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Original Article

The impact of short term, long term and intermittent *E. coli* infection on male C57BL/6J mouse prostate histology and urinary physiology

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Abstract: Prostatic inflammation and prostatic fibrosis are associated with lower urinary tract dysfunction in men. Prostatic inflammation arising from a transurethral uropathogenic *E. coli* infection is sufficient to increase prostatic collagen content in male mice. It is not known whether and how the sequence, duration and chronology of prostatic infection influence urinary function, prostatic inflammation and collagen content. We placed a transurethral catheter into adult male C57BL/6J mice to deliver uropathogenic *E. coli* UTI189 two-weeks prior to study endpoint (to evaluate the short-term impact of infection), 10-weeks prior to study endpoint (to evaluate the long-term impact of infection), or two-, six-, and ten-weeks prior to endpoint (to evaluate the impact of repeated intermittent infection). Mice were catheterized the same number of times across all experimental groups and instilled with sterile saline when not instilled with *E. coli* to control for the variable of catheterization. We measured bacterial load in free catch urine, body weight and weight of bladder and dorsal prostate; prostatic density of leukocytes, collagen and procollagen 1A1 producing cells, and urinary function. Transurethral *E. coli* instillation caused more severe and persistent bacteriuria in mice with a history of one or more transurethral instillations of sterile saline or *E. coli*. Repeated intermittent infections resulted in a greater relative bladder wet weight than single infections. However, voiding function, as measured by the void spot assay, and the density of collagen and ProCOL1A1+ cells in dorsal prostate tissue sections did not significantly differ among infection groups. The density of CD45+ leukocytes was greater in the dorsal prostate of mice infected two weeks prior to study endpoint but not in other infection groups compared to uninfected controls.

Keywords: *E. coli*, lower urinary tract infection, urethral catheterization, voiding dysfunction

Introduction

Americans live longer today than in previous decades, necessitating new research to identify and mitigate factors contributing to aging-related diseases, including lower urinary tract symptoms (LUTS) arising from lower urinary tract dysfunction. In 2030 it is predicted that over 20 million octogenarians will be living in the United States; over five million will be men experiencing LUTS [1]. Though many drugs are approved for male LUTS [2], some men never respond, and others respond initially but later fail therapy [3]. Without adequate treatment, lower urinary tract diseases associated with LUTS can worsen to include urinary retention, recurrent urinary tract infection, bladder calculi, and renal impairment [1, 4].

New evidence suggests male LUTS arise from multifactorial origins, with prostatic inflammation and fibrosis emerging as key drivers [5]. Histologically defined prostatic inflammation is common. A study of prostatic biopsy cores from over 8000 men identified acute inflammation in 15% and chronic inflammation in 78% of specimens [6]. Prostatic inflammation is also strongly associated with LUTS severity, especially among men with chronic prostatic inflammation [6]. Prostatic inflammation associates with col-

lagen accumulation and urinary dysfunction [7-12]. Prostatic tissue is stiffer and has higher collagen content in men with LUTS than in men without LUTS [13].

A single uropathogenic *E. coli* infection of the mouse prostate is sufficient to increase prostatic collagen content, including *Col1a1*, *Col1a2*, and *Col3a* isoforms [14, 15]. While previous studies involving *E. coli* infection of male mouse prostate examined responses to a single infection, whether repeated intermittent infection yields the fibrotic outcomes like that of a single infection has not been examined. This is noteworthy because men with a previous diagnosis of prostatitis, a clinical form of prostate inflammation, are likely to experience repeated episodes [16].

The goal of this study was to compare severity and duration of infection and prostate fibrosis in adult male C57BL/6J mice, two weeks after a single transurethral *E. coli* infection, ten weeks after a single transurethral UTI89 *E. coli* infection, or two weeks after three infections, each separated by two weeks. Protocols were designed so that all mice were the same age at study endpoint and all mice received the same total number of catheterizations and instillations with either sterile saline (control) or saline containing *E. coli*. We examined how the sequence, duration and chronology of prostate infection influences the severity of infection, urinary function, prostatic inflammation and collagen content. We found that *E. coli* persisted in the urine for a longer period and bacteriuria was more severe in mice that had been previously catheterized and instilled with either sterile saline or *E. coli* compared to mice that had not been previously catheterized before *E. coli* infection. We also found that repeated intermittent infections resulted in a greater relative bladder wet weight than single infections. However, voiding function, as measured by the void spot assay, and the density of collagen and ProCOL1A1+ in dorsal prostate tissue sections did not significantly differ among groups. CD45+ leukocytes are more frequent in the dorsal prostate of mice infected two weeks prior to study endpoint than in other infection groups compared to uninfected controls.

Materials and methods

Mice

All experiments were conducted under an approved protocol from the University of Wisconsin Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in Udel® Polysulfone microisolator cages on racks or in Innocage® disposable mouse cages on an Innorack®; room lighting was maintained on 12 h light and dark cycles; room temperature was typically 20.5 ± 5°C; humidity was 30-70%. Mice were fed 8604 Teklad Rodent Diet (Harlan Laboratories, Madison WI) and feed and water were available ad libitum. Cages contained corn cob bedding.

