Intraprostatic Reflux of Urine Induces Inflammation in a Rat

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Running head: Prostatitis by urine reflux

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Abstract

Background: We examined whether urine reflux into the prostate can induce prostatic inflammation in a rat and evaluated the effect of α1-adrenoreceptor antagonist.

Methods: Experiment 1: Male Sprague-Dawley rats were injected with 500 μL of Evans Blue through the urethral orifice. Intravesical pressure was measured, and the prostate was excised to evaluate urine reflux. Experiment 2: Rats were injected with 500 μL urine or saline (control) from the urethral orifice. Silodosin (200 μg/kg/day) was administered to the silodosin group. We evaluated histopathology, the expression of proinflammatory cytokines and oxidative stress markers of the prostate on day 7, after assessing the prostatic microcirculation and cystometrogram.

Results: Experiment 1: The histopathology showed that Evans Blue instilled through the urethral orifice entered the prostatic ducts. Intravesical pressure during Evans Blue instillation was 47.7 ± 1.6 cmH_2O (mean ± standard error). Experiment 2: On day 7 after urine instillation through the urethral orifice, histopathology showed infiltrated inflammatory cells in the peri-glandular stroma. Inflammation-associated proteins (IL-1α, IL-1β, IL-6, and TNFα) were upregulated in the urine-instilled rats but not in the silodosin group. Erythrocyte speed on the prostatic surface, immunostaining for hypoxyprobe, and quantification of oxidative stress markers (MDA and HIF-1α)

demonstrated prostatic hypoxia in the urine-instilled rats, which was ameliorated in the silodosin group. Cystometrogram revealed a shorter intercontraction interval in the urine-instilled rats, which was prolonged in the silodosin group.

Conclusions: Urine reflux into the prostatic duct induces abacterial prostatitis. Silodosin relieved prostatic inflammation and bladder overactivity by increasing microcirculation in the prostate.

Keywords: prostatitis, animal model, urine reflux, silodosin, microcirculation

Abbreviations

IL; interleukin, TNFα; Tumor necrosis factor α, MDA; malondialdehyde, HIF-1 α ; hypoxia inducible factor-1 α , CP/CPPS; chronic prostatitis/chronic pelvic pain symptoms, PDE; phosphodiesterase, NSAIDs; nonsteroidal antiinflammatory drugs, PBS; phosphate buffered saline, ELISA; enzyme-linked immunosorbent assay, CCD; charge-coupled device, LUTS; lower urinary tract symptoms.

Introduction

Prostatitis is categorized into four types based on the National Institutes of Health classification system; acute bacterial prostatitis, category I; chronic bacterial prostatitis, category II; chronic symptomatic abacterial prostatitis, category III; and chronic asymptomatic abacterial prostatitis, category IV.(1,2) Category III prostatitis is characterized by recurrent pelvic or perineal pain, dysuria, frequent urination, increased urgency, and unexplained fatigue. This type of prostatitis, which is a serious health concern that disturbs a patient's quality of life, is known as chronic prostatitis/chronic pelvic pain symptoms (CP/CPPS). The symptoms of category III prostatitis range from mild or negligible to severe; therefore, it is difficult to clearly distinguish category III from IV. Chronic abacterial prostatitis is the most common inflammatory prostatic disorder and exists widely among elderly men.(3) The Reduction by Dutasteride of Prostate Cancer Events trial suggested that chronic inflammation is found in >78% of men.(4) The Medical Therapy of Prostate Symptoms trial reported that about 40% of baseline biopsy specimens have chronic inflammatory infiltrates.(5)

Several mechanisms have been proposed to induce chronic inflammation in the prostate, including bacterial/viral infection,(6-10) changes in sex hormone secretion,(11) immunological reactions,(12) pelvic organ ischemia,(13) and urine reflux into the

prostatic ducts.(14) Reflux of urine into the prostatic ducts during micturition occurs in humans,(14) and can cause chemical irritation and inflammation. However, few studies have been conducted to examine the relationship between urine reflux and induction of inflammation.

