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Original Research

Clinical Assessment of Urinary Tract Damage during Sustained-Release Estrogen Supplementation in Mice

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Estrogen supplementation is a key component of numerous mouse research models but can adversely affect the urinary system. The goal of this study was to develop a clinical scoring system and identify biomarkers of occult urinary tract lesions prior to the development of systemic illness in mice. Ovariectomized or sham-surgery SCID mice were implanted subcutaneously with a placebo pellet or one containing sustained-release estradiol (0.18 mg 60-d release 17β-estradiol). Mice were assessed twice weekly for 4 to 6 wk by using a clinical scoring system that included body condition, general activity, posture, hair coat, hydration, abdominal distension, urine staining of coat and skin, and ability to urinate. Samples were collected weekly for urinalysis, BUN, creatinine, and serum estradiol levels. Terminal samples were analyzed for histopathologic lesions. Compared with placebo controls, estradiol-supplemented mice had higher serum estradiol levels at weeks 2 and 3; significant differences in total clinical scores by the 3-wk time point; and in body condition, general activity, posture, hair coat, and urine staining scores by the 6-wk terminal time point. Urinary tract lesions included hydronephrosis, pyelonephritis, cystitis, and urolithiasis. All mice with urolithiasis had crystalluria, and 5 of the 6 mice with pyelonephritis or hydroureter had dilute urine (that is, specific gravity less than 1.030). However, these findings were not specific to mice with lesions. A total clinical score of 3.5 (maximum, 24) identified estradiol-supplemented mice with 83% specificity and 50% sensitivity, but no single clinical parameter, biomarker, or the total clinical score accurately predicted occult urinary tract lesions. Considering the lesions we observed, prudence is warranted when using pelleted sustained-release estradiol in mice, and important parameters to monitor for animal health include urine staining, body condition score, urine sediment, and urine specific gravity.

Estrogen supplementation is a component of several mouse research models, including those used to study reproductive health,[26,31] tumorigenesis,[26] and osteogenesis.[2,31] However, supraphysiologic supplementation in mice has been associated with unintended and off-target effects including urinary retention and obstruction,[5,48] cystitis and urolithiasis,[16,38] hydronephrosis and hydroureter,[13,29] and vesicoureteral reflux and voiding disorders in males.[33] The incidence and severity of these adverse effects can vary by dose, route and duration of administration[25,29] in addition to the mouse strain or stock, and sex.[4,5,29] Other rodent species, including hamsters and guinea pigs, seem to be less susceptible to these effects, but these interspecies differences are not well understood.[5]

Currently, only a single commercially available sustained-release pelleted 17β-estradiol product is labeled for research use in rodents. The pellets can be purchased in doses of 0.18 to 1.7 mg. These formulations are designed to achieve maximal serum levels of 700 to 900 pg/mL, which are reflective of midcycle levels in women on the day before luteinizing hormone peaks and are much higher than the average circulating estrogen level in mice.[22] One study found significant differences in a rat ischemia model when using 2 different methods (commercial pellets compared with homemade silastic capsules) of chronic estradiol administration, thus giving rise to concerns regarding research reproducibility.[45] Sustained-release estradiol pellets can have highly variable release rates, which are exacerbated by any harsh manipulation of the product,[20] and several reports cite the clinical deterioration of estradiol-pellet–supplemented mice as a cause for early study termination and the loss of data.[10,16,38] Experimental requirements in addition to practical concerns related to ease of dosing have led to the continued used of sustained-release estradiol pellets despite recognized adverse effects.

Research and animal wellbeing concerns dictate the need for the identification of markers for monitoring the development and progression of urinary tract disease in estradiol-supplemented mice. Arguably the ability to develop model-specific scoring systems to identify early stages of disease would enhance animal wellbeing and enable researchers to control for a potential experimental confounder. Here, we determined whether a novel cageside clinical scoring system identified occult urinary tract disease in estradiol-supplemented mice. In addition, we evaluated whether additional relevant clinical pathology biomarkers

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were more sensitive or specific indicators of urinary tract lesions than the proposed clinical scoring system.

Materials and Methods

Animals. All mice used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*.[21] All procedures were reviewed and approved by the IACUC prior to initiation of experiments and were completed in accordance with federal policies and guidelines. Female SCID mice (CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl; age, 8 to 10 wk; Charles River Laboratories, Portage, MI) were housed 5 to a cage in autoclaved polysulfone IVC (Allentown Caging, Allentown, NJ) on a 12:12-h light:dark cycle in a temperature- and humidity-controlled room. Mice had unlimited access to a commercial rodent diet (PicoLab Laboratory Rodent Diet 5L0D, PMI Nutrition International, Brentwood, MO), autoclaved municipal water in bottles, irradiated corncob bedding (ScottPharma Solutions, Framingham, MA), and a nesting pad (Ancare, Bellmore, NY). Colony health was evaluated quarterly by sentinel exposure to dirty bedding. All sentinels were seronegative for mouse hepatitis virus, mouse parvovirus, minute virus of mice, epizootic diarrhea of infant mice, ectromelia virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus type 3, lymphocytic choriomeningitis virus, mouse adenovirus, polyoma virus, *Mycoplasma pulmonis*, and cilia-associated respiratory bacillus. Environmental and colony animal PCR testing for fur mites and pinworms were also negative. Mice were allowed to acclimate for 1 wk prior to any experimental manipulations.