All mice were sexually mature adult male wild-type C57BL/6J mice purchased from purchased from Jackson Laboratories (Stock #000664, Bar Harbor, ME) and transurethrally catheterized at eight weeks of age.

E. coli

Experiments involved *E. coli* UTI89 [17], a strain of uropathogenic *Escherichia coli* (UPEC) recovered from a human patient with cystitis [18] and transformed with a pCOMGFP plasmid [19] to confer green fluorescent protein expression and kanamycin resistance. Prior to inoculation, *E. coli* UTI89 were grown as a static culture for 18 hours in antibiotic free Luria-Bertani broth at 37°C. The optical density (OD) was determined prior to mouse inoculation. The culture was centrifugated for 15 minutes at 1157 rcf and the resulting pellet was resuspended into sterile PBS for instillation.

Transurethral instillation of sterile saline or E. coli

The 10-week experimental protocol began at 8 weeks of age and continued until 18 weeks of age. Starting at eight weeks of age, C57BL/6J male mice were anesthetized with isoflurane and a transurethral catheter (PE-10) was placed to instill sterile phosphate buffered saline (sterile PBS, control) or PBS containing *E. coli* UTI89 as previously described [15, 20]. All mice were catheterized a total of five times, twice during week 1, once during week 4, and twice during week 8. See **Figure 1** for the study timeline. The first exposure to *E. coli* for all infected mice involved two transurethral instillations given 48 h apart to prime infection. Uninfected control mice were transurethrally instilled with sterile saline twice on week one, once on week 4 and twice on week 8. To examine the long-term impact of infection (LT group), mice were trans-

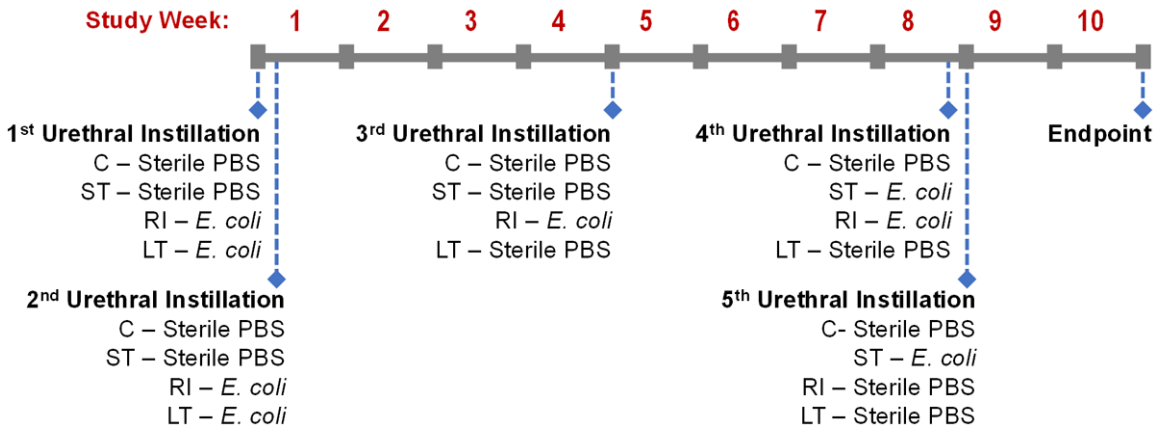

Figure 1. Study Design. All mice were catheterized five times, twice during week 1, once during week 4, and twice during week 8. The first *E. coli* exposure for all infection groups involved two transurethral instillations 48 h apart. For the long-term (LT) and repeated intermittent (RI) infection groups, the first *E. coli* exposure was on week one and for the short-term (ST) group, the first *E. coli* exposure was on week 8. The last two *E. coli* exposures for the RI group were single transurethral instillations. Sterile phosphate buffered saline (PBS) was delivered by a transurethral catheter at all time points when *E. coli* was not administered.

urethrally instilled with *E. coli* twice on week one, once per week with sterile PBS on weeks 4, and twice with sterile PBS on week 8. To examine the impact of repeated intermittent infection (RI group), mice were instilled with *E. coli* twice on week 1, and once on week 4 and week 8. To examine the short-term impact of infection (ST group), mice were instilled with sterile saline twice on week one, once on week 4 and twice with *E. coli* on week 8.

Free catch urine culture

Free catch urine culture was performed as previously described [15]. Mice were positioned above a sterile petri dish and the bladder expressed. Collected urine was serially diluted in sterile PBS and plated on Luria-Bertani (LB) agar containing kanamycin (KAN; 50 μg/mL). Colony forming units (CFU) were calculated per mL of urine.

