Long-lasting breaches in the bladder epithelium lead to storage dysfunction with increase in bladder PGE$_2$ levels in the rat

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Running head: urothelial injury and storage dysfunction

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ABSTRACT

Increase in bladder mucosal permeability can be reproduced by intravesical administration of protamine sulfate (PS); however, the influence of PS once administered into the bladder disappears within several days. We developed a chronic animal model of urothelial injury using PS. Insertion of a polyethylene catheter through the bladder dome was performed in female Wister rats. The other end of the catheter was connected to an osmotic pump for continuous delivery of PS or vehicle for 2 weeks. Urinary frequency (UF) and voided volume (VV) were measured in the metabolic cage. The 5th group of rats received a high dose (10 mg/ml) PS for 2 weeks and were followed for a further 2 weeks without PS. The 6th group received a high dose PS for 2 weeks and loxoprofen (0.1 mg/kg/day) for 4 weeks. UF was increased and VV was reduced in rats treated with a high dose PS, but not changed in rats treated with a vehicle or a low dose (1 mg/ml) PS. UF was further increased in the 5th group, while unchanged in the 6th group. Histological sections in rats treated with a high dose PS demonstrated a loss of the upper layer of urothelial cells and an increased number of mast cells. PGE2 level in the bladder was significantly elevated in the 5th group. These results indicate that chronic urothelial injury leads to an increase in UF and a decrease in VV. Increased PGE2 level in the bladder is likely to be associated with long-lasting storage dysfunction.

Key words: protamine, urothelium, detrusor overactivity, prostaglandin
INTRODUCTION

Painful bladder syndrome/interstitial cystitis (PBS/IC) is a chronic bladder disorder characterized by urinary frequency, urgency, nocturia, and bladder pain. The etiology of PBS/IC is unknown, although several theories have implicated various factors, including inflammation, autoimmune disorders, subclinical infections (viral and/or bacterial), epithelial dysfunction, neurogenic inflammation, allergies, and neuroendocrine conditions (2). It has been hypothesized that a defect in the glycosaminoglycan layer of the bladder results in the characteristic symptoms of pain, frequency, urgency, and nocturia experienced by patients with PBS/IC (9). The glycosaminoglycan layer may have importance in bacterial antiadherence and in prevention of urothelial damage by large macromolecules (16), however, there is no definite evidence that the glycosaminoglycan layer acts as the primary epithelial barrier between urine and plasma. A possible cause of increased bladder afferent sensitivity is that substances in the urine, which are normally prevented from contacting sensory afferents in the bladder wall by a relatively impermeable epithelial barrier, pass through a damaged or permeable urotherium. Increase in bladder mucosal permeability can be reproduced by intravesical administration of protamine sulfate (PS); however, the influence of PS once administered into the bladder disappears within 5 days (8). We have developed a chronic animal model of selective urothelial injury using PS. In the present study we determined if long-lasting breaches of the bladder epithelium lead to storage dysfunction, or morphological and biological disorders of the bladder in the rat.

MATERIALS AND METHODS
Seventy female Wister rats, weighing 230-277 g (mean = 248 g), were used in this study. They were housed at a constant temperature (23 ± 2°C) and humidity (50-60%) under a regular 12-hour light/dark schedule (lights on 7:00 AM - 7:00 PM). Tap water and standard rat chow were freely available. All experiments were performed in strict accordance with the guidelines of the Institutional Animal Care and Use Committee of our institution.

**Implantation of osmotic pump**

Surgical anesthesia was induced and maintained using 2% halothane. The bladder was exposed via a midline abdominal incision. The bladder end of a polyethylene catheter (size 3; i.d. 0.7 mm, o.d. 1 mm; Hibiki Co. Ltd., Tokyo, Japan) was heated to create a collar and passed through a small incision at the apex of the bladder dome, after which a suture was tightened around the collar of the catheter. Insertion of a polyethylene catheter through the bladder dome was performed in rats. The other end of the catheter was connected to a Model 2002 Micro-Osmotic Pump (Alzet Co., Palo Alto, California) fixed in the abdominal wall for continuous delivery of PS (1 or 10 mg/ml, 12 µl daily) or saline (vehicle) for 2 weeks. A Model 2004 Mini-Osmotic Pump (Alzet Co., Palo Alto, California) was implanted subcutaneously to deliver loxoprofen (0.1 mg/kg/day), nonsteroidal anti-inflammatory drugs for 4 weeks.