Experimental design. Cages were randomly allocated to experimental groups. Baseline urine and blood samples and clinical observations were obtained at 2 wk after arrival and prior to sham surgery or ovariectomy and dorsal subcutaneous implantation of either placebo or estradiol-containing pellets (60-d release 0.18-mg 17β-estradiol; Innovative Research of America, Sarasota, FL). For all surgeries, mice were anesthetized with isoflurane (Forene; Abbott Laboratories, Abbott Park, IL) delivered in 100% oxygen gas, and the surgical site prepared aseptically. Analgesia was provided once prior to surgery by using carprofen (5 mg/kg SC; Rimadyl, Pfizer, New York, NY). For ovariectomy and sham surgeries, flank laparotomies were performed, and the ovaries were visualized with or without subsequent resection, depending on the experimental group. A 2-layer closure was performed. For pellet placement, a small dorsal midline skin incision was made, which was closed with wound clips. Twice-weekly clinical observations and weekly sample collections were performed through postimplantation week 4 or 6, at which time necropsy was performed. Final experimental groups and numbers were: ovariectomized with estradiol supplementation and euthanized at week 4 (OVX-E4, $n = 9$); ovariectomized with estradiol supplementation and euthanized at week 6 (OVX-E6, $n = 10$); ovariectomized with placebo pellet and euthanized at week 4 (OVX-P4, $n = 9$); ovariectomized with placebo pellet and euthanized at week 6 (OVX-P6, $n = 9$); intact with estradiol supplementation and euthanized at week 4 (Sham-E4, $n = 5$); intact with estradiol supplementation and euthanized at week 6 (Sham-E6, $n = 5$); intact with placebo pellet and euthanized at week 4 (Sham-P4, $n = 5$); and intact with estradiol supplementation and euthanized at week 6 (Sham-P6, $n = 5$).

Clinical scoring system. Clinical observations were performed blindly and separately by a single veterinarian (DC) and research staff member (KM) using previously published scoring systems (body condition score, general activity level, posture, and hydration)[1,37,47] and known clinical parameters of urinary tract disease in mice (for example, abdominal swelling, ability to urinate, urine staining of fur and skin [Figure 1]). All parameters except for body condition score were evaluated on both a binary scale (0, normal; 1, abnormal) and an ordinal scale for severity (Figure 2) to evaluate interobserver replicability. To facilitate statistical analysis of the effect of urinary tract pathology on clinical scores, only the clinical score assigned by the veterinarian was used.

Hematology, serum biochemistry and serum estradiol levels. Blood (250 µL) was collected from the facial vein antemortem or by cardiocentesis postmortem into microtainer EDTA and serum-separator tubes (Becton Dickinson, Franklin Lakes, NJ) for hematology (HemaVet 950, Drew Scientific, Miami Lakes, FL), creatinine, and BUN levels (LIASYS 330 Analyzer, AMS Diagnostics, Weston, FL) and serum estradiol quantitative determination (Calbiotech, Spring Valley, CA). For hematology and serum biochemistry evaluation, vendor-provided colony animal reference values (female CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl mice; age, 8 to 10 wk; Charles River Laboratories) were used.[7] Small-volume samples were diluted 1:1 with normal saline before analysis. Terminal serum samples for BUN and creatinine analysis from the Sham-E6 group were not processed due to inappropriate sample storage conditions after collection.

Urinalysis. Urine was collected once each week by using manual expression, as previously described.[28] Specific gravity was assessed by using a veterinary refractometer for samples with appropriate volume (at least 60 µL) or by Multistix 10 SG (Siemens Healthcare, Malvern, PA) for samples of less than 60 µL.[12] Analysis was prioritized on the basis of volume in the order of specific gravity, pH, and microscopic sediment exam (Figure 3).

Necropsy and histopathology. Mice were euthanized at study endpoint by CO_2 asphyxiation followed by thoracotomy to induce a pneumothorax. Necropsy was performed, and urinary stones, if present, were collected and submitted for analysis to the Minnesota Urolith Center (College of Veterinary Medicine, University of Minnesota, St Paul, MN). Urinary tract tissues (kidneys, ureters, bladder, and urethra) were collected, fixed in 10% neutral buffered formalin, trimmed, routinely processed, paraffin-embedded, sectioned at 4 µm, and stained with hematoxylin and eosin. Lesions were scored according to severity grade (0, no lesion; 1, minimal; 2, mild; 3, moderate; and 4, marked severity) for inflammatory lesions (pyelonephritis or tubulointerstitial nephritis, ureteritis, cystitis, and urethritis) and lesions due to obstruction (hydronephrosis, hydroureter) by a board-certified veterinary pathologist (MH) who was blinded in regard to experimental group. Hucker–Twort Gram staining was performed on all slides that showed bacteria during histology.

Statistical analysis. Two-way ANOVA and Tukey multiple-comparison posthoc analysis were used to evaluate differences among groups for histopathology lesion severity scoring and among time points for serum estradiol levels and clinical scoring parameters. The Fisher exact test was used to analyze incidence data for histopathology (lesion present or absent) and urine specific gravity (concentrated or dilute). For direct comparison across groups, one-way ANOVA was used. These analyses were performed by using Prism (GraphPad, La Jolla, CA). Linear regression was used to evaluate differences among baseline and posttreatment values for all serum biochemical and urinalysis parameters. κ statistics were performed for ordinal and binary clinical scoring param-