Several types of drugs are used to treat CP/CPPS, including α1-adrenoreceptor antagonists, phosphodiesterase (PDE)-5 inhibitors, nonsteroidal antiinflammatory drugs (NSAIDs), and phytotherapeutic agents, but physicians occasionally encounter difficult-to-treat cases. A recent meta-analysis demonstrated that α1-adrenoreceptor antagonists is the most effective therapy among the above mentioned options,(15) however, the mechanism remains unknown. We hypothesized inflammation is diminished through the improvement of prostatic microcirculation.

In this study, we instilled urine into the prostatic duct and examined whether inflammation is induced. Also, we evaluated the effect of α1-adrenoreceptor antagonist on prostatic inflammation.

Materials and Methods

All animal experiments were performed in accordance with institutional guidelines and

were approved by the Nagoya University Institutional Animal Care and Use Committee.

Experiment 1

Intraprostatic reflux of transurethrally administered medium

Twelve-week-old male Sprague-Dawley rats (n = 6) were anesthetized with isoflurane. The bladder was exposed with a lower midline abdominal incision, PE-50 tubing (Becton Dickinson, Parsippany, NJ, USA) was inserted into the bladder through the dome, and urine in the bladder was aspirated to empty the bladder. The intravesical catheter was connected to a pressure transducer, and intravesical pressure was measured using the Chart 7 software package (AD Instruments, Milford, MA, USA). The genital area was cleaned with 70% alcohol and the rats were subsequently catheterized with a PE-10 tubing into the urethra by 2 cm length. Then, 500 μL of 2.5% Evans Blue in saline was infused in 5 sec using an insulin syringe. Ten min later, the prostate was dissected, rinsed with phosphate buffered saline (PBS), and frozen in compound. It was then cut into 8-μm-thick sections, mounted on slides, and counterstained with eosin.

Experiment 2

Twelve-week-old male SD rats were anesthetized with isoflurane in a supine position for

1 hour and the bladder was exposed through a lower abdominal incision. Next, 500 μL of urine was removed using a 24 G needle stuck via the bladder dome. The genital area was cleaned with 70% alcohol and the rats were catheterized with a PE-10 tubing into the urethra by 2 cm length. Then, 500 μL of urine or saline (control) was injected through the urethra in 5 sec using an insulin syringe, and the abdominal incision was closed. Osmotic pumps (Alzet; Durect Inc., Cupertino, CA, USA) containing silodosin (Kissei Pharmaceutical Co. Ltd, Matsumoto, Japan) (200 μg/kg/day when released at a rate of 1.0 μL/h for 7 days) were implanted subcutaneously in the back in the silodosin group.

Histological analyses

The excised prostate and bladder were frozen immediately in compound, cut into 8-μm-thick sections, and mounted on slides. The tissue sections were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. Histological changes in the prostate were graded according to the following scale: 0, no evidence of inflammatory infiltration or stromal edema; 1, mild (few inflammatory cells and little or no stromal edema); 2, moderate (infiltration of a moderate number of inflammatory cells and moderate stromal edema); and 3, severe (diffuse presence of many infiltrating

inflammatory cells and severe stromal edema). A single blinded observer graded the prostatic histological scores.

Hypoxyprobe-immunostaining

The rats were injected intraperitoneally with pimonidazole hydrochloride (Hypoxyprobe^{TM}-1, 60 mg/kg; Hypoxyprobe Inc., Burlington, MA, USA) 1 h before sacrifice. The excised prostate was frozen immediately in compound, cut into 10-μm-thick sections, and mounted on slides. The tissues were fixed with 4% paraformaldehyde for 10 min, rinsed with PBS, transferred to 3% hydrogen peroxide for 10 min to block peroxidase activity, and rinsed with distilled water and PBS. Next, the sections were blocked with 5% normal goat serum (Vector, Burlingame, CA, USA) for 1 h. Without rinsing, the tissues were reacted with anti-pimonidazole mouse IgG_1 monoclonal antibody for 40 min at room temperature and washed with PBS. The tissues were incubated for 10 min with biotinylated anti-mouse antibody (Dako, Carpinteria, CA, USA) at room temperature and rinsed with PBS. The tissues were stained with streptavidin-horseradish peroxidase (Dako) for 10 min followed by diaminobenzidine and nickel–ammonium sulfate with hydrogen peroxide (Vector). The tissues were counterstained in hematoxylin.