**Frequency and volume analysis**

Each rat was placed in a metabolic cage connected to a digital scale and personal computer. UF per day and VV per void were recorded continuously in the metabolic cage for 72 hours every 2 weeks. Each rat was provided with free access to food and water. Rats were divided into 6 treatment groups (fig. 1). In the 5th group rats received intravesical infusion of
a high dose (10 mg/ml) of PS for 2 weeks and were followed for a further 2 weeks without PS. In the 6th group rats received intravesical infusion of a high dose (10 mg/ml) of PS for 2 weeks and subcutaneous infusion of loxoprofen (0.1 mg/kg/day) for 4 weeks.

**Histological examinations**

The rats were sacrificed after frequency and volume analysis. Bladder samples were removed and weighed. One half of the bladder was processed for light microscopic investigations. Tissue sections (5µm) were stained with H&E and toluidine blue stains. Quantitative morphometric analysis of mast cell involvement was performed using a computerized digital analysis system (FUJIX digital camera, HC-2500; Photograb-2500 version 1.1, Fuji Photo film Co. Ltd, Tokyo, Japan). Ten samples were selected and mast cells containing metachromatic granules were counted separately at 400X magnification in the bladder wall by 1 or more observers blinded to the group from which each sample was obtained. Mast cell density is expressed as the cell number per unit area.

**Bladder PGE2 levels**

One half of the bladder sample was used for PGE2 determinations. The PGE2 content was determined spectrophotometrically using an EIA Kit Monoclonal LCP45 Cayman.

**Data analysis**

Data are presented as a mean ± the standard error (SE). The Mann Whitney’s U-test or Wilcoxon signed-ranks test was used for comparison of the two groups. A level of p <0.05 was considered statistically significant.

**RESULTS**
Differences in body and bladder weight between rats treated with saline, PS and loxoprofen were not found during the experiments.

Frequency and volume analysis

There were no differences in UF or VV between rats treated with a low dose (1 mg/ml) of PS and rats treated with saline. UF significantly increased in rats treated with a 2-week infusion of a high dose (10 mg/ml) of PS, when compared to rats treated with saline (p <0.05; fig. 2). UF was further increased in rats treated with a high dose of PS and followed for a further 2 weeks (p <0.01). However, UF remained control level in rats received intravesical infusion of a high dose (10 mg/ml) of PS for 2 weeks and subcutaneous infusion of loxoprofen (0.1 mg/kg/day) for 4 weeks.

VV in rats treated with a 2-week infusion of a high dose of PS was significantly lower than that in rats treated with saline (p <0.05; fig. 3). VV was further decreased in rats treated with a high dose of PS and followed for a further 2 weeks (p <0.01). VV in rats received intravesical infusion of a high dose (10 mg/ml) of PS and subcutaneous infusion of loxoprofen (0.1 mg/kg/day) was the same level as VV in rats treated with saline.

Histological examinations

In rats treated with saline or a low dose of PS normal mucosa was observed (fig. 4). In contrast, in rats treated with a high dose of PS mucosal ulceration (loss of the upper layer of urothelial cells) and submucosal hemorrhage were revealed by H&E stain. In rats treated with a high dose of PS for 2 weeks and followed for a further 2 weeks, submucosal lymphoid infiltration, decreased urothelial cell layer, and regeneration of urothelial cells were observed in part. Under toluidine blue stains, mast cells were predominantly found in
the submucosal and muscle layer in rats treated with a high dose of PS (fig. 5). These mast cells were not seen in the bladder treated with saline.

**Bladder PGE2 levels**

No increase in bladder PGE2 level was found in rats treated with 1 or 10 mg/ml PS for 2 weeks (fig. 6). Bladder PGE2 level was elevated in the bladder of rats treated with a high dose of PS for 2 weeks and followed for a further 2 weeks. PGE2 level was also elevated in the bladder of rats treated with 2 weeks-saline and followed a further 2 weeks; however, a significant difference was found between the 2 groups (p <0.05). PGE2 level in rats received intravesical infusion of a high dose (10 mg/ml) of PS and subcutaneous infusion of loxoprofen (0.1 mg/kg/day) was the same level as that in rats treated with saline.

**DISCUSSION**

The bladder is normally impermeable and resistant to hostile environmental factors. PS increases the apical membrane permeability to both monovalent cations and anions, and changes the permeability of different types of epithelium, as based on its concentration (20, 21). PS and urea given intravesically cause de-epithelization and inflammation of epithelial tissue (14). Ulcerated areas, an irregular glycosaminoglycan layer, an increased number of mast cells, etc have been reported to exist in rat bladders treated twice in 24 hours with PS (3). Prolonged exposure to PS (> 15 minutes) is poorly reversible and is thought to be caused by a decrease in paracellular resistance from cell lysis (16, 20, 21).