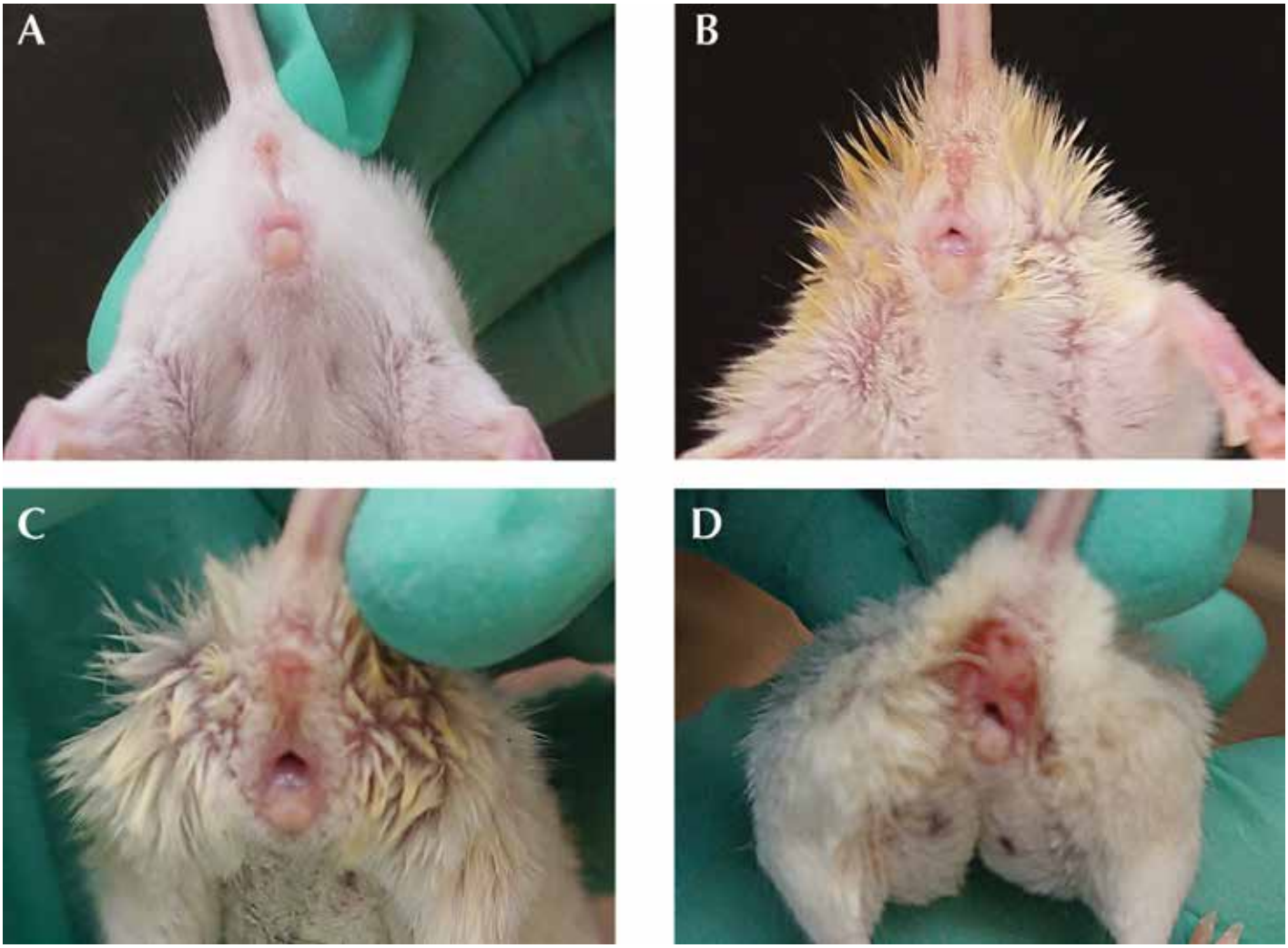

Figure 1. Examples of severity scoring for urine staining. (A) Normal animal with no urinary staining or perineal epithelial injury (score, 0). (B) Mild urinary staining with no lesion of underlying epithelium (score, 1). (C) Urinary staining with epithelium hyperemia and alopecia (score, 2). (D) Urine staining with ulceration and abrasion of perineal epithelium (score, 3).

eters to evaluate interobserver reliability, with composite κ scores constructed by using inverse variance weighting for all ratings through postimplantation weeks 4 and 6. These analyses were performed by using Statistical Analysis Software (SAS Institute, Cary, NC). For all statistical analyses, α was set at 0.05 for the level of significance.

Results

Estradiol levels. Compared with placebo-supplemented mice, estradiol-supplemented mice had significantly ($P < 0.05$) higher serum estradiol levels at weeks 2 and 3, with peak plasma concentration at week 2 (Figure 4). By week 4, serum estradiol was significantly ($P < 0.05$) higher in the OVX-E4 and OVX-E6 groups than in all nonsupplemented mice, but levels in the Sham-E4 and Sham-E6 mice were only significantly higher than those of the Sham-P4 and Sham-P6 mice. A return to consistent physiologic levels across groups did not occur until week 5.

Clinical scoring system. Figure 5 illustrates clinical observations by the veterinarian for all treatment groups over time. There were no significant differences between any of the groups in terms of hydration status or abdominal distension as scores greater than 0 in these categories were rare. Ability to urinate showed the greatest variability between successive scores, thus precluding statistical significance (data not shown). From week 3 onward, Sham-E4 mice had significantly higher ($P < 0.05$) total clinical scores than did OVX-P4 and Sham-P4 mice (Figure 5 A). These differences were driven by higher scores for posture (Figure 5 D), hair coat (Figure 5 E), and urine staining of fur and skin (Figure 5 F). In addition, by week 6, the Sham-E6 group had significantly ($P < 0.05$) higher total clinical scores than did the OVX-P6 and Sham-P6 mice (Figure 5 G). These differences were driven by increases in the body condition score (Figure 5 H), general activity (Figure 5 I), posture (Figure 5 J), hair coat (Figure 5 K), and urinary staining (Figure 5 L). Compared with OVX-P6 and Sham-P6 mice, the OVX-E6 group had significantly ($P < 0.05$) greater urine staining by week 6 and a transient increase in their total clinical score during week 5. Estradiol-supplemented and placebo groups were not significantly different, perhaps because of week 1 scores of 1 in a single OVX-P4 mouse and in 2 OVX-P6 mice.

General health parameters	
Body condition score (BCS)	0: BCS of 3 or greater 1: BCS of 2 2: BCS of 1
General activity	0: active curiosity 1: mild curiosity 2: loss of curiosity, decreased activity 3: active only when stimulated 4: inactive even when stimulated
Posture	0: normal 1: slightly hunched 2: moderately hunched but still able to rear 3: severely hunched and unable to rear
Hair coat	0: sleek, clean coat 1: mild scruffiness, some grooming behavior 2: obvious scruffiness 3: severe scruffiness,unkempt hair coat
Hydration	0: hydrated 1: mild dehydration, skin tent x > 1 s 2: moderate dehydration; skin tent of 1–2 s 3: severe dehydration, prolonged skin tent, enophthalmos
Urinary tract parameters	
Abdominal distension	0: normal 1: mild abdominal distension 2: moderate abdominal distension 3: abdomen severely distended and pendulous
Urine staining	0: none present 1: mild staining with no underlying irritation to the skin 2: staining with concurrent skin erythema present 3: anogenital sores present
Ability to urinate	0: bladder size normal or not palpable, easy to express 1: bladder enlarged or mildly difficult to express 2: bladder enlarged and very difficult to express 3: bladder enlarged and unable to be expressed

Figure 2. Clinical scoring parameters and scale.