Urinary function testing

Void spot assay (VSA) was performed and analyzed as previously described [21]. We followed the recommended guidelines of reporting VSA data [22-24]. VSA was performed in the vivarium where mice were housed. Whatman grade 540 (Fisher Scientific 057163-W) filter papers (27 × 16 cm) were placed in the bottom of Udel® Polysulfone microisolator cages. Mice were singly housed with food *ad libitum* but no water for four hours from 8 AM-12 PM GMT. Filter papers were dried and imaged with an Autochemi AC1 Darkroom ultraviolet imaging cabinet (UVP, Upland, CA) equipped with an Auto Chemi Zoom lens 2UV and an epi-illuminator. Image capture settings were adjusted using UVP VisonWorksLS image acquisition software. Images were captured using an Ethidium Bromide filter set (570-640 nm) and 365 nm epi-illumination. Void Whizzard was downloaded from http://imagej.net/Void_Whizzard and run according to the user guide [24]. Analyzed parameters included total spot count, total void area (cm^2), % area in center of paper, % area in corners of paper, and mass distribution of spots (0-0.1, 0.1-0.25, 0.25-0.5, 0.5-1, 1-2, 2-3, 3-4, 4+ cm^2).

Tissue preparation

Lower urinary tracts were collected for histology at week ten of the study. Tissues were prepared, fixed, and sectioned as described previously [15, 25-27].

Collagen quantification with picrosirius red staining

Picrosirius red staining (PSR), fluorescent imaging and quantification was performed as previously described [28]. Stained tissue sections were cleared with xylene and mounted with Richard-Allan toluene-based mounting medium. PSR staining was then imaged using Eclipse E600 compound microscope (Nikon Instruments Inc., Melville, NY) fitted with a 20x dry objective (Plan Fluor NA = 0.75; Nikon,

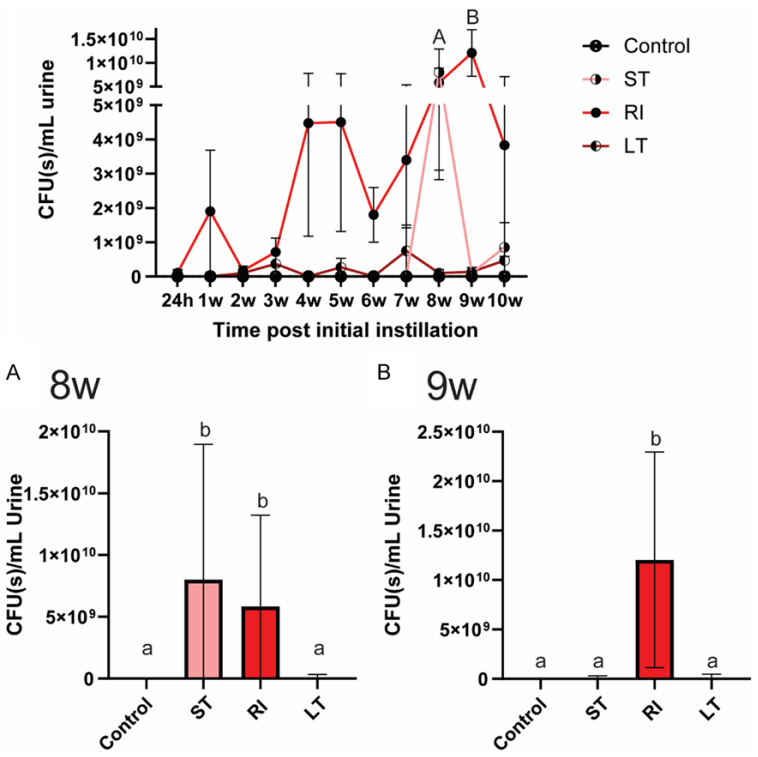

Figure 2. Impact of timing and frequency of transurethral *E. coli* instillation on urine bacterial load. Mice were catheterized once at time zero, once at 48 h, once at four weeks, and twice at eight weeks. See **Figure 1** for timing of sterile phosphate buffered saline or *E. coli* instillation. Free catch urine was collected weekly and plated on kanamycin agar plates to determine colony forming units (CFU) per mL of urine. (A) Differences among groups (six mice per group) were determined using two-way ANOVA followed by Tukey's multiple comparisons test. $P < 0.05$ was considered significant. There was a significant difference in CFUs over time ($P = 0.0053$) and between groups ($P < 0.0001$) and significant interaction between the variables of time and group ($P = 0.0062$). The short term (ST) and repeated intermittent (RI) infection groups had more CFUs per mL of free catch urine at 8 weeks than the long term (LT) infection and uninfected control groups (Tukey's multiple comparison test $P = 0.0008$ to 0.0009 and $P = 0.0167$ to 0.0194 respectively). (B) The RI infection group had more CFUs per mL of free catch urine at 9 weeks than all other groups (Tukey's multiple comparison test $P < 0.0001$). In (A) and (B) groups with a statistically similar CFU(s)/mL value are denoted with the same lower-case letter (ex. "a").