It seems that there is a population of PBS/IC patients with increased epithelial permeability. However, increased permeability is nonspecific and is a consequence of
bladder inflammation, and also occurs with cyclophosphamide-induced bladder injury, bacterial infection, and cystitis after intravesical challenge with antigen after sensitization (7). Whether the increased permeability represents a primary cause of PBS/IC or merely reflects the result of an unidentified source of inflammation is unclear. To verify this theory, further experiments are necessary concerning the permeability of the urothelium. In the present study we ascertained whether continuous damage of the epithelium with PS induced storage dysfunction, morphological, and biological disorder of the bladder of the rat, and whether storage dysfunction continued after intravesical instillation of PS.

In the present study, continuous infusion of PS induced storage dysfunction and histological disorder in the rat bladder, suggesting that continuous damage of the epithelium could be a major etiological factor for PBS/IC. To our knowledge, the possibility that a chronic animal model with damaged urothelium reveals storage dysfunction has never been reported.

Mast cells were predominantly found in rats treated with a high dose of PS in the present study. Mast cells are normally distributed throughout the body and are necessary for the development of allergic reactions, during which they secrete vasoactive and nociceptive molecules as well as numerous cytokines and inflammatory mediators including PGE\textsubscript{2} (5). Mast cells are especially prominent near surfaces exposed to the environment, including the skin, airways, and gastrointestinal tract, where pathogens, allergens, and other environmental agents are frequently encountered (5). There are twice as many mast cells in the urothelium and 10 times as many in the detrusor of patients with PBS/IC compared to controls (17). Bladder mast cells are located close to neurons and are activated by
neuropeptides and acute stress (19). Furthermore, the levels of urine substances, for example histamine, 1,4-methylimidazole acetic acid, and nerve growth factor, have been reported to increase in PBS/IC patients and to influence mast cell number and phenotype (4, 5).

How can we explain why the storage dysfunction continued after the discontinuation of intravesical PS? There may be 2 possible explanations. One is that regeneration of the urothelial barrier is not completed by 2 weeks after 2 weeks-instillation of PS. The other is the possibility that long-lasting detrusor overactivity might depend on the neural plasticity via the spinal reflex arc. Urinary PGE2 excretion has been reported to increase in PBS/IC patients (10). Elevated PGE2 level in the bladder wall in the present study seems to relate to development of detrusor overactivity because loxoprofen, cyclooxygenase inhibitor prevented PGE2 elevation and storage dysfunction. Prostanoids, in particular PGE2 have been implicated as endogenous modulators of bladder function in the normal physiological state and under pathophysiological conditions (1). Prostanoid synthesis occurs locally in the detrusor, urothelial, and inflammatory cells. It is initiated by various physiological stimuli, such as detrusor muscle stretch and nerve stimulation as well as by injuries and inflammation mediators, whose release is dependent on mast cell regulation. In vivo endogenous prostanoids enhance voiding efficiency through a direct or indirect effect on sensory nerves (11). Intravesical instillation of PGE2 in rats causes detrusor overactivity and stimulates reflex micturition (6, 22). Furthermore, cyclooxygenase-2 expression has been reported to be increased as a consequence of bladder outflow obstruction in rats, suggesting a possible role of prostanoids in the detrusor overactivity associated with outflow obstruction (15).
PGE$_2$ has been reported to play a critical role in the generation and maintenance of hyperalgesia that develops at sites of inflammation (18). Furthermore, by means of spinal microdialysis, it has been demonstrated that PGE$_2$ release from the dorsal horn of the spinal cord is immediately increased after nociceptive stimulation into the hindpaw of rats (12). Peripheral inflammatory nociceptive stimuli, which influence the upregulation of spinal cyclooxygenase and prostanoid synthesis, cause a combination of acute spontaneous nociception and persistent thermal and mechanical hyperalgesia (13). In the present study, long-lasting detrusor overactivity might depend on the neural plasticity via the spinal reflex arc, which was induced by the peripheral increase in PGE$_2$ level. Elevated PGE$_2$ level in the bladder wall might play an important role in the development of detrusor overactivity in rats treated with PS. Activation of bladder mast cells by urine substances through a damaged urotherium may lead to the release of PGE$_2$ which in turn results in overactivity and hyperalgesia of the bladder.