	Possible results
Color	Yellow, red, brown
Clarity	Clear, slightly cloudy, cloudy
pH	5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5
Specific gravity	<1.030 or ≥1.030
No. of RBC per HPF	None, 0–5, 6–10, >10
No. of WBC per HPF	None, 0–5, 6–10, >10
No. of bacteria per HPF	None, 0-5, 5-10, >10
Crystals	Absent or present (type: triple-phosphate, calcium oxalate, or other)
Casts	Absent or present (type: granular, hyaline, waxy, or other)

HPF, high-power (40×) field

Figure 3. Categories of results for each urinary parameter analyzed.

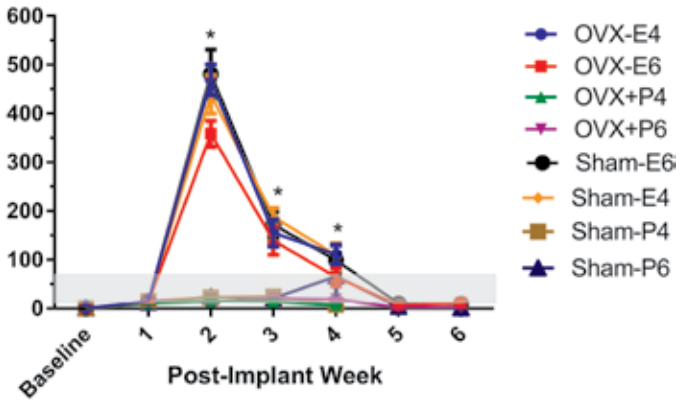

Figure 4. Serum estradiol concentrations (mean ± SEM) throughout the course of the study. The gray bar indicates the normal serum estradiol level in mice (10–60 pg/mL). *, $P < 0.05$ when compared with value for ovariectomized (OVX) or sham surgery (Sham) mice that received placebo pellets (P) at the same time point (week 4 or 6 after pellet implantation).

Interobserver agreement was used to assess the validity and reproducibility of the clinical scoring system (Table 1). When evaluated by using ordinal data, interobserver agreement was moderate ($0.41 < \kappa < 0.60$) or higher for all parameters of general health and urinary tract disease, except hair coat, posture, and ability to urinate which had fair ($0.21 < \kappa < 0.40$) interobserver agreement through postimplantation week 4. Interobserver reliability increased when these parameters were assessed until week 6 or when binary (normal compared with abnormal) rather than ordinal (0 to 3) data were evaluated. For all other parameters, the κ correlation did not increase or decrease with prolonged treatment times or the use of a binary rating system. Urine staining had substantial to excellent agreement ($\kappa > 0.61$) between observers when evaluated by using either ordinal or binary data.

Hematology, serum biochemistry, and urinalysis. The Sham-E6 group had a significantly ($P < 0.05$) decreased RBC volume compared with all other groups and a decreased ($P < 0.05$) Hct compared with all groups except Sham-E4. In addition, the final