Melville, NY) and equipped with NIS elements imaging software (Nikon Instruments Inc.). Fluorescence was detected using FITC, and Texas Red (Chroma Technology Corp, Bellows Fall, VT) filter cubes (Nikon, Melville, NY). Specific collagen staining was determined by subtracting the tissue autofluorescence (FICZ channel) from the PSR fluorescence (Texas Red channel) using the "image calculator" function of ImageJ. Images were analyzed using publicly accessible CT FIRE software developed at the University of Wisconsin-Madison [29]. This software detects and quantifies fiber-like structures in images. For this study, fiber density was measured.

Immunohistochemistry and cell counting

Immunohistochemistry (IHC) was conducted on dorsal prostate tissue sections from mice in all four groups. Tissue sections were deparaffinized with xylene and hydrated through a series of graded ethanol solutions. Tissues were immersed in citrate buffer pH 6.0 heated in a microwave for epitope decloaking. Tris-buffered saline containing 0.1% Tween 20 and 5% donkey serum was used as a blocking reagent, and primary and secondary antibodies were diluted in blocking reagent. Antibodies and dilutions are: procollagen type 1a1 (ProCOL1A1, Developmental Studies Hybridoma Bank, SP1.D8-c (concentrated, 238 µg/mL), 1:1000), protein tyrosine phosphatase, receptor type C (PTPRC, also known as CD45, Abcam, AB10558, RRID: AB 442810, 1:500), Anti-Goat 488 (Jackson ImmunoResearch, 705-545-003, RRID: AB_2340428, 1:250), Anti-Rabbit Cy3 (Jackson ImmunoResearch, 711-165-152, RRID: AB_2307443, 1:250), Anti-Mouse 647 (Jackson ImmunoResearch, 715-605-150, RRID: AB_2340846, 1:250), and DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine). Results validating specificity of antibody detection are available here: https://doi.org/10.25548/17-3ZTY [30]. Tissue sections were imaged using an Eclipse E600 compound microscope (Nikon Instruments Inc., Melville, NY) fitted with a 20x dry objective (Plan Fluor NA = 0.75; Nikon, Melville, NY) and equipped with NIS elements imaging software (Nikon Instruments Inc.). Fluorescence was detected using DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine), FITC, Texas Red (Chroma Technology Corp, Bellows Fall, VT), and CY5 filter cubes (Nikon, Melville, NY).

Collagen producing cells were defined as ProCOL1A1 immunopositive. ProCOL1A1 is the

