shows significant downregulation of urethral α₁ and α₂ adrenoceptors and upregulation of nNOS in the urethra of estrogen-treated groups. These studies question the use of HRT in the treatment of postmenopausal incontinence.

From the Knuppe Molecular Urology Lab, Department of Urology, University of California, San Francisco, Calif.

Acknowledgements: This work is supported by NIDDK/NIH grants: R01DK069655-01 and PS0DK64538. Dr. A.J. Bella is the American Urological Association Foundation Robert J. Krane Scholar and a Royal College of Physicians and Surgeons (Canada) Detweiler Travelling Fellow.

This article has been peer reviewed.

Competing interests: None declared.

References