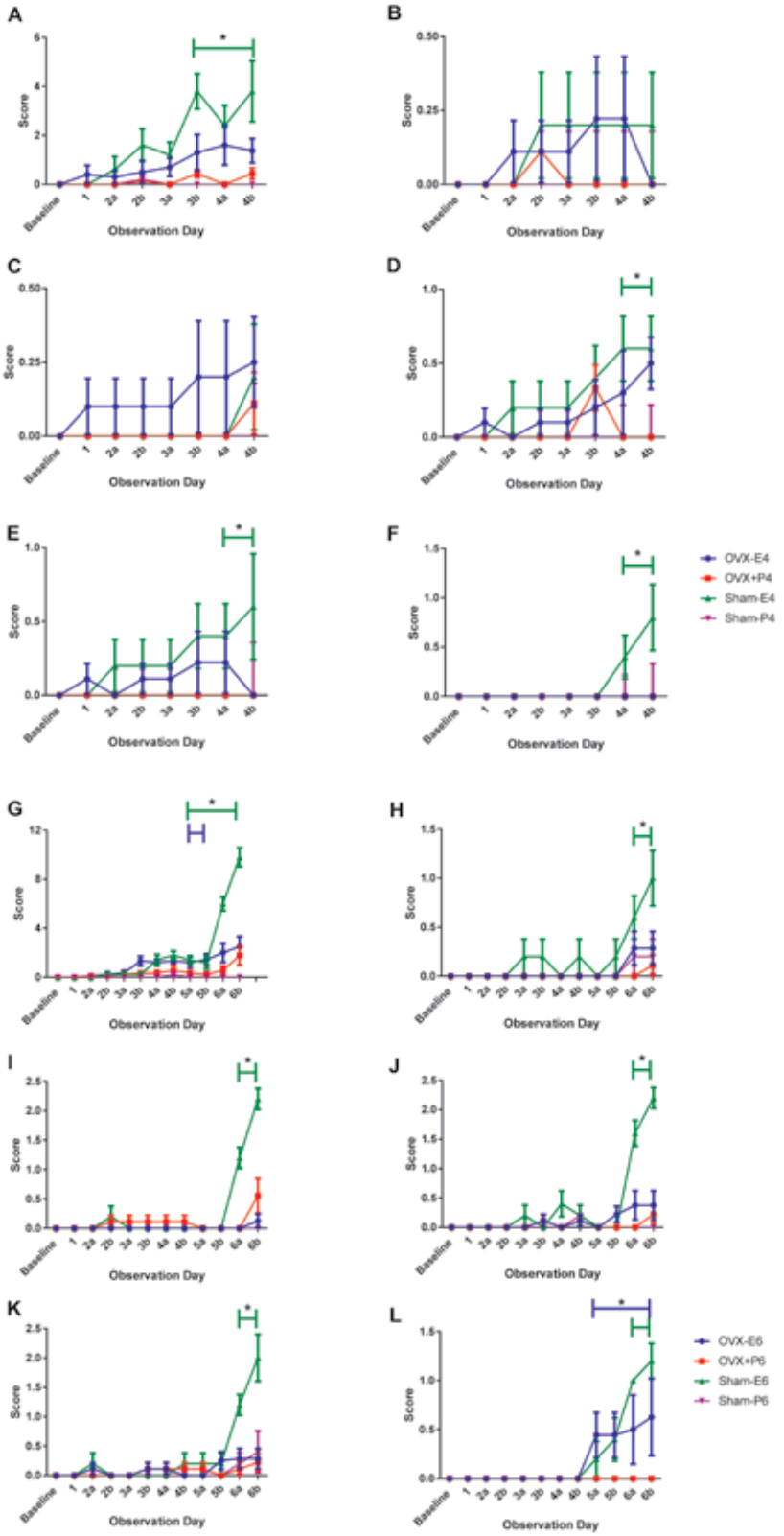

Figure 5. Longitudinal clinical scoring (mean ± SEM) by the veterinarian was performed on 2 d (a, Monday; b, Thursday) each week. Clinical scoring by the researcher is not displayed given the high interobserver correlation. Veterinarian-assigned scores are shown for the total clinical score (A and G), body condition score (BCS; B and H), general activity (C and I), posture (D and J), hair coat (E and K), and urine staining (F and L) for each group (at postimplantation weeks 4 and 6). Clinical scores for hydration, ability to urinate, and abdominal distension are not shown. Asterisks indicate *P* value of < 0.05 when compared with the OVX and Sham placebo groups at the same time point.

Table 1. Cohen κ coefficients of interobserver variability

	Assessment of ordinal data		Assessment of binary data	
	To week 4	To week 6	To week 4	To week 6
General health parameters				
Body condition score	0.58	0.66	—	—
General activity	0.44	0.44	0.5	0.5
Posture	0.38	0.4	0.4	0.4
Hair coat	0.33	0.44	0.44	0.44
Hydration	0.46	0.46	0.46	0.46
Urinary tract parameters				
Abdominal distension	0.52	0.52	0.52	0.52
Urinary staining	0.76	0.81	0.82	0.82
Ability to urinate	0.39	0.53	0.53	0.53

n = 443 and 613 observations for researcher compared with veterinarian for all observations until postimplantation week 4 or 6 respectively. The strength of agreement can be interpreted as follows: poor, κ <0.2; fair, 0.21–0.41; moderate, 0.41–0.60; substantial, 0.61–0.80; and almost perfect, >0.81.

Hgb measurement for the Sham-E6 group (11.42 ± 0.5 g/dL) was below the reference range (13.1 to 19.4 g/dL). All other hematology values were within reference ranges. Groups did not differ significantly in regard to deviations from baseline for BUN and creatinine (data not shown), and all values fell within normal limits. For the majority of animals in both estradiol-supplemented and placebo groups, urine was straw yellow and clear, with a pH range of 5.0 to 8.5. Microscopic examination of urine sediment after manual collection was within normal limits for all groups, with a range of 0 to 5 RBC, 0 to 10 WBC, and 0 to 10 bacteria per high-power (40×) field. All groups had animals with intermittent granular casts or triple-phosphate or amorphous crystals (0 to 5 per low-power field [10×]). Few animals also had single instances of waxy casts or ammonium urate crystals (0 to 5 per low-power field), which were not specific to any particular group. Linear regression analysis revealed no trends for urine pH or sediment exam according to group or individual animal. Urine specific gravity ranged from 1.008 to 1.045 and was 1.030 or higher in a majority of samples. Neither estradiol supplementation nor placebo administration had any effect on the incidence of dilute (less than 1.030) or concentrated (1.030 or greater) urine at any time point.

Histopathology. The ureter and urethra were not always present in tissue sections for evaluation; therefore, the incidence data for these tissues reflect the number of each tissue type examined (Table 2). Both kidneys and the urinary bladder were available for histologic examination from every animal. Hydronephrosis, defined as dilation of the renal pelvis associated with renal atrophy and cystic enlargement of the kidney (Figure 6 A and B), occurred in 3 OVX-E6 mice and a single OVX-P6 animal. Hydroureter, characterized by dilation of the ureteral lumen with urothelial attenuation, occurred in a single OVX-E6 animal. Primary inflammatory lesions in estradiol-supplemented mice included suppurative pyelonephritis, tubulointerstitial nephritis, cystitis, and urethritis. Pyelonephritis was characterized by a variably severe inflammatory infiltrate composed of mature and degenerate neutrophils that expanded the renal pelvis and infiltrated the urothelium, often extending into the underlying renal parenchyma (Figure 6 C), resulting in concurrent tubulointerstitial nephritis as a continuum of lesion development in kidneys. Tubulointerstitial nephritis was characterized by the presence of inflammatory cell infiltrates multifocally within renal tubules, accompanied by tubular degeneration and necrosis and extension of inflammatory cells into the renal interstitium. Within the urinary bladder, cystitis was characterized as variably severe neutrophil infiltrates within the suburothelial layers, often extending through the overlying urothelium into the bladder lumen (Figure 6 D).

Urethritis was characterized by the presence of neutrophilic infiltrates within the urethral lumen, often extending through the urothelium to the periurethral connective tissues. This response was sometimes accompanied by multifocal urothelial ulceration. Ureteritis was characterized by the presence of neutrophilic inflammatory infiltrates within the urothelium, occasionally extending to involve the connective tissues around the ureter. Inflammatory lesions in the kidney, urinary bladder, and urethra were associated with few to many colonies of gram-positive and gram-negative bacteria in all but one estradiol-treated animal; bacteria were not observed in any placebo mouse. In addition, one OVX-E6 mouse had squamous metaplasia of the urethral glands and unilateral hydronephrosis. Placebo-treated mice did not have lesions associated with obstruction or inflammation, except for one OVX-P6 mouse, which had unilateral hydronephrosis but no other notable histologic lesions within the urinary tract. Other lesions, which were of minimal to mild severity, occurred in individual mice, and were unrelated to treatment included minimal acute renal infarction (small focus of cortical necrosis with mineralization) and mild chronic renal infarction (focal renal parenchymal collapse and replacement by fibrosis), which occurred in one Sham-P6 and 2 OVX-P4 mice, respectively.