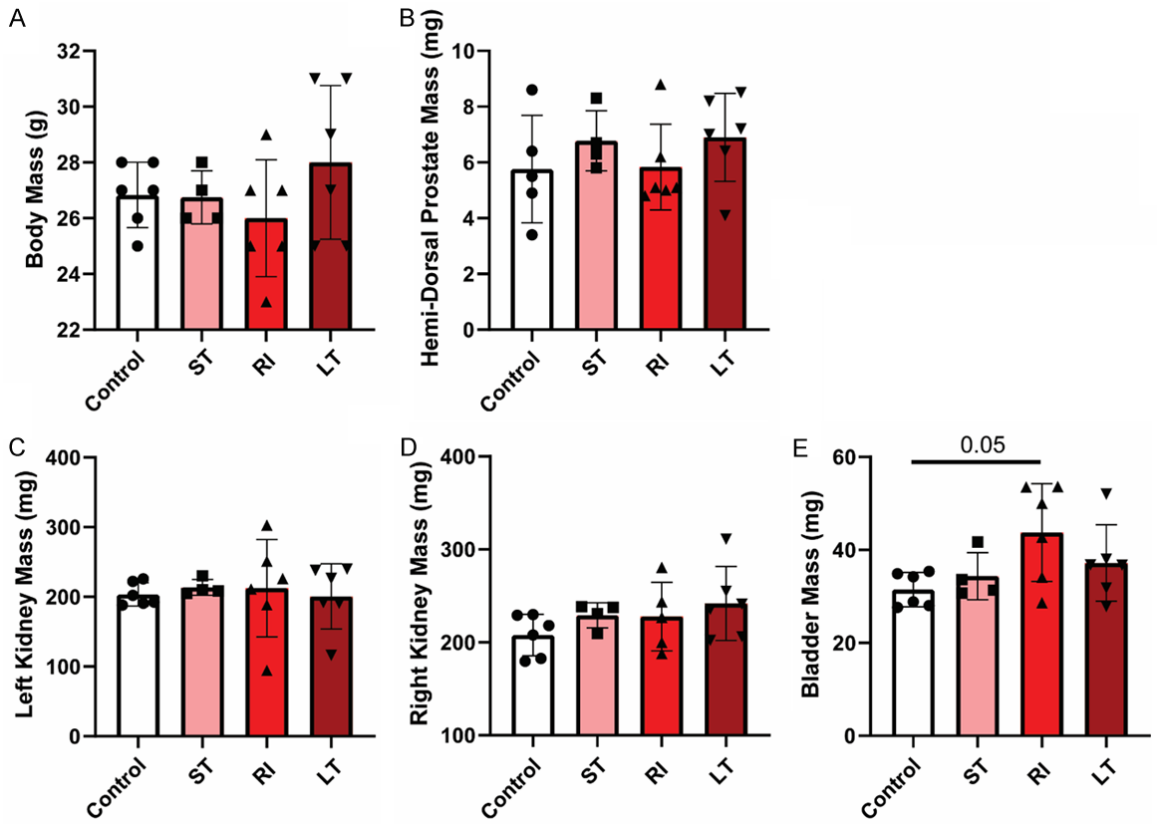

Figure 3. Impact of timing and frequency of transurethral *E. coli* instillation on genitourinary and body weights. Mice were catheterized once at time zero, once at 48 h, once at four weeks, and twice at eight weeks. See **Figure 1** for timing of sterile phosphate buffered saline or *E. coli* instillation. Differences among groups (six mice per group) were determined using one-way ANOVA in (A) and (C-E). Data in (B) were not normally distributed and were not normalized by transformation, so the Kruskal-Wallis test was applied. $P < 0.05$ was considered significant and *P*-values ≤ 0.05 are shown in graphs. (A) body mass, (B) hemi-dorsal prostate mass, (C) left kidney mass, and (D) right kidney mass did not differ between groups. (E) bladder mass was greater in the repeated intermittent infection group than the uninfected control group.

precursor to collagen 1A1, the most abundant collagen subtype in the extracellular matrix [31]. Bone marrow derived cells were defined as PTPRC immunopositive. Cells lying within blood vessels, prostate ducts, or prostate ductal epithelium were excluded from analysis. All remaining nucleated cells were counted and all cells that were partially or completely stained were considered positive. Cells were manually counted per image using Image J cell counter [32].

Statistical analysis

Statistical analyses were performed with Graph Pad Prism 8.0.2. Differences were considered significant at the $P < 0.05$ level. A Shapiro-Wilk test was used to test for normality and transformation was applied to normalize data. Bartlett's test was used to test for homogeneity of variance. For groupwise comparisons at a single timepoint, Welsh's ANOVA was applied when variance was unequal followed by Dunnett's T3 multiple comparisons test. When variance was equal, groupwise comparisons were made using ordinary one-way ANOVA followed by Tukey's multiple comparisons test. The Kruskal-Wallis test was applied when data could not be normalized through transformation. For groupwise comparisons with endpoints measured at multiple timepoints, a 2-way ANOVA was performed with Tukey's multiple comparisons test.

Results

Transurethral E. coli instillation causes more severe and persistent bacteriuria in mice with a history of one or more transurethral instillations of sterile saline or E. coli

The experimental design consisted of four groups: (1) a transurethral catheter was placed, and sterile saline infused for the uninfected