PERSPECTIVES AND SIGNIFICANCE

Our results support the existence of a mechanism in which continuous damage to the bladder permeability barrier and epithelium lead to storage dysfunction and histological changes in the bladder. Whether or not these persistent damages are sufficient to potentiate the storage dysfunction seen in PBS/IC patients, and elucidation of the additional mechanisms mediated by bladder wall PGE$_2$ remain interesting subjects for future experimentation. This model will be useful in defining the mechanisms governing the induction of PBS/IC and in investigating therapeutic modality.
REFERENCES


**FIGURE LEGENDS**

Fig. 1 Protocol for present experiment. Arrow heads indicate the time of tissue harvest. The fifth treatment group is composed of rats treated with a 2-week intravesical infusion of protamine sulfate (PS) and followed for a further 2 weeks. The sixth treatment group is composed of rats treated with a 2-week intravesical infusion of PS and subcutaneous infusion of loxoprofen for 4 weeks.

Fig. 2 Comparison of urinary frequency (UF) per day in rats treated with a continuous intravesical infusion of saline (white bars), a high dose (10 mg/ml) intravesical infusion of
protamine sulfate (PS) (black bars), or a high dose (10 mg/ml) intravesical infusion of PS and subcutaneous infusion of loxoprofen (0.1 mg/kg/day) (gray bars). UF significantly increased in rats treated with a 2-week intravesical infusion of PS, when compared to rats treated with saline (p < 0.01-0.05). Note that UF was further increased in rats treated with a 2-week infusion of PS and followed for a further 2 weeks (p < 0.01). Subcutaneous loxoprofen infusion prevented the increase in UF in rats treated with a 2-week intravesical infusion of PS.

Fig. 3 Comparison of voided volume (VV) in rats treated with a continuous intravesical infusion of saline (white bars), a high dose (10 mg/ml) intravesical infusion of protamine sulfate (PS) (black bars), or a high dose (10 mg/ml) intravesical infusion of PS and subcutaneous infusion of loxoprofen (0.1 mg/kg/day) (gray bars). VV significantly lowered in rats treated with a 2-week infusion of PS, when compared to rats treated with saline (p < 0.01-0.05). Note that subcutaneous loxoprofen infusion prevented the decrease in VV in rats treated with a 2-week intravesical infusion of PS.

Fig. 4 Histological findings of bladders in rats treated with saline (control) or a high dose (10 mg/ml) of protamine sulfate (PS). A and B: control bladder. H & E stainings. C and D: 10 mg/ml PS for 2 weeks and followed for further 2 weeks. H & E stainings. Note mucosal ulceration (loss of the upper layer of urothelial cells) and hemorrhage. Regeneration of urothelial cells is found in part (arrows). D: Note submucosal lymphoid infiltration. E, F: Toluidine blue stainings. Mast cells are predominantly found in the submucosal and
muscle layer in rats treated with a high dose of PS. None of these mast cells are seen in the controls.

Fig. 5 Number of mast cells in bladders in rats treated with saline (control) or a high dose (10 mg/ml) of protamine sulfate (PS). The number of mast cells increased in rats treated with PS for 2 weeks, when compared to the control rats. The number further increased significantly in rats treated with PS for 2 weeks and followed for a further 2 weeks (p< 0.05).

Fig. 6 Comparison of bladder tissue PGE\(_2\) content in each treatment group. PGE\(_2\) level significantly increased in rats treated with a high dose (10 mg/ml) of protamine sulfate (PS) and followed for a further 2 weeks, when compared to rats treated with saline and rats treated with a low (1 mg/ml) or a high dose of PS for 2 weeks (p< 0.01 – 0.05). PGE\(_2\) level in rats treated with a high dose (10 mg/ml) of PS and subcutaneous infusion of loxoprofen (0.1 mg/kg/day) was the same as that in rats treated with saline.
Fig. 1

- Control P saline
- Control P saline
- PS 1 mg/ml
- PS 10 mg/ml
- PS 10 mg/ml/2w
- PS 10 mg/ml/2w Loxoprofen 0.1 mg/kg/day

254x190mm (96 x 96 DPI)
Fig. 2

![Graph showing urinary frequency (per day) over 2 and 4 weeks for different conditions (Control saline, PS, PS + Loxoprofen). The graph indicates significant differences at 4 weeks with p < 0.01 for Control saline vs. PS and PS + Loxoprofen. The bars represent mean values with error bars showing standard deviation.](image-url)

254x190mm (96 x 96 DPI)
Fig. 3

![Graph showing voiding volume (ml) over time for different groups.]

254x190mm (96 x 96 DPI)
Fig. 4

A  × 25

B  × 100

C  × 25

D  × 100

E  × 25

F  × 100

254x190mm (96 x 96 DPI)
Fig. 5

Number of mast cells

- No manipulation
- Control (saline/2w-2w)
- PS (10 mg/2w)
- PS (10 mg/2w-2w)

$p < 0.05$

254x190mm (96 x 96 DPI)
**Figure 6**

254x190mm (96 x 96 DPI)