Analysis of urinary calculi. A total of 3 estradiol-supplemented mice (1 OVX-E6, 2 Sham-E6) had magnesium phosphate uroliths. All of these animals had phosphate crystalluria (1 to 5 phosphate crystals per low-power field) in the final urine sediment examination prior to necropsy. Histologically, the Sham-E6 animals had urethritis and cystitis with mixed gram-positive cocci and gram-negative rod-shaped bacteria, but there were no histologic lesions in the single OVX-E6 mouse with urolithiasis. The affected Sham-E6 animals had final total clinical scores of 9 and 10, which were some of the highest scores recorded throughout this study. Conversely, the OVE-E6 affected animal had a final total clinical score of 3.

Correlates between clinical and pathology score. Within a group, mice with urinary tract lesions at the final observation did not consistently have increased clinical scores compared with animals without histopathologic lesions (Table 2). Analy-

Table 2. Incidence of urinary tract lesions in estrogen-supplemented and placebo mice

	OVX-E4 (n = 9)	OVX-E6 (n = 10)	Sham-E4 (n = 5)	Sham-E6 (n = 5)	Sham-P4 (n = 5)	Sham-P6 (n = 5)	OVX-P4 (n = 9)	OVX-P6 (n = 9)
Kidneys								
Pyelonephritis–tubulointerstitial nephritis	2[a] (4, 4)[b]	1 (2)	0	0	0	0	0	0
Hydronephrosis	0	3 (4, 4, 4)	0	0	0	0	0	1 (4)
Ureters (no. examined)[a]	6	8	5	4	4	5	9	8
Inflammation (ureteritis)	0	0	0	0	0	0	0	0
Hydroureter	0	1 (2)	0	0	0	0	0	0
Urethra (no. examined)[a]	7	6	2	5	4	5	5	2
Inflammation (urethritis)	3 (3, 3, 1)	1 (1)	0	2 (1, 4)	0	0	0	0
Bladder								
Inflammation (cystitis)	3 (3, 3, 2)	1 (4)	0	2 (1, 4)	0	0	0	0
	Clinical score[c]							
	OVX-E1	OVX-E2	Sham-E1	Sham-E2	Sham-P1	Sham-P2	OVX-P1	OVX-P2
Mice without lesions	1.3 ± 1.7	2.2 ± 2.5	3.7 ± 2.7	8 ± 1.8	0 ± 0	0.1 ± 0.2	0.2 ± 0.3	0.7 ± 1.3
Mice with lesions	3.3 ± 2.6	2.1 ± 1.4	NA	7.8 ± 0.3	NA	0	0.5	3.5

NA, not available

Both kidneys and ureters were collected from each animal, but due to the plane of section or the small size of the tissue, areas of interest were not always present postprocessing. Therefore, the incidence data are provided for each tissue per group.

Except where noted, [a]data given are the number of mice with the lesion; [b]individual severity scores are given in parentheses

Number of mice for which target tissue was present in the section is indicated by n.

[c]Data are given as group mean ± 1 SD of the final 2 clinical scores prior to euthanasia as assessed by the veterinarian

sis of inflammatory lesion severity by tissue (row factor) and experimental group (column factor) revealed experimental group as a significant source of variation. However, there was no statistically significant interaction between the placebo and estradiol-supplemented groups, and posthoc analysis did not reveal significant differences between experimental groups for inflammatory lesion severity. In addition, neither obstructive lesion severity scores nor total lesion severity score differed between experimental groups: a P value of 0.07 was obtained when total lesion severity scores were compared across E4, E6, P4, and P6 groups regardless of surgical manipulation. When the data were analyzed by lesion incidence instead of severity, there were no significant differences in the incidence of lesions when separated by tissue distribution, surgical manipulation, or treatment groups or by lesion type (inflammatory compared with obstructive) when stratified by duration of supplementation (E4 and P4 mice compared with E6 and P6 animals).

However, comparing all estradiol-supplemented (E4 and E6) groups and all placebo-supplemented (P4 and P6) groups disclosed a significant increase in histologic lesion incidence and severity for all urinary tract lesions ($P < 0.05$) and for lower urinary tract inflammatory (cystitis and urethritis) lesions ($P < 0.05$) in the estradiol-supplemented animals compared with the placebo mice, indicating that urinary tract lesions were effective in discriminating animals with E2 supplementation. Similarly, when used as a predictive test, the clinical score discriminated between estradiol-supplemented and placebo groups at a cut-off of a total clinical score of greater than 3.5. This value was predictive of mice receiving estradiol supplementation at 50% sensitivity and 83.33% specificity. However, no such cut-off point for the prediction of urinary tract lesions could be determined on the basis of the clinical scoring system, indicating a lack of correlation between clinical scores and pathology scores.