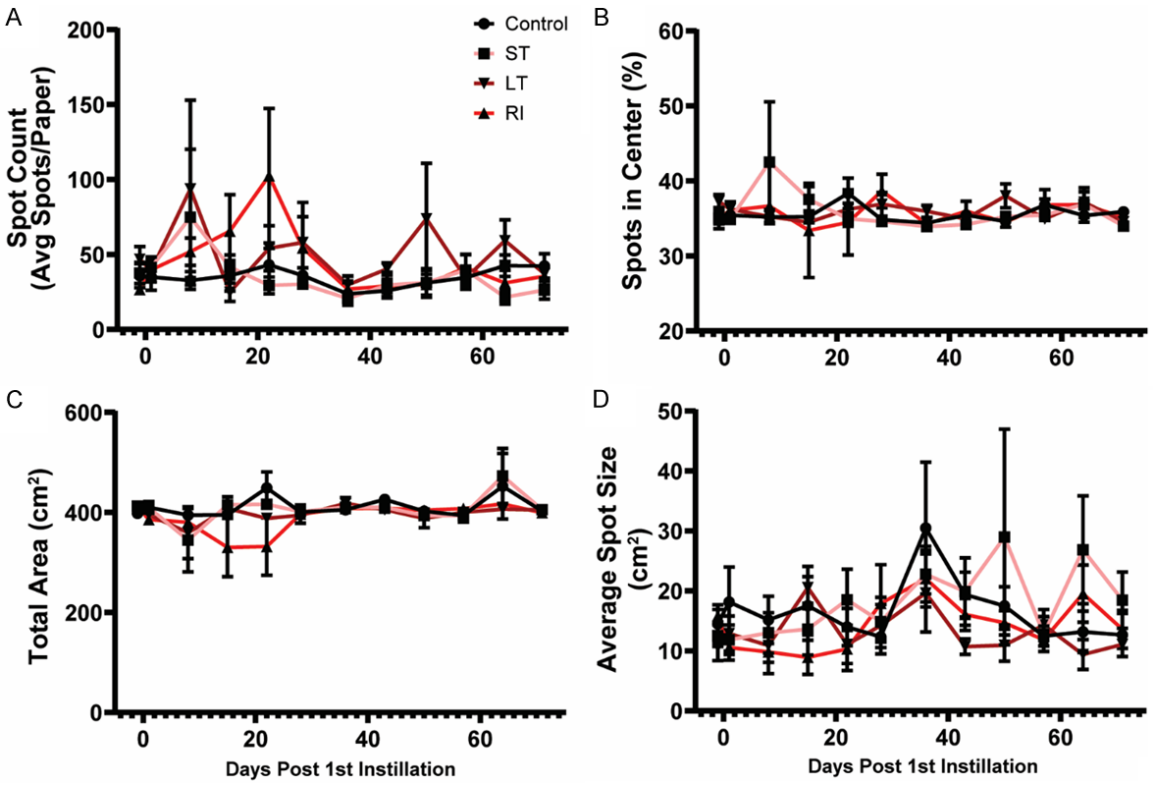

Figure 4. Impact of timing and frequency of transurethral *E. coli* instillation on urinary voiding function as measured by the void spot assay. Mice were catheterized once at time zero, once at 48 h, once at four weeks, and twice at eight weeks. See **Figure 1** for timing of sterile phosphate buffered saline or *E. coli* instillation. The void spot assay was performed weekly. Papers were dried, imaged, and analyzed using the Void Whizzard plugin for ImageJ. A two-way ANOVA was used to compare groups and $P < 0.05$ was considered significant. (A) spot count, (B) spots in center, (C) total area, and (D) average spot size did not significantly differ among groups at any timepoint.

control (C) group, or *E. coli* UTI189 was delivered (2) two-weeks prior to study endpoint for the short term (ST) group or (3) 10-weeks prior to study endpoint for the long term (LT) group, or 10-, six-, and two-weeks prior to endpoint for the repeated intermittent (RI) infection group. Mice were catheterized the same number of times across all experimental groups and instilled with sterile saline when not instilled with *E. coli* to control for the variable of catheterization. Free catch urine was collected weekly and plated on LB plates to determine colony forming units (CFU) per mL of urine. ST and RI mouse urine contained significantly more *E. coli* CFUs at study week 8 than LT and C mouse urine ($P = 0.0008$ to 0.0009 and $P = 0.0167$ to 0.0194 respectively; **Figure 2A**). RI mouse urine contained significantly more *E. coli* CFUs at study week 9 than ST, LT and uninfected control mouse urine ($P < 0.0001$ for all; **Figure 2B**). Notably, bacteriuria was observed for two weeks after the second and third infection in the RI group, but only for one week after the first infection in the RI group, or after the only infection in the ST and LT groups. These results are consistent with the notion that a previous transurethral catheterization causes more persistent bacteriuria after *E. coli* infection.

Despite greater bladder weight in the RI group than ST, LT, and uninfected control groups, there were no differences in voiding function between groups

Body mass, hemi-dorsal prostate mass, left kidney mass, and right kidney mass did not differ among groups (**Figure 3A-D**). Bladder mass was greater in the RI group than the ST, LT and uninfected groups ($P = 0.05$; **Figure 3E**). Void spot assay was performed weekly to evaluate the impact of infection on urinary voiding function. Filter papers were placed in cage bottoms for 4 hours and analyzed using the Void Whizzard. There were no differences among groups at any time point (**Figure 4**).