Measurement of inflammatory markers and oxidative stress markers

Prostate tissues (n = 6 per groups) were homogenized in RIPA lysis buffer. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatants were stored at −80°C until assayed. Interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α levels were determined on the MAGPIX (Millipore, Billerica, MA, USA) using the MILLIPLEX MAP Rat Cytokine/Chemokine Panel (Millipore). Malondialdehyde (MDA) and hypoxia inducible factor-1α (HIF-1α) expression levels were measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Nikken, Shizuoka, Japan and Cell Biolabs, Tokyo, Japan, respectively). Protein concentration was determined using a Bio-Rad kit (Hercules, CA, USA) with bovine serum albumin as the standard. The concentrations of the target proteins were standardized to tissue protein levels and expressed as pg/mg total protein for IL-1α, IL-1β, IL-6, TNF-α, and HIF-1α and as n mol/mg total protein for MDA.

Measurement of erythrocyte speed on the prostate surface

The rats (n = 6 per groups) were anesthetized with isoflurane on day 7. The ventral lobes of the prostate was exposed with a lower midline abdominal incision, and the erythrocyte

speed on the prostate surface was measured using the pencil lens charge-coupled device (CCD) microscopy system; a video recorder (DV-CAM, Sony Co., Tokyo, Japan) and monitor, lighting apparatus, controller, camera, lighting guide, and pencil lens with a spatial resolution of 0.86 μm and time resolution of 33 ms. Using an adjustable micromanipulator with a precision of a few tens of microns in the X, Y, and Z axes, the tip of the conical lens was secured in a position 30–50 μm from the prostate surface, allowing blood flow in the prostatic surface capillaries to be observed in real time. The reflected image was captured and passed through a green filter and converted to electrical signals in a CCD camera. The velocity at which red blood cells travelled through single capillaries of the prostate was measured from sequences of pictures.(16)

Conscious cystometry

The rats (n = 6 per groups) were anesthetized with isoflurane on day 7. The bladder was exposed with a lower midline abdominal incision, and PE-50 tubing was inserted into the bladder through the dome. The anesthesia was turned off and conscious rats were placed in a recording cage (Natsume Seisakusho Co., Tokyo, Japan). Saline was infused transvesically at 0.04 mL/min after 2 h of accumulation, and the rats voided spontaneously through the urethra. At least four reproducible micturition cycles were

recorded after an initial stabilization period (60 min). Baseline pressure, voiding pressure threshold, peak voiding pressure, and intercontraction interval were evaluated.(17,18)

Statistics

All data are expressed as means ± standard errors. The nonparametric Mann–Whitney U-test was used to assess differences between groups. All tests were two-sided, and p-values < 0.05 were considered statistically significant. All statistical analyses were performed using the SPSS software package (SPSS Inc., Chicago, IL, USA).

Results

Experiment 1

Reflux of trans-urethrally instilled fluid into the prostatic ducts

First, we instilled 500 μL of 2.5% Evans Blue through the urethral orifice in 5 s. The maximum intravesical pressure during instillation was 47.7 ± 1.6 cm H_2O. Histology of the excised prostate showed that the Evans Blue occupied the prostatic ducts and was exuded into some parts of the stromal area (Fig. 1). This result showed that 500 μL of trans-urethrally instilled fluid inflew into the prostatic ducts.

Experiment 2

Histological examination

Next, we instilled 500 μL of urine or saline through the urethral orifice. On day 7, the stromal area expanded apparently in the urine-instilled rats with polymorphological cells diffusely throughout the prostate. There were no inflammatory cells in the acini in contrast to the bacterial prostatitis model. No histological changes were observed in the bladder. In the silodosin group, stromal inflammation was observed, but at the lesser extent (Fig. 2).

Inflammation- and hypoxia-associated protein expression levels

ELISA showed that tissue levels of the proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α were upregulated in the prostates of the urine-instilled rats, but tended to be decreased in the silodosin-treated rats. We observed similar changes in the expression of the hypoxia-associated proteins, MDA and HIF-1α (Table 1). No group differences in the levels of these proteins in the bladder were observed.