Discussion

Similar to previously published reports,[20,46] our study showed that pelleted estradiol supplementation in mice does not reliably establish elevated serum estradiol levels for the full period of time advertised (8 wk for the product in this study). In comparison, alternative administration methods (oral administration,[20,25] nanoemulsion injection,[42] or subcutaneous silastic capsule[8,41]) result in more stable serum concentrations with fewer documented adverse effects. Compared with a previous study using the same 60-d release 0.18-mg 17β-estradiol pellet,[20] peak concentrations occurred later in our study (postimplantation week 2 compared with week 1), but the return to baseline levels was similar between studies, occurring at approximately week 5. This discrepancy could be due to the use of different serum estradiol detection methods: marked differences (more than 640%) in serum estradiol measurements can arise between different serum immunoassay kits analyzing the same sample.[44] Results from the commercial assay we used in the current study are reported to closely parallel those from gas chromatography–tandem mass spectrometry, which is considered the 'gold standard' for estradiol measurement in mouse serum.[19] In addition, we noted a delay between peak serum estradiol levels and the observation of clinical signs. Whereas peak estradiol levels occurred in supplemented ani-

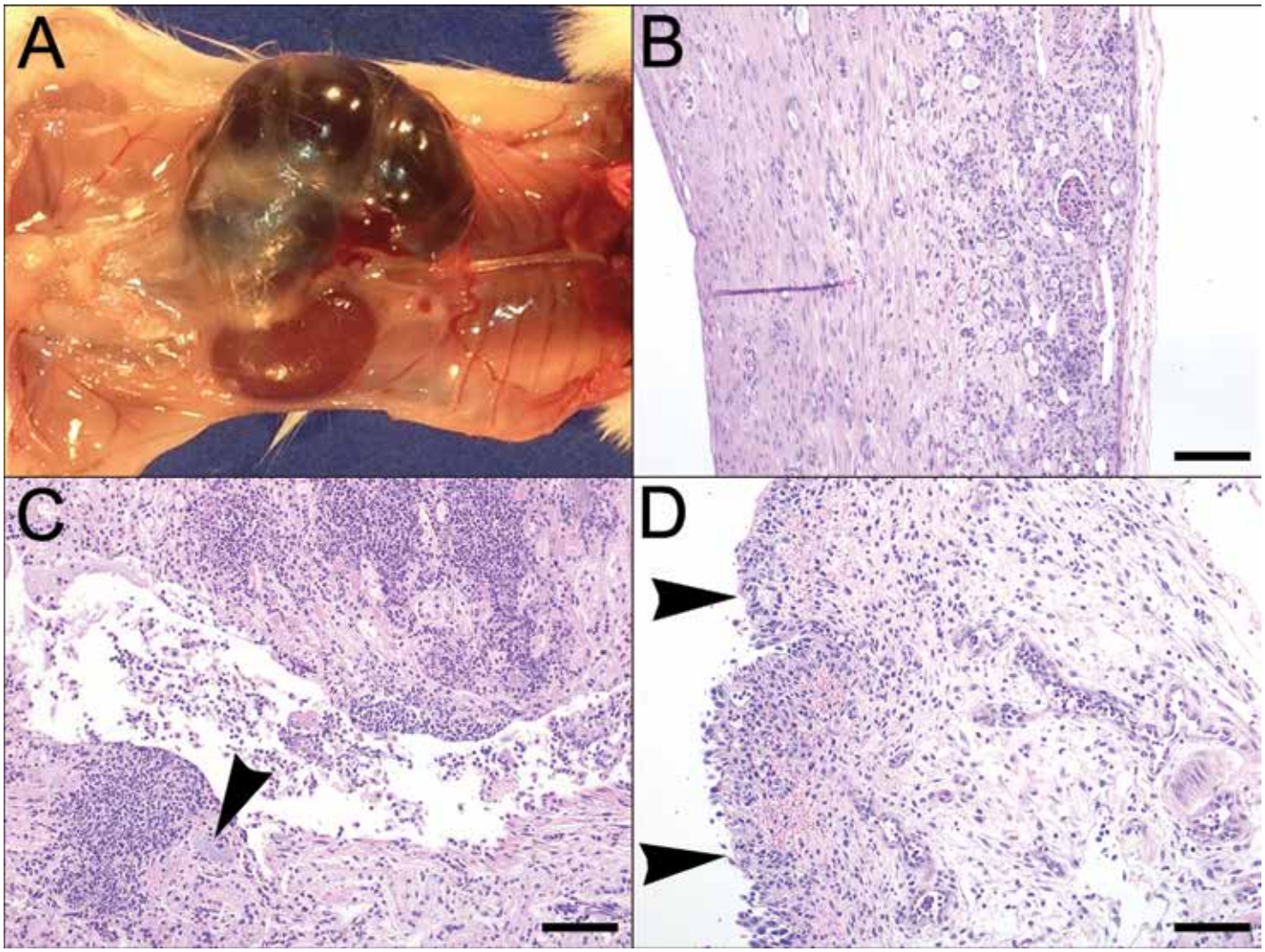

Figure 6. Select gross and histopathologic lesions in estradiol-supplemented mice. (A) Gross photograph illustrating severe unilateral cystic dilation and hemorrhage of the right kidney. (B) Photomicrograph illustrating marked renal cortical atrophy and thinning with loss of renal tubules and glomeruli and replacement by fibrous connective tissue. Magnification, 20×. (C) Suppurative pyelonephritis characterized by numerous neutrophils and sloughed cellular debris within the renal pelvis, with extension of neutrophilic infiltrates into the adjacent renal medullary parenchyma and associated bacterial colonies (arrowhead; magnification, 20×). (D) Suppurative cystitis characterized by urothelial erosion and ulceration (arrowhead) and expansion of the submucosa with edema, granulation tissue, and neutrophilic infiltrates. Magnification, 20×; bar, 50 µm.

mals at week 2, appreciable clinical signs according to behavioral scoring were not documented in most mice until week 4 to 6. This 2- to 4-wk delay indicates that even after return to baseline physiologic levels, pathologic changes (which we ultimately observed histologically) may already be present in a proportion of estradiol supplemented mice.