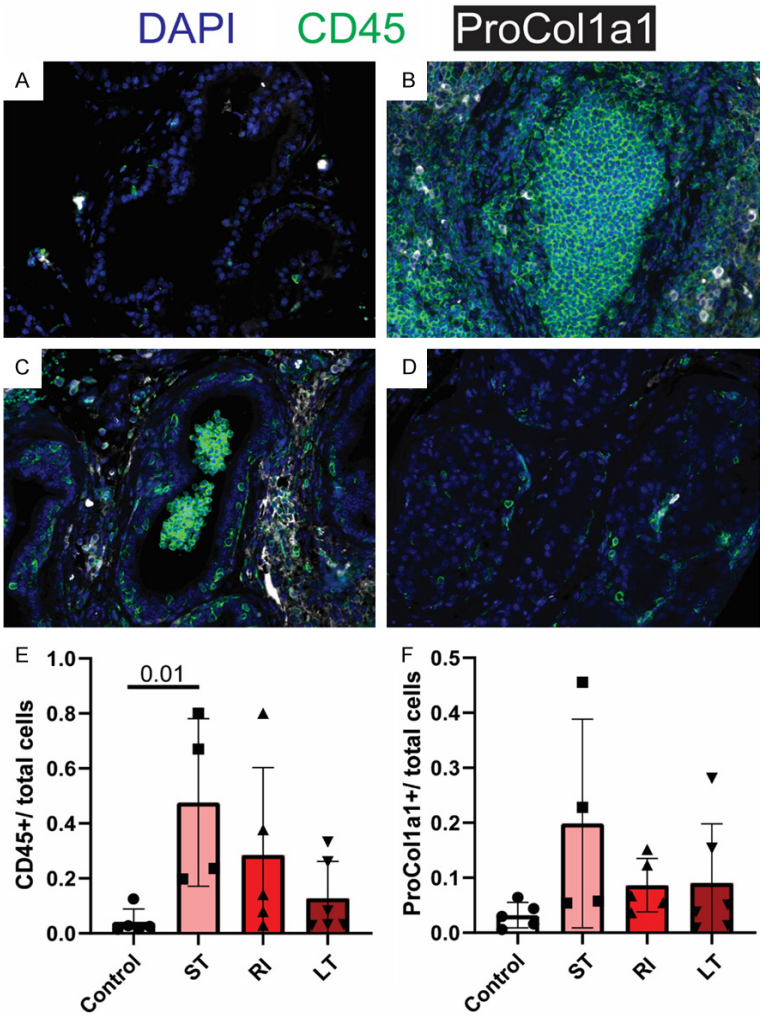

Figure 5. Impact of timing and frequency of transurethral *E. coli* instillation on dorsal prostate CD45+ leukocyte frequency (index of inflammation) and pro-collagen 1A1 (proCOL1A1) cell frequency (index of fibrosis). Mice were catheterized once at time zero, once at 48 h, once at four weeks, and twice at eight weeks. See **Figure 1** for timing of sterile phosphate buffered saline or *E. coli* instillation. Dorsal prostates were fixed, dehydrated, cleared, embedded in paraffin and cut into five-micron sections. Immunohistochemical staining for CD45 (green) and ProCOL1A1 (white) was performed. Tissue sections were also stained with DAPI (blue) to visualize cell nuclei. All slides were imaged at 200X. Representative images are from (A) the uninfected control group, (B) the short term (ST) infection group (C) the repeated intermittent (RI) infection group, (D) the long-term (LT) infection group. Differences among groups were identified using the Kruskal-Wallis test in (E) and a one-way ANOVA in (F). $P < 0.05$ was considered significant and any *P*-values ≤ 0.05 are shown in graphs.

The percentage of CD45+ cells in the dorsal prostate of ST mice (an index of infection severity) is greater than that of RI, LT and uninfected control groups at study endpoint while collagen fiber density in the dorsal prostate does not differ among groups

Immunohistochemical staining was conducted for the leukocyte marker CD45 and the collagen pro-peptide ProCOL1A1 and DAPI was used to stain nuclei and CD45+, ProCOL-1A1+, and total cell nuclei were counted (**Figure 5A-D**). The percentage of CD45+ cells in dorsal prostates of ST mice, an index of inflammation severity was greater than that of controls at study week 10 ($P = 0.01$; **Figure 5E**). The percentage of ProCOL1A1+ cells in the dorsal prostate did not differ among groups (**Figure 5F**). Picrosirius red staining was performed to mark collagen fibers. And CT-Fire was used to determine number of collagen fibers per image. Collagen fiber density in the dorsal prostate did not differ among groups (**Figure 6**). Thus, although a history of prior transurethral catheterization increased the severity and duration of bacteriuria in adult male mice, it did not increase the prostate inflammatory or fibrotic response to uropathogenic *E. coli* infection.

Discussion

Histological prostate inflammation is common in older men and occasionally observed in young men even though LUTS and LUTD are rare before the sixth decade of life. We used a mouse model of transurethral *E. coli* infection with the primary goal of understanding how timing and frequency of infection influence inflammation, prostatic collagen abundance and voiding function. We hypothesized that recurrent intermittent infections with uropathogenic *E. coli*, to model the repeated episodes of prostate inflammation experienced by some men, would result in more severe inflammation and prostate fibrosis than single infections. There was no evidence to support this hypothesis. We used CD45+ leukocyte density in the prostate as a proxy for severity of inflammation and observed that CD45+ leukocyte cell density