Quantification of prostate microcirculation

The diameter of microvessels on the prostatic surface was smaller in the urine-instillation group and was normalized in the silodosin group (Fig. 3). Erythrocyte speed on the prostatic surface was 166.9 ± 6.2 μm/s in the control group; the speed was significantly slower in the urine instillation group (142.9 ± 3.0 μm/s, $p = 0.034$ compared to controls) but did not differ from that of the silodosin group (150.9 ± 4.0 μm/s, $p = 0.192$ compared to controls).

Hypoxyprobe staining

Hypoxyprobe immunostaining demonstrated weak positivity, mainly in the epithelium of the control rats. Much stronger positive staining was seen in the urine-instilled rats, which was normalized in the silodosin-treated rats (Fig. 4).

Cystometric analysis of bladder function

The intercontraction interval in control rats was 1818 ± 126 sec. The urine-instilled rats exhibited bladder overactivity, as evidenced by frequent micturition (993 ± 58 sec, respectively, $p < 0.001$ compared to controls). The intercontraction intervals were prolonged in the silodosin group (1607 ± 150 sec, $p < 0.001$ compared to controls; Fig. 5 and Table 2).

Discussion

This study revealed five major findings. First, when rats were instilled with 500 μL of medium from the urethral orifice, some of the medium flew into the prostatic duct. Intravesical pressure during instillation was not as high as it injured the bladder. Second, abacterial prostatitis was induced by inflow of urine into the prostatic ducts, which was characterized by inflammatory cell infiltration in the expanded stroma. Third, microcirculation was disturbed on the surface of the inflamed prostate. Fourth, urine-instilled rats showed bladder overactivity, as evidenced by frequent micturition. Finally, silodosin suppressed prostatic oxidative stress and relieved the bladder overactivity.

There is some evidence that urine reflux occurs during normal voiding in humans.(14) Kirby et al. instilled a carbon particle suspension in the bladders of male human cadavers and found that carbon flew into the prostatic duct in all cases after 20 min of maintaining intravesical pressure of 50 cmH_2O. They also instilled 400 mL of the carbon particle suspension into the bladders of patients with benign prostatic hyperplasia prior to transurethral resection of the prostate. They asked the patients to void and found

intraductal carbon present in 70% of cases. They also showed that prostatic calculi are composed partly of urine ingredients. The constituents of refluxing urine may contribute to the formation of prostatic stones by precipitation of minerals upon existing corpora amylacea.(19) The association between high urate concentration in an expressed prostatic secretion and the symptoms of patients with nonbacterial prostatitis is also evidence of urine reflux into the prostatic ducts.(20)

Several prostatitis models have been reported, including spontaneous, bacteria, auto-immune, thymectomy, hormone, stress, chemical, and diet-induced models.(21) Some of these models are based on the concept of transurethrally instilled medium upflow into the prostate. E. coli administered through the urethra causes bacterial prostatitis.(22) Transurethral ethanol/dinitrobenzenesulfonic acid-mediated mucosal injury results in acute prostatitis that peaks at 24–48 h.(23) These models irritate both prostate and bladder; therefore, they are not appropriate to evaluate the effect of prostatic inflammation on bladder function. We instilled rat urine and no injuries were observed in the bladders, therefore, we can conclude that prostatic inflammation causes bladder overactivity.

Some other studies reported prostatitis rat models of intraprostatic urinary inflow secondary to partial urethral obstruction. Takechi et al. ligated the proximal

urethra and reported that low molecular weight substances in the urine penetrated prostatic stromal tissue, which resulted in prostatic lymphocytic infiltration and interstitial edema that was most prominent on day 3.(24) After releasing the nylon ligature from the urethra, the prostatic inflammatory changes disappeared gradually. Melman et al. compared two partial urethral obstruction techniques, namely bulbous urethral obstruction through a perineal incision and midprostatic obstruction using a retropubic approach.(25) They reported that a midprostatic urethral obstruction caused bacterial prostatitis and a bulbous urethral obstruction induced a few areas of mild inflammation in the acini and adjacent interstitium with most of the normal prostatic tissue.