The urinary, serum biochemical, and hematologic markers we assayed were not predictive of estradiol administration or pathologic changes. This finding is not surprising for serum creatinine measurements, given that alterations in this enzyme do not occur until late in the disease process when approximately 75% of renal function is lost.[40] Furthermore, 2 studies on estradiol supplementation in female mice both suggest that the renal pathology is secondary to ascending lower tract infection.[13,16] Therefore elevations in BUN and creatinine likely would not be seen until late in the course of disease and may not be elevated at all in mice with lower urinary tract disease. Normal BUN levels have been documented in the face of severe urinary retention[17] and significant elevations have been observed in moribund estradiol-supplemented animals only.[48] However, although we observed no differences in urinary concentrating ability across groups, 5 of the 6 mice with hydronephrosis or pyelonephritis–tubulointerstitial nephritis had dilute urine that ranged in specific gravity from 1.008 to 1.025 at final collection. These results indicate that alterations in urine specific gravity may be a sensitive but non-specific indicator of urinary tract disease. Urine specific gravity must be interpreted with caution, given that urinary concentrating ability can fluctuate greatly with regard to various biologic factors like circadian rhythm, and hydration status.[36] Other, more sensitive biomarkers of kidney disease such as kidney injury molecule 1 (KIM1)[40] or symmetric dimethylarginine (SDMA)[3,32] should be considered for future studies to detect upper urinary tract disease earlier in the clinical course. The levels of these markers might correlate more accurately with pathology findings in estradiol

supplemented mice. However, their use as clinical monitoring tools is limited due to lack of widespread availability as rapid in-house diagnostic tests.

With regard to hematology, only the Sham-E6 group had microcytic anemia, even though serum estradiol levels were comparable across all supplemented groups. Microcytic anemia (decreased Hct and reduced RBC volume) due to estrogen-induced suppression of erythropoietin production and direct suppression of hematopoiesis within the bone marrow is well characterized.[9,30,39] Perhaps the Sham-E6 mice were in a prolonged proestrus, during which endogenous circulating levels are at a peak. This endogenous difference might have been sufficiently large to cause a physiologic response but not a statistically significant difference when compared with other estradiol supplemented groups.

Regarding our clinical scoring system, the fair to substantial interobserver agreement obtained for all parameters indicates that the scoring criteria were reliable and reproducible. We hypothesized that the ability to urinate scores would provide a reliable clinical parameter, given prior papers that cite consistently increased bladder size and pressure in estradiol treated mice.[11,33] However, evaluation of this parameter was confounded in that the mouse typically completely voided during the first examination, thus complicating evaluation by the second observer and resulting in a low κ coefficient. For all other parameters, discrepancies in observational scores were often for very low categorical scores. For example, regarding a mouse's general activity, the researcher might have assigned a score of 0, whereas the veterinarian assigned a score of 1. Conversely, for posture scoring, often the researcher scored the degree of kyphosis at a higher level than did the veterinarian. We attribute these discrepancies to differences in observer experience regarding the range of severity in rodent clinical disease presentations. However, interobserver correlation was greater with worsening clinical disease (data not shown).

In the current study, pathologic changes within the urinary tract were similar to what has been previously reported in estradiol supplemented mice.[13,16,29,38] In general, pathology successfully predicted which mice were estradiol-supplemented. Only those animals with supplementation developed inflammatory lesions (Table 2). However, lesions varied between estradiol-supplemented groups. For example, OVX-E groups developed upper urinary tract lesions (pyelonephritis–tubulointerstitial nephritis and hydronephrosis) and lower urinary tract lesions (urethritis, cystitis), whereas Sham-E6 animals had only lower urinary tract lesions with urolithiasis. Furthermore, only a subset of estradiol-supplemented animals (1 to 3 affected per group) developed lesions rather than whole cohorts, suggesting biologic variation in susceptibility between animals.

The outcome of similar clinical scores across groups despite significant differences in pathologic lesions precluded determining a predictive threshold score to identify estradiol-supplemented animals with urinary tract lesions according to the proposed clinical scoring system. One potential explanation for this result is individual animal susceptibility to estrogen-induced urinary tract disease. Susceptibility to estrogen effects can be dose-dependent[31,43] and influenced by relative estrogen receptor expression (α and β), concentration, and tissue distribution.[27] We used the lowest dose of 60-d release pellet currently available, and higher doses might be required to produce pathologic changes in the majority of estradiol-supplemented mice.[18,24,29] No prior studies regarding the urinary effects of estrogen have been published for SCID mice, but other studies have demonstrated intrastrain differences in urinary tract disease presentation secondary to estrogen supplementation. In 2 previous studies using the same estradiol pellet (60-d release 1.7 mg 17β-estradiol) in the same strain (athymic nude mice), mice in one study developed hydroureter and hydronephrosis, whereas those in the other developed cystitis, urolithiasis, and pyelonephritis.[13,16] This difference suggests that study-specific parameters might also affect the susceptibility of a cohort of animals to varying urinary tract lesions secondary to estradiol supplementation. Despite the presence of a variety of potential confounding factors, we believe that the primary limitation in our current study is the small sample size of each group. This factor likely resulted in a type 2 statistical error and an inability to recognize differences in clinical scores for animals with and without histopathologic evidence of urinary tract disease.

Micturition is a highly complex process that involves the coordination of local and CNS inputs in addition to hormonal influences including estrogens.[34,35] Estrogen supplementation is a therapeutic intervention for incontinence in postmenopausal women and spayed dogs.[35] Mechanistically, studies support the local suppression of inducible neuronal nitric oxide due to exogenous estrogen supplementation leading to increased urethral sphincter tone, reduced urination frequency, and urine retention.[6,14,15] Others have suggested structural or physiologic effects of estrogen on the urinary bladder, thus decreasing its contractile ability.[11,27] Finally, urethral gland squamous metaplasia, a novel pathologic finding in one of our estradiol-supplemented mice with unilateral hydronephrosis, suggests that pathologic changes in the urethral gland may contribute to urinary retention. The significance of this pathologic finding is difficult to assess because, for most animals, urethral glands were not in the tissue sections evaluated. Urethral gland squamous metaplasia has been observed in women with local, vaginal estrogen supplementation,[23] and one can hypothesize that urethral gland pathology might cause functional dysregulation leading to urine retention. Whatever the mechanism, our studies indicate that supraphysiologic estrogen supplementation has long-term effects, given that clinical scores were increased in supplemented mice both with and without lesions even after return to normal physiologic estradiol levels.

In conclusion, our results indicate that mice being implanted with pelleted sustained-release estradiol should be evaluated frequently at the first sign of urinary retention, because our study indicates that their clinical condition can change rapidly. Parameters that were significantly different between groups, such as urine staining and body condition score, can be used as clinical monitoring tools.

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