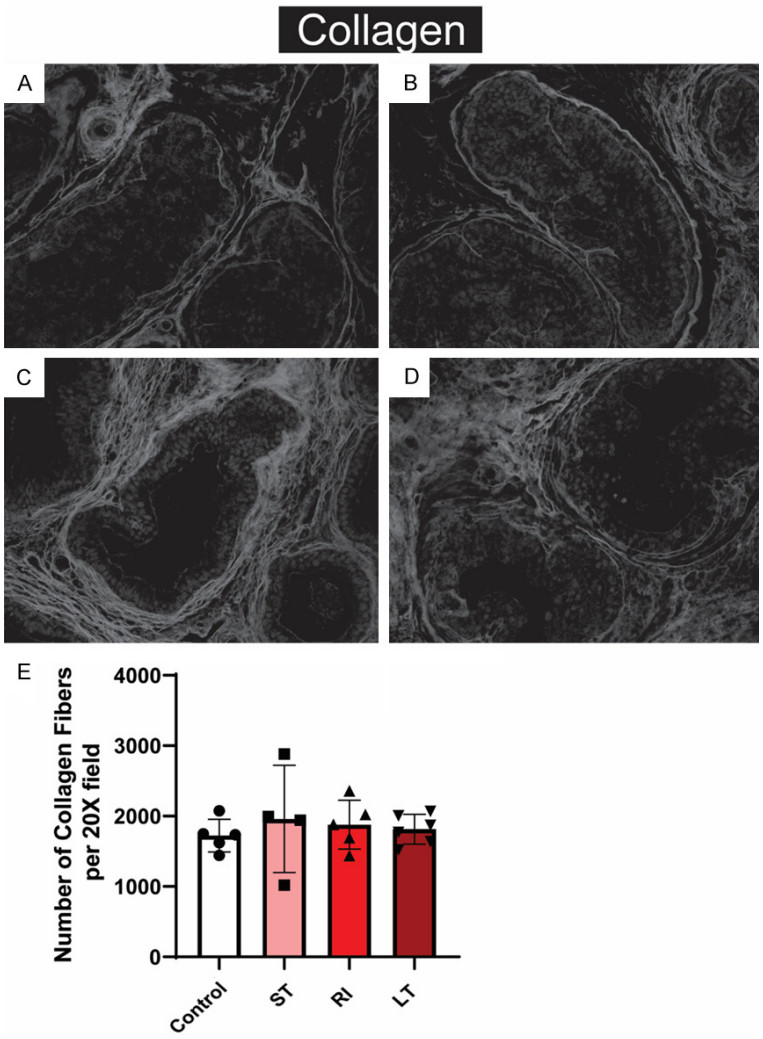

Figure 6. Impact of timing and frequency of transurethral *E. coli* instillation on picrosirius red stained collagen fibers in the dorsal prostate (index of fibrosis). Mice were catheterized once at time 0, once at 48 h, once at four weeks, and twice at eight weeks. See **Figure 1** for timing of sterile phosphate buffered saline or *E. coli* instillation. Dorsal prostates were dissected, fixed in formalin, dehydrated cleared, embedded in paraffin and sectioned to a thickness of five microns. Tissue sections were stained with Picrosirius Red to reveal collagen fibers and images were captured at 200× and analyzed with CT-Fire to count stained collagen fibers. Representative images are from (A) the uninfected control group, (B) the short term (ST) infection group (C) the repeated intermittent (RI) infection group, (D) the long-term (LT) infection group. A one-way ANOVA was used to evaluate for differences among groups (six mice per group) and $P < 0.05$ was considered significant and any *P*-values ≤ 0.05 are shown in graphs. (E) There were no differences in collagen fiber density among groups.

was greater 2 weeks after a single infection than 10 weeks after a single infection or after repeated intermittent infections. However, the weight of the dorsal prostate (the lobe most inflamed after transurethral *E. coli* infection) and the frequency of proCOL1A1+ cells did not differ among groups. There were no differences in spontaneous voiding function, as assessed by the void spot assay, among experimental groups. The only measured difference among groups was bladder weight, which was greater in the repeated infection group than other groups. Bladder weight is known to increase as a hypertrophic response to bladder outlet obstruction [33], but we did not obtain other evidence of overt bladder outlet obstruction; bladder volumes appeared similar among groups upon visual inspection and spontaneous voiding behaviors did not differ between groups. Thus, it does not appear that prostate fibrosis and/or LUTD emerge uniquely because of repeated intermittent prostate infections. It is important to note that the outcomes of the current study, conducted using young adult male mice, may not be generalizable to older males. Whether repeated intermittent infections drive more severe prostate inflammation and fibrosis in geriatric males, when the immune function declines with age, remains to be determined. We also examined just one mode of prostate inflammation-infection with uropathogenic *E. coli*. Prostate inflammation can arise from multiple sources, not and it remains to be determined whether the timing and frequency of non-bacterial inflammatory episodes, including autoimmune prostatitis [34], influence the severity of prostate fibrosis and LUTD.

We introduced *E. coli* into the urinary tract by transurethral catheterization, a procedure for which we controlled by catheterizing mice the same number of times to deliver sterile saline as a vehicle control or saline containing *E. coli*. We found that mice catheterized at least once prior to instillation of *E. coli* experienced more

severe and longer lasting bacteriuria than mice that were not previously catheterized. The influence of catheterization on rates and duration of bacteriuria was not a focus of our study; however, our finding is potentially relevant to humans who intermittently catheterize for neurogenic bladder management. Some patients using intermittent catheterization develop bacteriuria [35]. Bacteriuria can lead to life threatening pyelonephritis and sepsis and is a significant contributor to morbidity and mortality in these patients. One mechanism by which catheterization can sensitize to bacteriuria is the transfer of bacteria from genital skin to catheter and then to the urethra or bladder during catheter placement. We infected mice with a strain of uropathogenic *E. coli* (UTI89) transfected with a plasmid conferring kanamycin resistance, and counted bacterial colonies grown on kanamycin agar. Thus, the bacteria we counted in urine after transurethral *E. coli* infection were not from genital skin. Our results suggest the physical act of catheter placement is a risk factor for future infection in mice by a mechanism that is not entirely clear.

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Disclosure of conflict of interest

None.

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