Some authors have reported decreased prostatic blood flow in disease conditions in both humans and animals. Berger et al. found significantly reduced prostatic perfusion in men at high risk for lower urinary tract symptoms (LUTS) using contrast-enhanced color Doppler ultrasonography.(13) Zarifpour et al. demonstrated that chronic prostatic ischemia significantly increases the contractile response of isolated prostatic tissue to electrical and pharmacological stimulation, as well as smooth muscle α-actin and collagen deposition.(26) Thurmond et al. found that oxidative modifications of cellular and subcellular elements increase contractions of prostate tissue under ischemic

conditions. They found that cultured human prostate smooth muscle cells, epithelial cells, and stromal cells were highly sensitive to hypoxic and oxidative stress conditions.(27) Our data also showed that the prostate microcirculation was disturbed in the abacterial prostatitis model, probably due to compression of the microvessels by edematous stroma.

Several drugs are used to treat chronic prostatitis, including α1-adrenoreceptor antagonists, PDE-5 inhibitors, NSAIDs, and phytotherapeutic agents.(15) A meta-analysis demonstrated that α1-adrenoreceptor antagonists are more effective than NSAIDs or phytotherapy(15) and a recent guideline recommended α1-adrenoreceptor antagonists as the initial treatment option.(28) Shimizu et al. showed that silodosin increased prostatic blood flow and suppressed oxidative stress, inflammatory cytokines, growth factors and morphological abnormalities in the ventral prostate of spontaneously hypertensive rats.(29) Although the mechanisms by which α1-adrenoreceptor antagonists relieve prostatitis symptoms have not been fully elucidated, this study demonstrated the possibility that the improvement in prostate blood flow that results from blocking the α1A subtype in vascular smooth muscle might be one mechanism.

This study has several limitations. We evaluated histological and hemodynamic changes only in the ventral lobe of the prostate. We did not examine the dose-response relation or time-course change of silodosin. Instilled urine may not be homogenous in

gradients among rats and the urine ingredient that was acting as an irritant was not unknown. Uric acid crystals result in the development of chronic prostatic inflammation by activating the NALP3 inflammasome;(30) normalization of uric acid levels is effective for improving prostatitis symptoms.(31) Proton ions, potassium ions, and ammonium in urine are also possible candidates. Further studies to identify the irritant or to use standardized urine sample are required in the future.

Conclusions

We evaluated the influence of urine reflux into the prostatic duct on the prostatic inflammatory reaction and voiding function. Urine reflux into the prostatic duct induced abacterial prostatitis and frequent micturition. Silodosin improved prostatic hypoxia by increasing blood flow, which suppressed prostatic inflammation and bladder overactivity.

Conflicts of interest

This study was supported by Dai-ichi Sankyo Pharmaceutical.

Figure Legends

Figure 1. Evans-Blue reflux into the prostate

The prostate was evaluated histologically after instillation of 500 μL of Evans Blue through the urethra. Evans Blue was observed in the prostatic duct (white arrowhead in A) and spilled out in some parts (black arrow head in B). Scale bar: 100 μm.

Figure 2. Hematoxylin and eosin staining of the prostate and bladder

Compared with control prostate (A), increased submucosal edema and inflammatory cell infiltrates were observed in the urine-instilled rats (B). Diffuse inflammation was observed in the silodosin group also (C). No inflammation was observed in the bladder of any of the groups (D; control, E; urine-instilled group, and F; silodosin group). Scale bar indicates 100 μm (A to C) and 200 μm (D to F).

Figure 3. Microvessels on the prostate surface

Pencil lens CCD microscopy directly visualized the movement of each erythrocyte. Compared to the control (A), the microvessels had smaller diameters in the urine-instillation group (B), which was larger in the silodosin group (C). Scale bar: 50 μm.

Figure 4. Immunohistochemistry for hypoxyprobe

Hypoxyprobe staining was stronger mainly in the epithelium of the urine-instilled rats, which was normalized in the silodosin-treated rats. Scale bar: 100 μm.

Figure 5. Cystomegrogram

Representative intravesical pressure was demonstrated. Compared to the control (A), the intercontraction interval was shorter in the urine-instilled group (B) and was prolonged in the silodosin-treated group (C). Scale bar: 10 min.

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